

# **Differential gene expression in rodent embryo fibroblasts upon replicative senescence**

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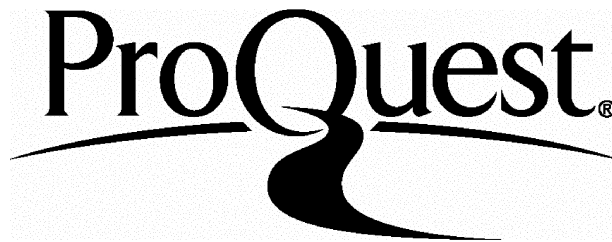
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## Enclosed Unbound Material

### Publications:

Benvenuti, S., Cramer, R., Bruce, J., Waterfield M.D. and Jat P.S. (2002) Identification of novel candidates for replicative senescence by functional proteomics. *Oncogene* (in press)

Benvenuti, S., Cramer, R., Quinn, C.C., Bruce, J., Zvelebil, M., Corless, S., Bond, J., Yang, A., Hockfield, S., Burlingame, A.L., Waterfield, M.D. and Jat, P.S. (2002) Differential proteome analysis of replicative senescence in Rat Embryo Fibroblasts. *Molecular and cellular proteomics* 1, 280-292.

A CD containing the raw microarray data.

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## Abstract

Cancer arises as a consequence of the accumulation of multiple independent mutations in genes that regulate cell proliferation and survival. The acquisition of an unlimited proliferative potential is probably a critical step in this process since normal somatic cells can only undergo a finite number of divisions. One approach that has been extensively used to study this phenomenon has been the serial cultivation of normal somatic cells. When these cells are cultured *in vitro*, they undergo a finite number of divisions and then cease dividing and enter a state of replicative senescence, in which they are viable and remain metabolically active, but cannot be induced to undergo mitosis, re-enter the cell cycle and divide.

To identify the underlying mechanisms that determine the finite mitotic potential of normal somatic cells, and how they may be abrogated in cancer cells, we first undertook a high-resolution differential proteome analysis aimed at identifying proteins that are differentially expressed upon replicative senescence.

Since replicative senescence in primary fibroblast cultures is asynchronous, two complimentary systems were used. We analysed a group of conditionally immortalised rat embryo fibroblast cell lines that have previously been shown to undergo synchronous senescence upon inactivation of SV40 tsA58 large T antigen. Moreover since it has been shown that rodent embryo fibroblasts only become dependent upon the immortalising gene when their finite life span has elapsed, we complemented these studies by analysing replicative senescence in the asynchronous serially passaged rat embryo fibroblast cultures.

In these two proteome analyses a number of features have been identified as differentially expressed upon replicative senescence; 49 features were found to be up or down-regulated upon serial passaging of the primary rat embryo fibroblasts, and 44 were found to be differentially expressed in the conditionally immortalised cell lines. Some of the identified changes were also

shown to occur in two other models of senescence: oncogene induced premature senescence of REF52 cells and replicative senescence of mouse embryo fibroblasts.

Comparison of the identity of the features found to be differentially expressed in the two complimentary studies identified 9 differential features that were in common.

Although the majority of the identified proteins have previously not been recognised to be involved with replicative senescence, they are likely to represent novel starting points for elucidating the underlying mechanisms that regulate the finite mitotic life span of somatic cells and how it can be overcome in cancer cells.

We then extended our studies by comparing the mRNA level of growing versus senescent cells using mouse cDNA microarrays to determine if there was any correlation between differential protein expression and RNA expression, and also to achieve higher sensitivity for identifying changes in gene expression.

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## **Statement concerning collaborations**

The work presented in this thesis is the work of the author except where indicated.

The high-resolution 2-D gels were run in collaboration with OGS (Oxford GlycoSciences).

The mass spectrometry analysis was performed by the Analytical Biochemistry Laboratory of the Royal Free University College Branch of the Ludwig Institute for Cancer Research.

## Abbreviations

The following is a list of abbreviation used through the thesis.

%H:	percentage of homology
1-D:	one-dimensional
2-D:	two-dimensional
ABC:	ammonium bicarbonate
ACN:	acetonitrile
AD:	Alzheimer's disease
A2M:	$\alpha$ -2-macroglobulin
AP-1:	activator protein 1
APS:	ammonium persulphate
Arp:	actin related protein
ATM:	ataxia telangiectasia mutated protein
ATP:	adenosine triphosphate
b:	<i>Bos taurus</i>
BSA:	bovine serum albumin
c:	<i>Gallus gallus</i>
CAK:	CDK- activating kinase
CBP:	CREB binding protein
Cdk:	cyclin dependent kinase
cDNA:	complementary deoxyribonucleic acid
ch:	<i>Chinese hamster</i>
CHIMAP:	change in intensity of multiply associates PEMs
CIP:	calf intestinal alkaline phosphatase
CKIs:	cdk inhibitors
cl:	<i>Cricetulus longicaudatus</i>
CR:	caloric restriction
CRMP:	collapsin response mediator protein
Cts d:	cathepsin D
Da:	daltons

DEPC:	diethyl pyrocarbonate
DHFR:	dihydrofolate reductase
DMEM:	Dulbecco's modified eagle medium
DMSO:	dimethyl sulphoxide
DNA:	deoxyribonucleic acid
DNAse:	deoxyribonuclease
DTT:	dithiothreitol
E:	embryonic day
ECM:	extracellular matrix
EDTA:	ethylenediaminetetraacetic acid
EF1:	elongation factor 1
EPC-1:	early population doubling level cDNA-1
ER:	endoplasmic reticulum
ERF:	eukaryotic releasing factor
ERK-2:	extracellular signal-regulated kinase-2
ESI:	electrospray ionisation
EST:	expression sequence tag
F.C.:	fold change
FCS:	foetal calf serum
FN:	fibronectin
FR:	Fischer rat
GSE:	genetic suppressing element
h:	<i>Homo sapiens</i>
H <sub>2</sub> O <sub>2</sub> :	hydrogen peroxide
HAT 1:	histone acetyltransferase 1
HDAC:	histone deacetylase activity
HDF:	human diploid fibroblasts
Hmg1:	high mobility group 1
HPLC:	high-performance affinity chromatography
HRP:	horseradish peroxidase
HSP:	heat shock proteins

IAA:	iodoacetic acid
ID:	identity number
IEF:	isoelectric focusing
IGF-BP3:	insulin-like growth factor binding protein 3
IPG:	immobilized pH gradient
kb:	kilobase
kDa:	kilodalton
LC:	liquid chromatography
LT-ag:	Large T-antigen of Simian virus 40
M:	molar
m:	<i>Mus musculus</i>
MALDI:	matrix-assisted laser desorption/ionization
MAP:	mitogen-activated protein kinase
MCI:	molecular cluster index
MEFs:	mouse embryo fibroblasts
min:	minutes
MKK:	MAP kinase kinase
M-MLV RT	Moloney Murine Leukaemia Virus Reverse Transcriptase
MMPs:	matrix metalloproteinases
MOPS:	[3 - (N-morpholino) propanesulphonic acid]
Mot:	mortalin
MPF:	Maturation Promoting Factor
MRE:	metal-responsive element
mRNA:	messenger ribonucleic acid
MRP:	migration inhibitory factor-related protein
MS/MS:	tandem mass spectrometry
MS:	mass spectrometry
MT:	metallothionein
MTF-1:	metal-responsive element-binding transcription factor-1
MW:	molecular weight
NaDOC:	sodium deoxycholate



NCBI:	National Centre for Biotechnology Information
NEFGE:	non-equilibrium pH gradient electrophoresis
NGF:	nerve growth factor
NLS:	nuclear localization signal
NTP:	nucleotide triphosphate
oc:	<i>Oryctolagus cuniculus</i>
OD:	optical density
P1:	passage 1
P2:	passage 2
P3:	passage 3
P4:	passage 4
P5:	passage 5
P6:	passage 6
P7:	passage 7
P8:	passage 8
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCNA:	proliferating cell nuclear antigen
PCR:	polymerase chain reaction
PD:	population doublings
PDGF:	platelet-derived growth factor
PEDF:	pigment epithelial cell derived factor
PEM:	protein expression map
pI:	isoelectric point
pRB:	retinoblastoma susceptibility protein
r:	<i>Rattus norvegicus</i>
R:	restriction point
RA:	retinoic acid
REFs:	rat embryo fibroblasts
RMM:	relative molecular weight
RNA:	ribonucleic acid

RNAse:	ribonuclease
ROS:	reactive oxygen species
rpm:	revolutions per minute
RT:	reverse transcription
SA- $\beta$ -gal:	senescence associated- $\beta$ -galactodidase
SAGE:	serial analysis of gene expression
SCC:	squamous cell carcinoma
SD:	Sprague-Dawley
SDS:	sodium dodecyl sulphate
sec:	seconds
SIPS:	stress induced premature senescence
SRE:	serum response element
SRF:	serum response factor
Ss:	single stranded
SV:	control cell line for the conditionally immortalised cell lines
SV40:	Simian virus 40
<i>t</i> -BHP:	<i>tert</i> -butylhyroperoxide
TE:	Tris-EDTA buffer
TEMED:	N,N,N',N' tetraethylenemethyldiamine
TFA:	trifluoroacetic acid
TGF- $\beta$ 1:	transforming growth factor- $\beta$ 1
TE:	Tris-EDTA
Timp:	tissue inhibitors of metalloproteinases
tk:	thymidine kinase
TNF- $\alpha$ :	tumour necrosis factor $\alpha$
TOAD:	turned on after division
TOF:	time-of-flight
Tris:	tris (hydroxymethyl) aminomethane
tRNA:	transfer RNA
ts:	temperature sensitive
tsa:	conditionally immortalised cell line

TUC:	TOAD-64/Ulip/CRMP
UTR:	un-translated region
UV:	ultra violet radiation
xl:	<i>Xenopus laevis</i>
WS:	Werner syndrome
WT:	Wilms' tumour

## **Aim of the research**

Replicative senescence is a complex, multigenic phenomenon, which has been extensively investigated but which underlying mechanisms still remain largely unknown, probably due to the fact that the variety of traditional approaches utilised so far to try to identify the underlying changes that are the causes of replicative senescence have been insufficient to comprehensively analyse such a complex biological process.

Recently new approaches directed to address complex biological systems are becoming available. These novel techniques are known as “post-genomic era techniques” and they have the potential to monitor global changes in gene expression. Proteomics and microarrays are two of the most powerful “post-genomic era” techniques. The former allows all the proteins expressed by a certain cell type under a particular condition (“proteome”) to be studied. The proteins are first separated by 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and then identified by mass spectrometry (MS). The latter allows the study of several thousand transcripts at the same time.

The aim of my research was to identify genes, which are differentially regulated upon replicative senescence both at the protein and at the transcriptional level using proteomics and microarrays.

I have used two complementary model systems to study replicative senescence: replicative senescence of primary rodent embryo fibroblasts and conditionally immortalised cell lines (tsa). The combination of these two models will allow the identification of changes in gene expression associated with both the counting mechanism and the entry into the post-mitotic state, which have been proposed to underlie replicative senescence.

The work described in this thesis represents one of the first attempts to study replicative senescence in its entirety combining two of the most powerful post-genomic era techniques together with two commonly used model systems

# 1 Introduction

## 1.1 Replicative senescence

More than 30 years ago Hayflick (Hayflick, 1965; Hayflick, 1961) observed that human diploid fibroblasts show a spontaneous decline in growth rate when kept in culture, which was not related to the elapsed time in culture but to an increasing number of population doublings (PD). Eventually the cells stop dividing after 50-70 PD, and remain in a quiescent but viable state called replicative or cellular senescence (Wynford-Thomas, 1999).

Since then various other normal human cell types have been found to undergo replicative senescence, including epidermal keratinocytes, smooth-muscle cells, lens epithelial cells, glial cells, endothelial cells and melanocytes (Berube *et al.*, 1998), as well as fibroblasts of other species. It is now well established that normal somatic cells have a limited proliferative life span even when cultured under ideal growth conditions (Wynford-Thomas, 1999).

Senescent cells can be kept in culture for long period of time, remaining metabolically active, as shown by their ability to synthesize mRNAs and proteins (Lumpkin *et al.*, 1986). However even though they retain some responsiveness to mitogens these cells cannot be induced to re-enter the cell cycle and divide (Matsumura *et al.*, 1979). The block in the cell cycle is irreversible (Cristofalo *et al.*, 1989), and it is not associated with programmed cell death.

### 1.1.1 *In vitro* replicative senescence and *in vivo* aging

It was first suggested that the finite proliferative potential exhibited by cells cultured *in vitro* was simply an artefact due to the cell culture

environment. This hypothesis was based on the suggestion that *in vitro* it is impossible to mimic the natural environment of cells within the organism. In culture cells do not retain their three-dimensional environment and their original cell-cell interactions, which are critical for functional cooperation among cells. It was also suggested that the explantation of cells from the organism, their serial sub-cultivation and the enzymatic treatment, in addition to the cell culture conditions *per se*, were severely stressful factors (Rubin, 1997). These stressful conditions were believed to cause different kinds of cellular damage whose accumulation resulted in a progressive loss of proliferative potential, leading to replicative senescence.

However, even though physiological conditions cannot be perfectly duplicated in cell culture, it has been proposed that the finite replicative lifespan of normal cells in culture is the manifestation at the cellular level of *in vivo* organismal aging (Campisi *et al.*, 1996; Smith and Pereira-Smith, 1996).

Several studies have shown an inverse relationship between donor age and fibroblast culture replicative lifespan. Cells taken from older donors were shown to undergo fewer PD in culture than cells taken from younger donors (Bruce *et al.*, 1986). However several findings have recently been reported that raise questions about the validity of the relationship between replicative senescence of cells in culture and the lifespan of the organism. When the earlier studies were re-analysed and statistic methods employed, a donor age-dependent correlation could no longer be observed. Recently Cristofalo *et al.* (Cristofalo *et al.*, 1998) have published a re-evaluation of the concept of inversion correlation between donor age and replicative life. They determined the replicative lifespan of 124 human skin fibroblasts cell cultures established from donors of different ages, which were declared “healthy” at the time of the transplant, and they were able to conclude that if health status and biopsy conditions were controlled, the replicative lifespan of fibroblasts in culture did not correlate with donor age (Cristofalo *et al.*, 1998).

Also the statement that the maximum replicative potential of cells in culture seems to be directly proportional to the life span of the donor species (Stanulis-Praeger, 1987) has been recently revisited.

Therefore in light of the most recent studies the reproducible loss of proliferative potential *in vitro* may not reflect changes in replicative capacity that occur *in vivo*; however fibroblast culture remain a powerful model for the age-related studies (Cristofalo *et al.*, 1998).

However evidence to support the theory that *in vitro* replicative senescence is the manifestation at the cellular level of *in vivo* organismal aging was provided when it was shown that human fibroblasts from patients affected by the hereditary premature aging syndrome, Werner syndrome (WS), have a shorter *in vitro* life span than cells taken from normal individuals of the same age (Brown, 1990) (Ostler *et al.*, 2002).

Another strong evidence that senescent cells exist and accumulate with age *in vivo* was provided by Dimiri *et al.* (Dimiri *et al.*, 1995). They showed several human cell types express an acidic  $\beta$ -galactosidase that is histochemically detectable at pH 6, upon senescence in culture. This marker was expressed by senescent, but not presenescent, fibroblasts and keratinocytes and was absent in quiescent fibroblasts and terminally differentiated keratinocytes. It was also absent in immortal cells while it was induced by genetic manipulations that reversed immortality. Dimiri *et al.* showed also that in skin samples from human donors of different age, there was an age-dependent increase in  $\beta$ -galactosidase, which therefore remains to date the only marker, which provides *in situ* evidence that senescent cells may exist and accumulate with age *in vivo*.

### ***1.1.2 Replicative senescence, quiescence, terminal differentiation and apoptosis***

The senescent state is distinct from other ways in which cells can escape the cell cycle which are quiescence, terminal differentiation and programmed cell death also called apoptosis.

When cells undergo senescence they mainly exit the cell cycle in late G<sub>1</sub> phase or at the G<sub>1</sub>/S phase boundary (Sherwood *et al.*, 1988). More recently however Gonos *et al.* (Gonos *et al.*, 1996) showed that senescent cells could also be arrested in G<sub>2</sub> phase of the cell cycle.

Quiescence is a very specific state in which the growth arrest is the consequence of depriving cell cultures of mitogenic stimuli (it can be achieved either by serum starvation or by growing cells to confluence); quiescent cells leave the cell cycle in G<sub>1</sub> and enter the G<sub>0</sub> phase. Quiescence is a reversible state, and cells in G<sub>0</sub> can be induced to re-enter the cell cycle by stimulation with suitable growth factors and replating (Pardee *et al.*, 1978) whereas no known combination of mitogens can stimulate senescent cells to divide again (Cristofalo *et al.*, 1989).

Cellular senescence shares quite few features with the terminal differentiation status. Both senescent and terminally differentiated cells undergo irreversible growth arrest and alter their phenotype (Bayreuther *et al.*, 1988; Peacocke and Campisi, 1991). In addition, the two states share a common molecular profile. Cell cycle proteins with a regulatory key role, such as the cyclin dependent kinase (cdk) inhibitors p21<sup>WAF1/Sdi1/CIP1</sup> and p16<sup>INK4a</sup> as well as pRB, which are involved in cellular senescence, have been shown to be associated with the induction of terminal differentiation (Harvat *et al.*, 1998). However, even though senescence and terminal differentiation share common features, evidence suggests that they are independent cellular pathways. While terminal differentiation can be induced by various signals, replicative senescence is a consequence of completing the finite number of divisions.



Finally senescent cells exhibit many fundamental differences to cells undergoing programmed cell death (apoptosis). In contrast to apoptotic cells senescent cells are viable and metabolically active in culture for long periods of time (Matsumura *et al.*, 1979); moreover it has been suggested that senescent cell have acquired resistance to apoptosis (Wang *et al.*, 1994).

### ***1.1.3 Replicative senescence: a stochastic or a programmed event?***

Even though the actual cause of senescence remains unknown, many different theories have been proposed to explain the loss of proliferative potential during replicative senescence; they generally can be grouped in two broad categories, which suggest that senescence is either a stochastic or a programmed event.

The “stochastic theories” suggest that cellular senescence occurs as a result of the random accumulation of cellular damage. When cells divide *in vitro* they accumulate various forms of damage like mutations, karyotypic alterations and loss of DNA methylation. These damages lead to genomic instability and to the altered expression of key growth regulatory molecules, which result in the loss of proliferative potential (Sherwood *et al.*, 1988).

Accumulation of cellular damage is also the mechanism proposed by the “oxidative stress” theory of aging. According to this hypothesis, respiration and other physiological processes within cells, generate a variety of products, which are toxic for cells themselves including some highly reactive oxygen species (ROS). Anti-oxidant cellular defences are not always sufficient to completely eliminate ROS from cells, and ROS can therefore oxidate both DNA and protein molecules, resulting in irreversible cellular damage that causes senescence.

The “mitochondrial theory” is based on the accumulation of cellular damage. It suggests that aging is caused by the accumulation of defective mitochondria with time; while the second proposes that the rate of aging is in

direct correlation with the metabolic rate. The “oxidative stress theory” may underlie this theory. Mitochondria are the sites where oxidative phosphorylation, a process that generates many ROS, takes place; therefore the accumulation of mitochondrial alterations may be the result of continuous exposure to oxygen free radicals.

Another damage theory that has been proposed to account for the gradual loss of proliferation capacity during senescence is the “telomere hypothesis”, which predicts that the telomeres progressively shorten throughout the cellular life span. Telomeres represent specialised nucleotide structures at the end of the linear chromosomes which stabilise the chromosomes, ensure a correct segregation of the sister chromatids at mitosis and protect the chromosomes from end-to-end fusions (Greider, 1994). In normal human fibroblasts in culture the telomere length decreases by about 50bp per population doubling, due to the “end-replication problem” (Goyns, 2002), whereas in immortalised cells the telomere length is stabilised due to the expression of telomerase (Kim *et al.*, 1994). Thus during the proliferative life span of normal cells telomeric sequences shorten progressively until a critical amount is missing, which cells recognise as a cellular damage. Since somatic cells do not express the catalytic subunit of the telomerase enzyme (hTERT), while they still express the telomerase mRNA component (mTR), they are not able to repair this damage and therefore exit the cell cycle, entering the senescent state. On the contrary, immortalised cells express telomerase and therefore retain unlimited cell division potential.

Nevertheless there are at least four main concerns regarding this theory. First, telomerase-negative immortal cells with much longer telomeres than immortalised cell lines with telomerase have been identified (Bryan *et al.*, 1995). Second, some senescent hybrids, despite expressing telomerase, still shorten their telomeres during their replicative life span (Broccoli *et al.*, 1995); moreover telomerase negative mice were shown to be viable for 5 generations of homozygosity and their fibroblasts undergo normal senescence. Finally species such as *Mus musculus* with a shorter life span than humans, have 5-20

times longer telomeres (Blasco *et al.*, 1997; Prowse and Greider, 1995). Therefore, telomerase is not required for the immortalisation and transformation of rodent cells where senescence is most likely to be regulated by a telomere-independent clock (Wright and Shay, 2000).

Thus telomere shortening might play an important role in regulating the replicative potential of some cells, but it is not the only mechanism underlying senescence and other mechanisms must exist.

The second group theories of senescence includes those that propose that cells age according to an intrinsic genetic program, and suggests, in contrast to the damage theories, that cells are active participants in their own aging (Berube *et al.*, 1998).

Pereira-Smith and Smith demonstrated in 1983 (Pereira-Smith and Smith, 1983) not only that replicative senescence is under genetic control, but also that it is a dominant phenotype. Somatic cell fusion experiments between normal diploid fibroblasts and various immortal cell lines yielded hybrids, which only proliferated for a limited period of time and then underwent senescence (Pereira-Smith and Smith, 1988). However treatment of the primary cells with inhibitors of protein synthesis prior to fusion prevented senescence in the resulting hybrids suggesting that senescence is mediated via one or more proteins. Moreover Lumpkin *et al.* (Lumpkin *et al.*, 1986) showed that microinjection of poly(A)<sup>+</sup> RNA prepared from senescent human diploid fibroblasts into proliferation competent cells inhibited DNA synthesis. This inhibition was blocked if the RNA was digested with ribonuclease prior to injection. In contrast poly(A)<sup>+</sup> RNA from young cells made quiescent by serum starvation had only a minor inhibitory effect. These results implied for the first time that the failure of senescent cells to proliferate was due to a genetic component, and not a deficiency in a positive growth signal or critical cellular components.

To try to establish how many different genes were directly involved in replicative senescence Smith and Pereira-Smith (Smith and Pereira-Smith, 1996) fused a series of different human immortal cell lines with each other.

They showed that the immortal cell lines became mortal when fused, and they grouped the 40 or more immortal human cell lines studied into 4 different complementation groups (Pereira-Smith and Smith, 1988; Smith and Pereira-Smith, 1996). When cells were fused within a complementation group the hybrids were immortal whereas they were mortal when fused across complementation groups. Moreover they suggested that some human chromosomes, such as 1, 4, 6, 7, 11, 18 and X, encoded senescent genes and in fact when these were introduced into immortal cell lines they were able to suppress the immortal phenotype (Sugawara *et al.*, 1990).

#### ***1.1.4 Characteristics of replicative senescence***

In addition to their irreversible growth arrest, senescent cells have various other characteristics. Major molecular differences have been shown between young and senescent cells cultured *in vitro*, both at the mRNA and at the protein level (Goldstein, 1990; Lumpkin *et al.*, 1986; Seshadri and Campisi, 1990; Smith and Pereira-Smith, 1996). These differences include components of the cell cycle and extra-cellular matrix, mitochondrial proteins, and also chromosomal DNA structure.

Several markers of mortality have been identified by comparative studies on mortal, immortal and hybrid cells. Over-expressed genes described during replicative senescence and aging, include prohibitin (Nuell *et al.*, 1991), interleukin-1 $\alpha$  (Maier *et al.*, 1990), vimentin (Satoh *et al.*, 1994), fibronectin (Kumazaki *et al.*, 1993), p21<sup>WAF1/Sdi1/CIP1</sup> (Noda *et al.*, 1994) and mortalin (mot-1) (Wadhwa *et al.*, 1993).

However even though many genes have been identified whose expression or regulation is altered during senescence it still remains to be demonstrated whether those changes are the result of the growth arrest or if they are the cause of it.

Although senescent cells cannot be induced to re-enter the cell cycle and undergo any more rounds of divisions by any combination of growth factors (Cristofalo *et al.*, 1989) they possess normal receptors for all the major growth factors. This implies that the mitogenic signal pathways are selectively blocked during senescence. For example it is known that senescent human fibroblasts do not phosphorylate ERK-2 (extracellular signal-regulated kinase-2) while they constitutively phosphorylate ERK-1 (Afshari *et al.*, 1993).

Despite their inability to stimulate a re-entry of senescent cells into the cell cycle serum growth factors can induce many early response genes, such as *ras* and *c-myc* but not *c-fos* (Seshadri and Campisi, 1990). Upon replicative senescence serum response factors (SRFs) are hyper-phosphorylated and therefore fail to bind serum-response elements (SREs), which results in *c-fos* transcriptional repression.

Senescent cells are also characterised by a change in cell size and shape. All senescent cells assume the characteristic “fried egg” morphology, which correlates with the over-expression of extracellular matrix proteins like fibronectin (Kumazaki, 1992; Kumazaki *et al.*, 1993), collagenase and stromelysin (Millis *et al.*, 1992).

Senescent cells also show mitochondrial changes like the accumulation of mitochondrial deletions (Cortopassi *et al.*, 1992), and the changes in expression of certain mitochondrial proteins.

For a discussion of the changes in the cell cycle machinery occurring upon replicative senescence see Cell cycle section (Paragraph 1.2.4).

### ***1.1.5 Stress induced premature senescence (SIPS)***

It has been shown that normal human diploid fibroblasts (HDFs) exposed to various types of noncytotoxic oxidative stress display a senescent phenotype called “stress induced premature senescence” or SIPS (Toussaint *et al.*, 2000). Such stressful conditions include exposure to hydrogen peroxide

(H<sub>2</sub>O<sub>2</sub>), *tert*-butylhydroperoxide (*t*-BHP), hyperoxia, UV light and radiation (Dumont *et al.*, 2000). Many of the biomarkers of cellular senescence appear in SIPS: enlarged and flat morphology (Toussaint *et al.*, 1992), irreversible growth arrest, lack of response to mitogenic stimuli (Chen and Ames, 1994), decrease of DNA synthesis and increase in cells positive for the senescent-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) (Dumont *et al.*, 2000). An up-regulation of the CKI p21<sup>WAF1/Sdi1/CIP1</sup> (Dumont *et al.*, 2000), together with a hypophosphorylation of pRB was also observed, explaining the block of the cell cycle.

The mechanisms responsible for induction of SIPS are still not known, however there are several observations, which suggest an involvement of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). Toussaint *et al.* showed that IMR-90 HDFs which develop SIPS phenotype after exposure to H<sub>2</sub>O<sub>2</sub> have higher steady-state levels of TGF- $\beta$ 1 mRNA and secrete increased levels of TGF- $\beta$ 1. In addition stimulation of IMR-90 HDF with TGF- $\beta$ 1 triggers the appearance of biomarkers of SIPS, while antibodies against TGF- $\beta$ 1 or TGF- $\beta$ 1 receptor II abrogate the over-expression of these gene observed after subcytotoxic H<sub>2</sub>O<sub>2</sub> stress (Fripiat *et al.*, 2001).

### **1.1.6 Immortalisation and cancer**

Further insights into the mechanisms of senescence may be found by the study of the opposite phenomenon, which is immortalisation. Cells that have overcome senescence and have acquired an infinite proliferative potential, are said to have become immortal. Immortal cells have a reduced requirement for mitogens, but still remain dependent on external stimuli. They cannot overgrow a confluent monolayer or cause tumours if introduced into nude mice, characteristics, which distinguish them from tumourigenic cells. Immortal cells

can either arise spontaneously, or be induced by radiation, chemical carcinogens, or the introduction of viral and cellular oncogenes.

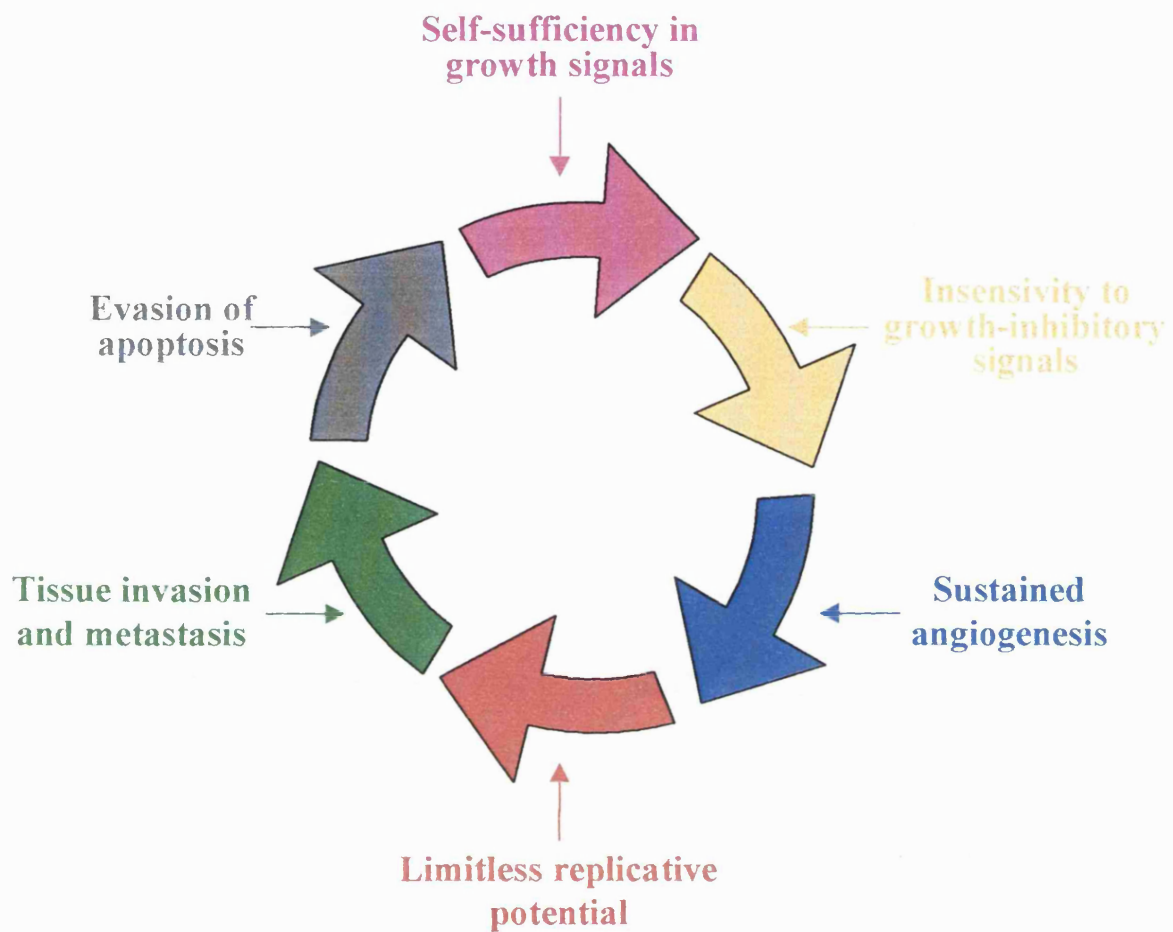
One of the differences in the behaviour of normal versus tumour cells in culture is that normal cells divide for a finite number of times whereas tumour cell lines are immortal. Cellular senescence is a major tumour suppressive mechanism.

Transformation of immortalised cells into malignant cells requires the introduction of a second oncogene or another spontaneous mutational event leading to activation of a transforming oncogene. The concept that tumourigenesis is a multistep process is well established. Weinberg and Hanahan (Hanahan and Weinberg, 2000) have recently suggested that to become tumourigenic normal cells have to undergo six essential alterations which are: evasion from programmed cell death (apoptosis); self-sufficiency in growth signals; insensitivity to growth-inhibitory signals; sustained angiogenesis; limitless proliferative potential, and tissue invasion and metastasis (Fig. 1.1).

#### ***1.1.7 Simian virus 40 (SV40)***

Simian virus 40 (SV40) is a member of the papovavirus family of small icosahedral DNA viruses, which contain a double-stranded DNA genome (Tooze, 1981).

SV40 is able to induce a lytic infection in its permissive host, which is the African green monkey. The lytic cycle of the virus results in the production of new viral particles and eventually cell lysis. During this kind of infection, the viruses adsorb to the cells and penetrate the nucleus where the viral genomes are uncoated and the early genes expressed. The lytic infection can be divided into two phases, early and late. During the early phase the viral genes from the early region are expressed. These genes block any cellular antiviral



**Fig. 1.1** Acquired capabilities of cancer.

Hanahan and Weinberg (Hanahan and Weinberg, 2000) have proposed that there are six alterations in cell physiology that generate cancers: evasion of apoptosis, self-sufficiency from growth signals, insensitivity to growth-inhibitory signals, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis.



system and recruit cellular proteins for viral replication and virus production. Therefore they are able to drive the infected cells into the cell cycle in order to ensure the availability of the cellular proteins required for viral replication, transcription and production. The SV40 early region encodes three proteins called large T (LT-ag), small t and tiny t antigens; they are produced from differentially spliced mRNAs of the same coding sequence. Following the replication of viral DNA the late region genes are expressed. The SV40 late region encodes for three proteins VP1, VP2 and VP3, which are generated from alternatively spliced mRNAs and alternative initiation codons, and which are the structural components of the virions.

When SV40 infects non-permissive cells (such as rat or mouse cells) the infection is abortive and it results in a lysogenic cycle. SV40 cannot replicate and produce new virions, but the viral DNA may integrate into the host genome and the early genes can be expressed.

#### 1.1.7.1 SV40 large T antigen (LT-ag)

LT-ag is a 708 amino acid protein. It mostly localises to the nucleus via a nuclear localization signal (NLS) in the N-terminus region (Soule and Butel, 1979), although a very small fraction is bound to the plasma membrane.

LT-ag has various biochemical functions (Fanning, 1992) including ATPase activity (Tjian and Robbins, 1979), DNA and RNA helicase activity (Scheffner *et al.*, 1989) and topoisomerase activity (Marton *et al.*, 1993). It was shown to be able to bind RNA covalently (Carroll *et al.*, 1988), and also DNA both specifically and non-specifically (Carroll *et al.*, 1974). LT-ag also has the ability to activate and repress transcription from various viral and cellular promoters.

LT-ag can associate with a number of host cellular proteins (Lane and Crawford, 1979), which include p53 (Lane *et al.*, 1985), pRB (DeCaprio *et al.*, 1988), p107 (Dyson *et al.*, 1989), p130 (Hannon *et al.*, 1993), DNA polymerase

$\alpha$  (Smale and Tjian, 1986), heat shock protein hsc70 (Sawai and Butel, 1989), p185 (Kohrman and Imperiale, 1992), CREB-binding protein (CBP), p300 (Avantaggiati *et al.*, 1996), p400 (Lill *et al.*, 1997) and SUG1 regulatory component of the proteasome (Grand *et al.*, 1999).

Introduction of LT-ag into primary rodent cells enables those cells to overcome senescence and to acquire an infinite proliferative potential (Jat and Sharp, 1989) but does not necessarily results in their transformation. Only a small proportion of the immortalised cells progress to the transformed state. Inactivation of LT-ag in these immortal cells results in a rapid and irreversible loss of the proliferative potential in either G<sub>1</sub> or G<sub>2</sub> phases of the cell cycle showing that LT-ag protein is continuously required to maintain the proliferative state (Gonos *et al.*, 1996; Jat and Sharp, 1989). However introduction of LT-ag into immortalised cell lines can result in a fully transformed phenotype (Jat *et al.*, 1986; Jat and Sharp, 1986).

### ***1.1.8 Rodent versus human immortalisation***

Continuous passage of senescent cultures under appropriate conditions often leads to the outgrowth of variant population of cells with renewed and infinite proliferative potential (Curatolo *et al.*, 1984). The exact nature of the lesions enabling these cells to escape senescence is not yet defined.

Moreover a sub-group of viral and cellular oncogenes have shown to be able to overcome senescence in primary rodent fibroblasts. Such immortalised cells exhibit an infinite life span and have acquired one property of tumour cells, in that the subsequent introduction of an activated oncogene, such as Ha-ras, results in fully transformed cells. The viral oncogenes that are capable of immortalising rodent cells include the early region 1A (E1A) gene of adenovirus (Ad) (Ruley, 1983), the E7 gene of human papillomavirus type 16 (HPV16) (Kanda *et al.*, 1988) the large tumour antigen (LT-ag) of Simian virus

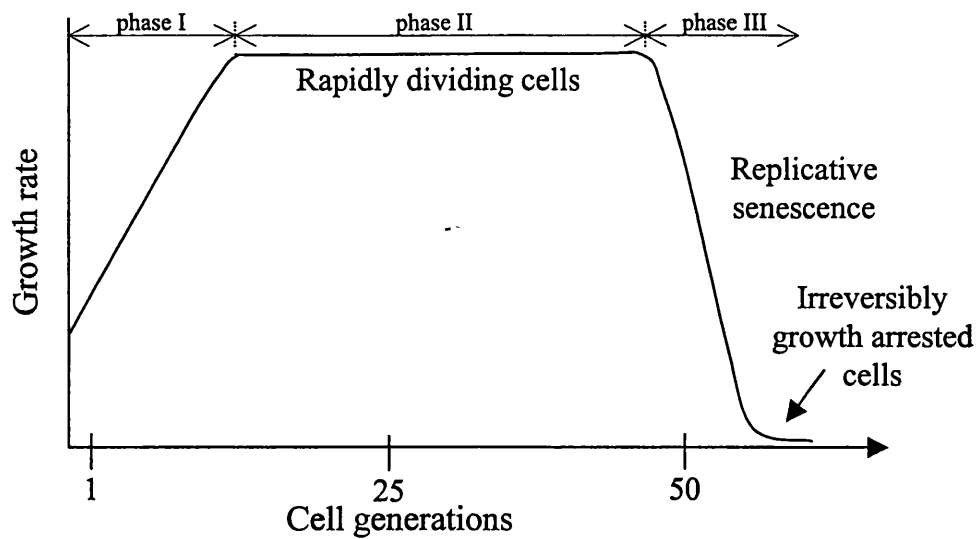
40 (SV40) and the large tumour antigen of polyoma virus (Py) (Jat and Sharp, 1986).

In contrast to rodent fibroblasts where spontaneous immortalisation is a rare but measurable event, spontaneous immortalisation of human fibroblasts is extremely rare, and immortal cells, even after the introduction of oncogenes, are obtained at a very low frequency (Fig. 1.2).

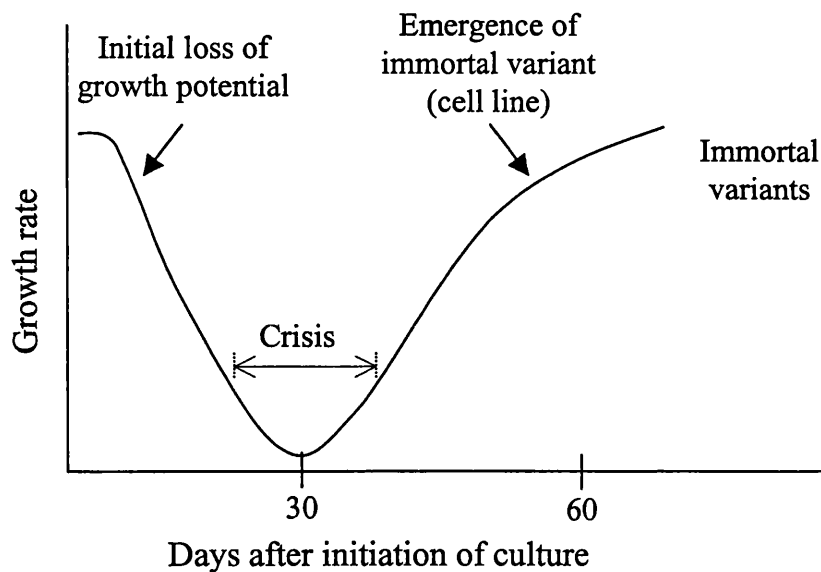
The low frequency of immortalisation suggests that multiple genetic alterations involving both activation of dominant oncogenes and repression of growth suppressor genes are required to overcome senescence in the human system. The difference in the immortalisation rate between the two species can be explained with the two-stage model of senescence for human cells (Wright *et al.*, 1989). According to this model, when normal human fibroblasts are grown in culture, they undergo a limited number of divisions before entering cellular senescence, called Mortality stage 1 (M1). Cells in M1 however can be pushed to re-enter the cell cycle and divide by the introduction of viral oncogenes, such as SV40 LT-ag, which is capable of stimulating cellular DNA synthesis. However their life span is extended only by a few PD, after which almost all cells undergo a second crisis, which is called Mortality stage 2 (M2) and cease dividing, (Fig. 1.3). Therefore, immortalisation of human cells requires the bypass of two mortality stages and spontaneous immortalisation becomes an extremely rare event. Very rarely a clone of immortal cells may arise spontaneously from a long-term culture of normal human cells (Baden *et al.*, 1987; Briand *et al.*, 1987) indicating an establishment frequency less than 1 per  $10^9$  cells (Holliday, 1987).

Establishment of primary mouse cells, in contrast to human cells, is a frequent and reproducible phenomenon, with approximately one immortalisation event per  $10^4$  to  $10^6$  cells (Curatolo *et al.*, 1984), suggesting that they either lack M2 or they have a greater capacity to overcome it. Rat and hamster cells spontaneously immortalise somewhat less readily, while human cells almost never immortalise *in vitro* (Macieira-Coelho and Azzarone, 1988).

### A) Human cells

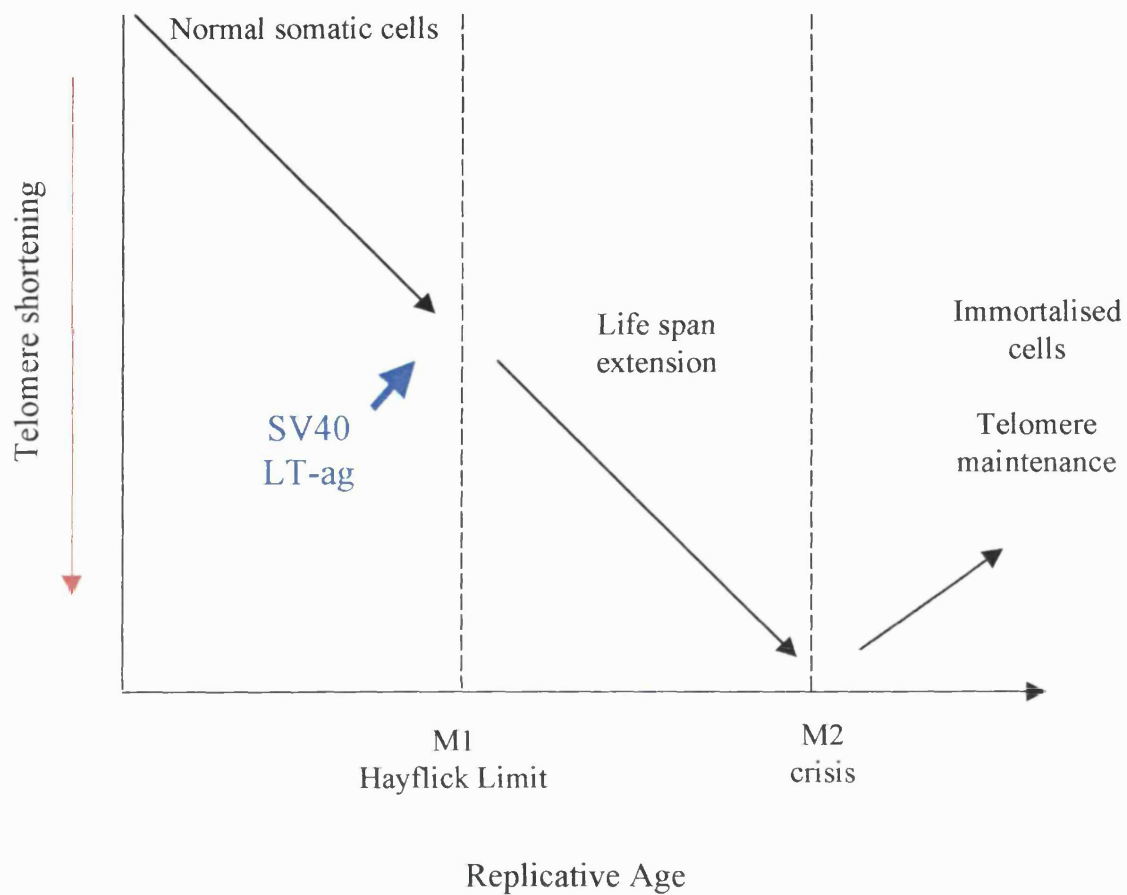


### B) Mouse cells



**Fig. 1.2** Life span (human versus mouse).

A) Human cells. When an explant is made some cells die and others start to grow; overall the growth rate increases (phase I). If the remaining cells are diluted the cell strain grows at a constant rate for 50-60 cell generations (phase II), after which growth begins to slow (phase III), until ultimately the cells become growth arrested. B) Mouse cells. In a culture prepared from rodent embryo cells there is an initial cell death followed by the emergence of healthy growing cells. When these cells are diluted and allowed to grow they soon lose their growth potential and most cells die (crisis). However some cells do not die and continue growing (they become a cell line).



**Fig. 1.3** Mortality stages 1 and 2.

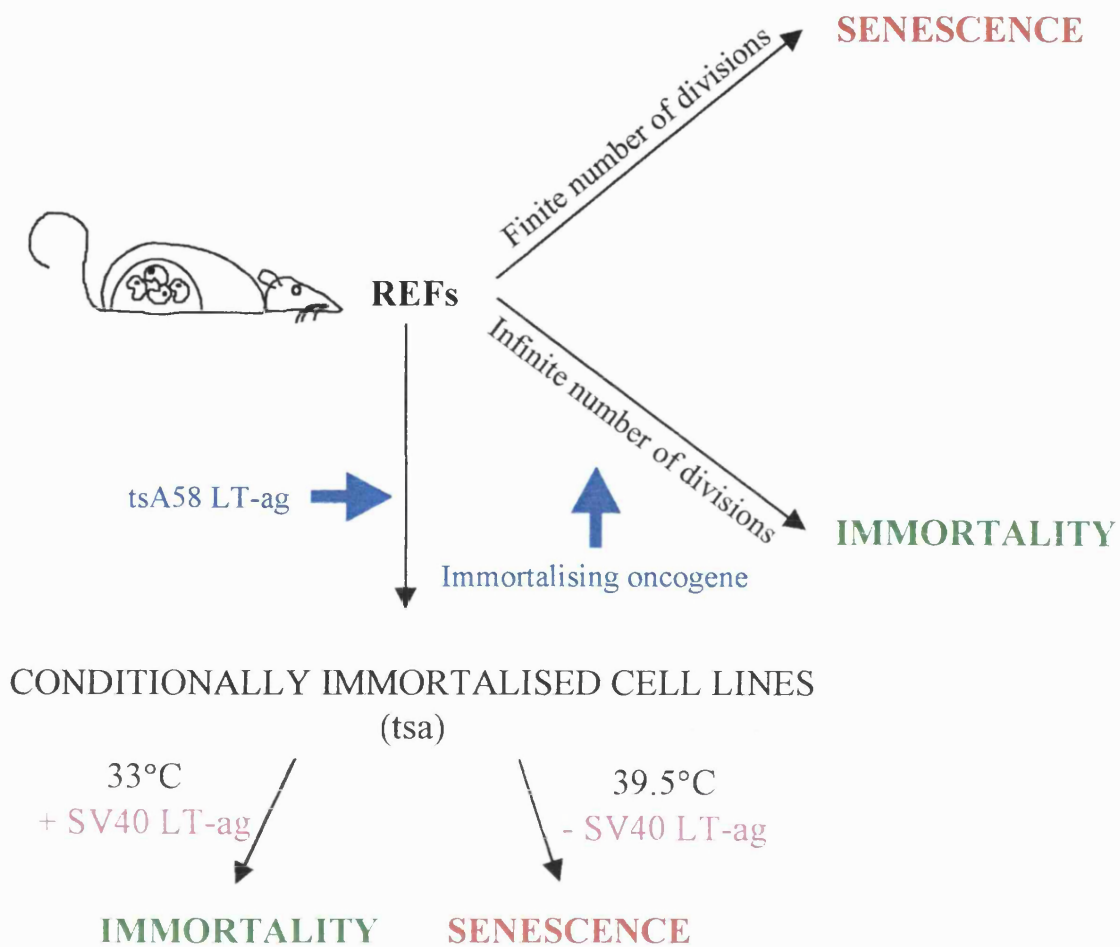
Normal human fibroblasts in culture undergo a finite number of divisions before entering cellular senescence, also called mortality stage 1 (M1) or Hayflick limit. Cells can be pushed to bypass M1 with the introduction of viral oncogenes (such as SV40 LT-ag), however their lifespan is extended only by a few population doublings, after which almost all cells undergo crisis and cease dividing, mortality stage 2 (M2). Cells that bypass M2 are immortal.

Rodent and human cells differ also in the biological counting mechanism. The progressive shortening of telomeres throughout the cellular life span has been proposed to be the mitotic clock in human cells (Harley *et al.*, 1990) while, as previously discussed in Paragraph 1.1.3, in rodent cells senescence is most likely to be regulated by a telomere-independent mechanism (Wright and Shay, 2000).

### **1.1.9 Model systems**

#### **1.1.9.1 Primary rat embryo fibroblasts (REFs)**

Replicative senescence is an asynchronous process whereby a growing culture gives rise to an irreversibly arrested culture. The model systems that are commonly used to study replicative senescence involve the isolation and serial *in vitro* cultivation of primary fibroblasts (Fig. 1.4 and 1.5). Initially these cells proliferate exponentially, but cease dividing after some passages, at which point the cell number no longer increases. The loss of proliferative potential in such heterogeneous cultures of primary cells is asynchronous. When cells have reached the end of their *in vitro* mitotic life span, they can be maintained, remaining metabolically active, but cannot be induced to undergo new rounds of cell division (Cristofalo *et al.*, 1989). The loss of proliferative potential in such heterogeneous primary cultures occurs after differing numbers of passages depending upon the cell type and the donor species (Cristofalo and Pignolo, 1993; Stanulis-Praeger, 1987). In such model systems, the culture as a whole divides initially and undergoes growth arrest towards the end; however there can be growth arrested cells in the early passages and dividing cells towards the later passages.



**Fig. 1.4** The model systems used to study replicative senescence.

One model uses primary rat embryo fibroblast (REFs) which undergo senescence after a finite number of divisions. The second model uses conditionally immortalised cell lines which are immortal at the permissive temperature of 33°C, where SV40 LT-ag is active, but rapidly undergo senescence upon switch to the non-permissive temperature (39.5°C) at which LT-ag is inactivated.

#### 1.1.9.2 Conditionally immortalised cell lines (tsa)

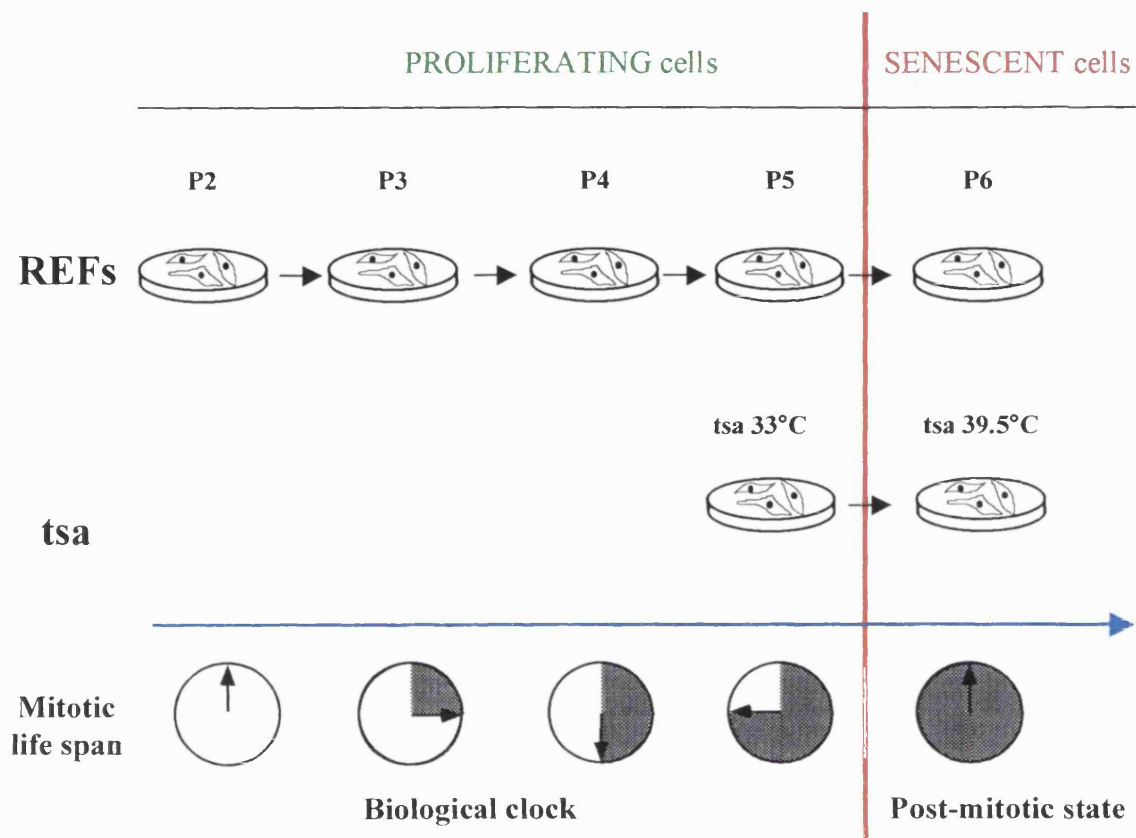
As previously described serial cultivation of primary cells is the most commonly used model to study replicative senescence. However the discovery that certain viral oncogenes have the capacity to confer indefinite growth in various cell types has been utilised to develop a system in which senescence can be induced synchronously by altering the growth temperature (Fig. 1.4 and 1.5).

As previously described, the introduction of SV40 LT-ag alone into rodent fibroblasts gives rise to cells that can proliferate indefinitely but are dependent upon it for maintenance of their growth once the normal mitotic life span has elapsed (Conzen and Cole, 1995; Darmon and Jat, 2000; Jat and Sharp, 1989). A thermolabile SV40 LT-ag, tsA58 T antigen, was used to isolate conditionally immortal cell lines derived from primary REFs. The conditionally immortalised cell lines (tsa) grow continuously at the permissive temperature, 33°C, where the LT-ag is active, but rapidly undergo a synchronous growth arrest upon shift up to the non-permissive temperature, 39.5°C, due to inactivation and probably degradation of the tsA58 T antigen (Gonos *et al.*, 1996).

Analysis of these conditionally immortalised tsa cell lines showed that upon shift up to the non-permissive temperature they show a loss of proliferative potential that has characteristics very similar to replicative senescence of normal REFs upon serial cultivation (Gonos *et al.*, 1996). However, in contrast to replicative senescence upon serial passaging of primary REFs, senescence in these tsa cell lines occurs synchronously within 72 hours (one or two generations) (Gonos *et al.*, 1996) (Fig. 1.5).

It has been shown that cells become dependent upon LT-ag for maintaining growth only when their normal mitotic life span has elapsed and that the biological clock that limits the mitotic potential continues to function normally, even in the presence of the immortalising gene (Ikram *et al.*, 1994).





**Fig. 1.5** Primary cells versus conditionally immortalised cell lines.

The primary cells are a suitable model system to investigate both mechanisms that have been suggested to regulate replicative senescence: the biological clock and the entry into the post-mitotic state. The conditionally immortalised cell lines can be only used to study the entry into the post-mitotic state.

To determine when cells became dependent upon the SV40 LT-ag to continue dividing, the H-2K<sup>b</sup>tsA58 strain of transgenic mice was developed (Jat *et al.*, 1991). These mice harbour the tsA58 T antigen under the control of the  $\gamma$ -interferon inducible H-2K<sup>b</sup> class 1 promoter, and allow the preparation of cell populations in which the expression of LT-ag can be induced or suppressed in every cell by manipulating the growth conditions (Jat *et al.*, 1991). Analysis of embryonic fibroblasts prepared from these mice has demonstrated that cells become dependent upon LT-ag for maintaining growth only when their normal mitotic life span has elapsed and that the biological clock that limits the mitotic potential continues to function normally, even in the presence of the immortalising gene (Ikram *et al.*, 1994). Thus cell division in rodent cells is not regulated by random accumulation of cellular damage and neither is it simply a culture shock but involves a genetic program comprising two components: a counting mechanism that measures the finite mitotic life span, and a process of entry into the post-mitotic state.

As previously discussed the counting mechanism in mouse cells is not dependent upon shortening of telomeres (Wright and Shay, 2000), as has been proposed for human cells (Blasco *et al.*, 1997; Serrano and Blasco, 2001), but is temperature sensitive (Ikram *et al.*, 1994). SV40 LT-ag blocks the entry into the post-mitotic state and it is for this reason that the cells undergo synchronous senescence upon its inactivation. Therefore the tsa cell lines should represent an excellent model system for identifying the changes that are critical for the entry into senescence.

More recently a conditionally immortalised system derived from human cells has been developed (O'Hare *et al.*, 2001). O'Hare *et al.* showed that the catalytic subunit of human telomerase (hTERT) together with a temperature-sensitive mutant (U19tsA58) of SV40 LT-ag resulted in the efficient generation of immortal cell lines when ectopically expressed in freshly isolated normal adult human mammary fibroblasts and endothelial cells. However only a combination of both genes, irrespective of the order in which they were

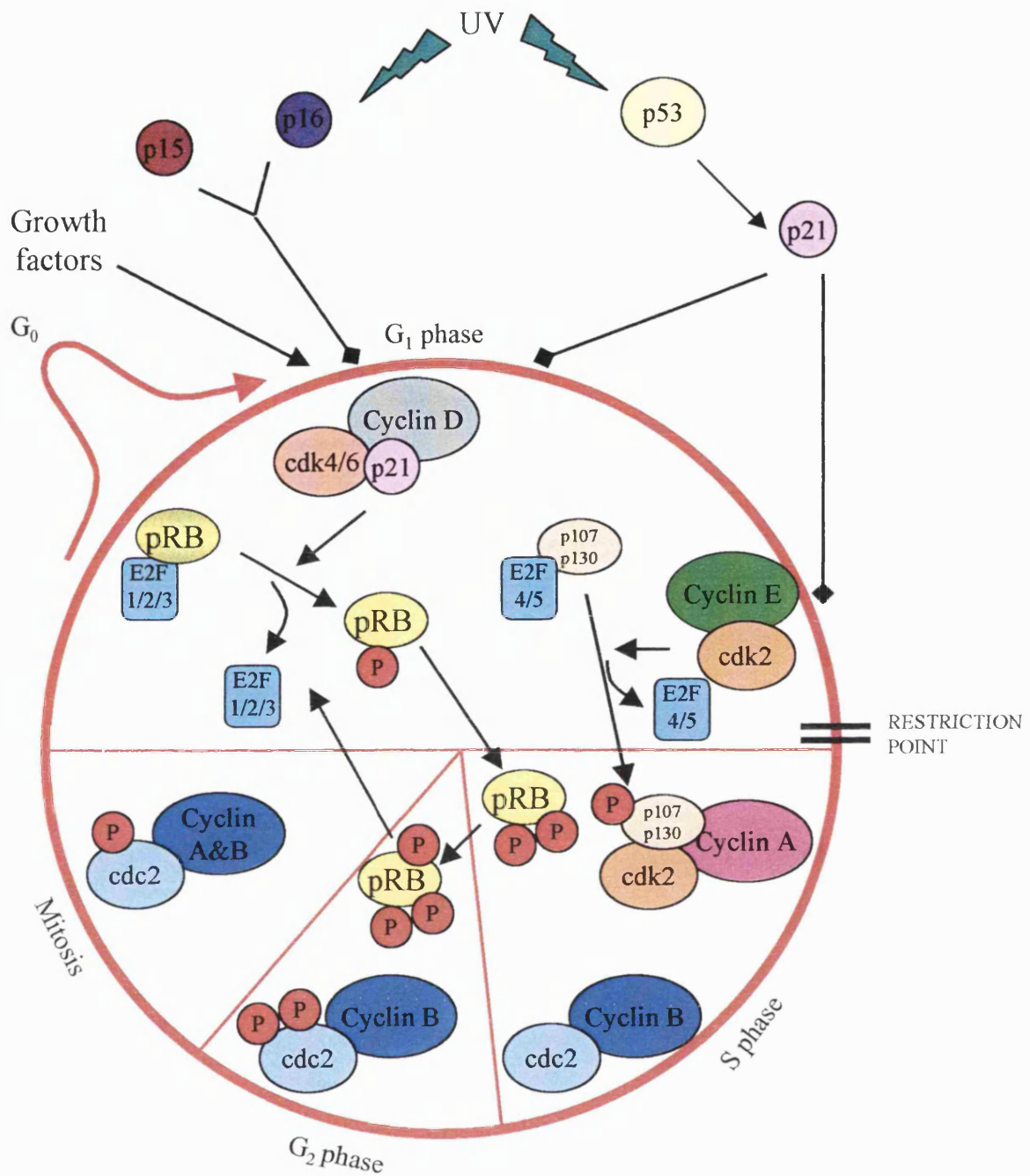
introduced or whether they were introduced early or late in the normal proliferative life span of the cultures, was required for immortalisation. Temperature-shift experiments revealed that maintenance of the immortalised state depended on continued expression of functional U19tsA58 LT-ag, with hTERT alone unable to maintain growth at non-permissive temperatures for U19tsA58 LT-ag.

## 1.2 Cell cycle

The ability of a multicellular organism to grow and divide is a critical concept in biology. During their lifetime cells have different options, they can proliferate and increase their number by duplicating their genetic content and segregating their chromosomes into their daughter cells. During their life span cells can arrest reversibly or irreversibly their growth and stop dividing, while still remaining viable and metabolically active. As previously discussed a reversible growth arrest can be achieved by depleting cells of mitogens or growing them to confluence. Cells exit the cell cycle with unduplicated DNA content and persist in a quiescent state ( $G_0$ ) in which their basic cellular functions are reduced. With the addition of mitogens however the  $G_0$  block is relieved and cells re-enter the cell cycle and undergo new rounds of divisions. In contrast cells in a state of irreversible growth arrest, such as senescence or terminal differentiation, cannot re-enter the cell cycle. Another option cells have is to die by programmed cell death, also called apoptosis.

The eukaryotic cell cycle can be divided into four different phases: the period associated with DNA synthesis is called S phase (S) which is separated from the mitotic phase (M), where the duplicated genome gets divided, by a gap of varying length designated Gap2 ( $G_2$ ). The M phase and the next S phase are also separated by an interval called Gap1 ( $G_1$ ).

Cells in  $G_1$  have to decide whether to divide and therefore continue



**Fig. 1.6** Cell cycle regulation.

A simplified summary of eukaryotic cell cycle regulation is shown and the major players represented. The progression through the cell cycle is regulated by sequential assembly and activation of cyclin-dependent kinases (cdk) and cyclin complexes.

through the different phases of the cell cycle to M, or to become quiescent and enter  $G_0$ . This decision is made by cells at the “restriction point” (R) (two thirds of the way through  $G_1$ ) (Pardee, 1989) by assessing the availability of extracellular mitogenic signals and the generic state of the cell such as DNA damage which would signal the cell to arrest and even enter an apoptotic program if the damage is too vast. After the R point cells commit to traverse the rest of the cell cycle and no longer depend on mitogens.

The order and the timing of the cell cycle phases is critical for the accurate transmission of genetic information, therefore a number of biochemical pathways have evolved to ensure that the initiation of a particular phase occurs only after the successful completion and assessment of the preceding one (Fig. .1.6).

### ***1.2.1 Cyclins, cyclin-dependent kinases (cdks) and cyclin-dependent kinase inhibitors (CKIs)***

The progression through the cell cycle is regulated by a complex network of regulatory proteins, the more important of which are the cyclins and cyclin-dependent kinases (cdks) complexes. Within each complex the cyclins are the regulatory subunits while the cdks are the catalytic subunits, which catalyse the phosphorylation of specific substrates important for cell cycle progression. The levels of the cyclin components oscillate through the cell cycle and are both regulated by modulation of transcription and degradation rates. Cdk expression levels are generally constant throughout the cell cycle and their activity is regulated by binding with a cyclin subunit, association with specific cdk inhibitors (CKIs) (Morgan, 1997) and by phosphorylation and de-phosphorylation of specific residues.

Different cyclin-cdk complexes are required in the different phases of the cell cycle. As an early response to mitogens, cells in  $G_0$  enter  $G_1$  phase and express genes encoding the D-type cyclins (cyclins D1, D2 and D3), which are

expressed as long as the growth factor stimulation persists. During early G<sub>1</sub> D-type cyclins associate with their catalytic partners, cdk4 and cdk6. Assembled cyclin D-cdk4/6 complexes then enter the cell nucleus where they are phosphorylated by a cdk-activating kinase (CAK) (Kaldis, 1999). Cyclin D-dependent kinases become activated and initiate phosphorylation and inactivation of pRB (Kato *et al.*, 1993) (see Paragraph 1.2.2). It has been shown that the activity of the cyclin D-cdk4/6 complexes is critical in middle and late G<sub>1</sub>, since injection of anti cyclin D1 antibodies in normal fibroblasts during G<sub>1</sub> prevents cells from entering the S phase (Baldin *et al.*, 1993).

Cyclin E assembles with cdk2 and is required for the G<sub>1</sub>/S transition (Dulic *et al.*, 1993). Cyclin E-cdk2 complex complete pRB phosphorylation, initiated in mid G<sub>1</sub> by cyclin-D dependent kinase, on additional sites (Mittnacht *et al.*, 1994). Cyclin E gets degraded in S phase and the free cdk2 can associate with cyclin A. Cyclin A is mainly active during the S phase and is also required for the G<sub>2</sub>/M transition. The entry into mitosis (G<sub>2</sub>/M transition) is triggered by the Maturation Promoting Factor (MPF), which is composed of cyclin B and cdc2. The cdc2 kinase is kept in an inactive state during S and G<sub>2</sub> phases by the phosphorylation on two residues: Tyr15 and Thr14 (Hoffmann *et al.*, 1993). At the end of G<sub>2</sub> the phosphatase cdc25 dephosphorylates these two residues and the dephosphorylation results in cdc2 activation. Ultimately the degradation of cyclin B is necessary to exit M phase and start a new cell cycle.

As mentioned before, in response to a variety of extracellular and intracellular signals, cells decide whether to exit the cell cycle and maintain a non-proliferative state or to start a new round of division. Most of these signals are integrated through the activity of the cdks and the different checkpoint genes.

The kinase activity of cyclin-cdks is inhibited by two families of CKIs. The Cip/Kip family consists of p21<sup>WAF1/Sdi1/CIP1</sup>, p27<sup>KIP1</sup> and p57<sup>KIP2</sup>; and the Ink4a family consists of p15<sup>INK4b</sup>, p16<sup>INK4a</sup>, p18<sup>INK4c</sup> and p19<sup>ARF</sup>. In general these proteins inhibit cdk-dependent phosphorylation and arrest proliferation when highly expressed. The Cip/Kip proteins are potent inhibitors of cyclin E

and A dependent cdk2, which is involved in the G<sub>1</sub> and G<sub>1</sub>/S control, although, when present at very low levels, they can act as positive regulators of cyclin D dependent kinases (Sherr and Roberts, 1999). p21<sup>WAF1/Sdi1/CIP1</sup>, a p53-inducible transcript (el-Deiry *et al.*, 1992; Harper *et al.*, 1993), together with p27<sup>KIP1</sup> can contact both cyclin D and cdk4/6 to stabilise them in an active ternary complex (Sherr and Roberts, 1999).

The Ink4 family specifically inhibits cdk4/6-cyclin D complexes (Morgan, 1997). The Ink4a locus encodes from alternative reading frames two different growth inhibitors: p16<sup>INK4a</sup>, which is a component of the pRB pathway, and p19<sup>ARF</sup>, which has been functionally linked with p53 (Kamijo *et al.*, 1997). p16<sup>INK4a</sup> inhibits cyclin D-cdk4/6 complexes leading to a hypophosphorylation of pRB and therefore cell cycle arrest. It has been shown by Serrano *et al.* (Serrano *et al.*, 1995) that enforced expression of p16<sup>INK4a</sup> induces G<sub>1</sub> arrest. p19<sup>ARF</sup> is the alternatively spliced product of the p16<sup>INK4a</sup> genomic sequence. p19<sup>ARF</sup> is not a cdk inhibitor (Quelle *et al.*, 1995) but can induce a cell cycle arrest in a p53-dependent manner (Kamijo *et al.*, 1997). p19<sup>ARF</sup> interacts directly with the p53 inhibitor mdm2 and promotes degradation of mdm2 and therefore stabilisation of p53 resulting in cell cycle arrest (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998) (see Paragraph 1.2.3).

### 1.2.2 pRB

Transition through R coincides with the phosphorylation of the retinoblastoma susceptibility protein pRB (Weinberg, 1995). pRB undergoes cell cycle-dependent phosphorylation, which depends on the activity of several cdks (Kato *et al.*, 1993) and occurs in a stepwise manner. pRB is phosphorylated in at least three steps when cells are driven into the cell cycle. The first event occurs during middle to late G<sub>1</sub> phase by cyclin-D dependent kinases (Hinds *et al.*, 1992), the second at the G<sub>1</sub>/S boundary by the cyclin-A and E dependent kinases (Hinds *et al.*, 1992) and the third in G<sub>2</sub>/M. It has been

shown that the different phosphorylation events occur (DeCaprio *et al.*, 1992), at least in part, on different residues in pRB (Mittnacht, 1998; Zarkowska and Mittnacht, 1997), while in quiescent cells and early G<sub>1</sub> pRB is present in cells in a hypophosphorylated state.

When hypophosphorylated, pRB associates and sequesters the E2F family of transcription factors (Chellappan *et al.*, 1991; Hiebert *et al.*, 1992). Upon transition through R and switch to the hyperphosphorylated state, pRB releases E2F, which becomes free and can transactivate genes containing an E2F site in their regulatory region (Sladek, 1997; Suzuki-Takahashi *et al.*, 1995). E2F targets include genes important for DNA synthesis such as: dihydrofolate reductase, thymidine kinase, DNA polymerase  $\alpha$ , proliferating cell nuclear antigen (PCNA), as well as cell cycle genes like cyclin A, cyclin E and cdc2 (Hinds *et al.*, 1992; Sladek, 1997).

The importance of the pRB/E2F interaction in regulating the progression through the cell cycle was demonstrated by over-expression of E2F1 in quiescent cells, which resulted in a progression through G<sub>1</sub> and S phases (Singh *et al.*, 1995).

Two other pRB-related proteins are p107 and p130 and together with pRB are members of the “pocket” family of proteins (Ewen *et al.*, 1991). p107 and p130 are also putative substrates of cdk-cyclin complexes and have similar biological properties to pRB, suggesting a role in the cycle control. p107 (Ewen *et al.*, 1991) and p130 (Li *et al.*, 1993) associate with E2F via their pocket domain, highly conserved within the family, but have been found to regulate the transcription of different E2F target genes than pRB (Hurford *et al.*, 1997).

The E2F transcription factors are heterodimers of E2F and DP proteins. There are five E2F and three DP family members (La Thangue, 1994; Martin *et al.*, 1995). Hypophosphorylated pRB associates with E2F-1, E2F-2 and E2F-3, p107 associates with E2F-4 (Ginsberg *et al.*, 1994) and p130 with E2F-4 and E2F-5 (Hijmans *et al.*, 1995). Each pRB protein may control a different cell cycle step: p130 is the predominant E2F associated protein in G<sub>0</sub> and early G<sub>1</sub>



phases; pRB associates with E2F in early to middle G<sub>1</sub> while p107 is the predominant E2F associated protein at the G<sub>1</sub>/S boundary. Thus, it seems that pRB proteins associate with various E2F proteins to regulate different sets of genes (Hurford *et al.*, 1997); (Cloud *et al.*, 2002).

### 1.2.3 p53

At the G<sub>1</sub>/S boundary in the progression of the cell cycle, R checkpoint, prior to duplicating their DNA, cells check that everything is in order; p53 is the main protein responsible for the G<sub>1</sub>/S checkpoint (Levine, 1997). Damage in the DNA results in the stabilisation of the normally short-lived p53, triggering different outcomes in cells depending on the extent and the type of the damage (Kastan *et al.*, 1991). A small amount of damage results in p53-induced cell cycle arrest, which may occur in any phase of the cell cycle and which allows cells to repair the damaged DNA before proceeding through S phase, and therefore ensuring that no mutations are passed to the daughter cells. However if the damage is extensive and cannot be repaired, p53 induces cell cycle exit and apoptosis (Chen *et al.*, 1996).

Damage of genomic DNA and hypoxia are distinct signals that converge on p53 and result in cell cycle arrest, until the defects are fixed, or alternatively in apoptosis. For this reason p53 has been called “the guardian of the genome” (Lane, 1992). Oncogenic activation and infection by certain viruses however can lead to p53 inactivation and bypass of this control checkpoint.

The effects of p53 are mediated through transcriptional activation of several genes containing p53-responsive elements. The list of p53-inducible genes includes: cdk inhibitor p21<sup>WAF1/Sdi1/CIP1</sup> (el-Deiry *et al.*, 1994), nucleotide excision repair proteins ERCC3 (Schaeffer *et al.*, 1994) and GADD45 (Kastan *et al.*, 1992), p53 inhibitor mdm2 (Barak *et al.*, 1993), apoptotic proteins Bax (Miyashita and Reed, 1995) and ASPP2 (Samuels-Lev *et al.*, 2001), cyclin G, a novel cyclin which plays a role in G<sub>2</sub>/M arrest, (Zauberman *et al.*, 1995);

(Okamoto *et al.*, 2002) and the insulin-like growth factor binding protein 3 (IGF-BP3) which blocks the signalling of a mitogenic growth factor (Levine, 1997).

Some of these genes are involved in the cell cycle checkpoint pathway, while others are not. ERCC3 and GADD45 are both involved in DNA repair. ERCC3 is associated with the nucleotide excision repair mechanism (Schaeffer *et al.*, 1994), while GADD45 was found to bind the proliferation cell nuclear antigen (PCNA), a component of the DNA replication machinery required for both DNA replication and repair, and thereby it stimulates DNA repair and inhibits the entry into S phase (Kastan *et al.*, 1992).

mdm2 (murine double minute) is a unique p53-regulated gene. It is an important regulator of p53 function and stability; it inactivates p53-mediated transcription and generates an auto-regulatory loop with p53 activity (Levine, 1997). mdm2 is an E3 ubiquitin ligase for p53. Ubiquitin (Ub) conjugation is a general targeting modification and poly-ubiquitin chains specifically target proteins to the proteasome for degradation (Lai *et al.*, 2001). mdm2 can bind the N terminus of p53 and promote its ubiquitination and proteasomal degradation (Inoue *et al.*, 2001), both in the nucleus and in the cytoplasm, provided both proteins are co localised (Xirodimas *et al.*, 2001). While binding to the N-terminal of p53 mdm2 also masks the transcription activation domain and therefore inhibits p53 transcriptional and tumour suppressor functions (Henning *et al.*, 1997).

p53 induces G<sub>1</sub> arrest through transactivation of p21<sup>WAF1/Sdi1/CIP1</sup>. p21<sup>WAF1/Sdi1/CIP1</sup> inhibits cyclin D-cdk4/6 complexes and therefore results in a hypophosphorylated pRB, which sequesters E2F and blocks the E2F-responsive genes required for progression into S phase. Moreover p21<sup>WAF1/Sdi1/CIP1</sup> can induce a block in any phase of the cell cycle inhibiting all the different cdk kinases. In addition, p21<sup>WAF1/Sdi1/CIP1</sup> binds and inhibits PCNA; when PCNA is associated with p21<sup>WAF1/Sdi1/CIP1</sup> the PCNA-dependent DNA replication is inhibited but DNA repair is promoted.

p53 induces the apoptotic pathway mainly through the transactivation of Bax (Miyashita and Reed, 1995). Bax promotes apoptosis by heterodimerising with Bcl-2. This complex is responsible for the release of cytochrome c from the mitochondria, which results in activation of the caspases, a family of cysteine proteases that are mediators of apoptosis.

The last checkpoint of the cell cycle, after the ones that control the progression through S and G<sub>2</sub> phases, occurs during the M phase, where cells have to ensure that all DNA had been replicated and that no abnormalities occurred during the process, and they are called mitotic checkpoints. They are: the DNA damage checkpoint and the spindle checkpoint.

The first checkpoint prevents mitosis in the event of DNA damage, which can result from mistakes introduced by the polymerases during the replication process, by incomplete DNA replication, by ionising radiation, by free radicals, or by incomplete DNA repair. Key proteins of the DNA damage checkpoint include the ataxia telangiectasia mutated protein (ATM) which is involved in the recognition and repair of damaged DNA, as well as the Chk1 kinase, which in the presence of DNA damage inhibits the activation of the cyclin B-cdc2 complex and therefore blocks the entry into mitosis.

The spindle checkpoint monitors the proper assembly of the mitotic spindle and it is essential for ensuring the accurate segregation of chromosomes, thus preventing any gain or loss of genetic material (aneuploidy). When a spindle defect, like failure of chromosomes to attach to the spindle is detected, the checkpoint mechanism generates a signal that blocks cell cycle progression until the defect is repaired (Amon, 1999).

#### ***1.2.4 Senescence and cell cycle***

The inability of senescent cell to progress through the cell cycle is indicated in their failure to express several cyclins and cdks.

Senescent human fibroblasts under express cdk2 mRNA (Afshari *et al.*, 1993), as well as cyclins A and B (Stein *et al.*, 1991) and cyclins E and D (Dulic *et al.*, 1993).

The protein expression of the cdk inhibitor p21<sup>WAF1/Sdi1/CIP1</sup> increases significantly upon senescence. During a screen of a senescence cell-derived cDNA library Noda *et al.* found that p21<sup>WAF1/Sdi1/CIP1</sup> mRNA was 10-fold up-regulated in non-proliferating cells (Noda *et al.*, 1994). Also the CKI p16<sup>INK4a</sup> mRNA and cellular protein levels gradually rise, with the protein levels in senescent HDFs nearly 40-fold higher than early passages, and complexed with both cdk4 and cdk6. It has also been shown that over-expression of both these CKIs in normal cells in their early passages results in a premature senescence phenotype (McConnell *et al.*, 1999; Serrano *et al.*, 1996).

As mentioned above cyclin D-cdk4/6 activity is modulated by p21<sup>WAF1/Sdi1/CIP1</sup> and p16<sup>INK4a</sup>. Upon senescence both p21<sup>WAF1/Sdi1/CIP1</sup> and p16<sup>INK4a</sup> are up-regulated and cyclin D-cdk4/6 activity is impaired. This results in a constitutively hypophosphorylated pRB in senescent cells (Stein *et al.*, 1990). Hypophosphorylated pRB has been observed not only in growth arrested cells (Stein *et al.*, 1990) but also cells which are terminally differentiated (Thomas *et al.*, 1991). As previously described hypophosphorylated pRB inactivates the E2F transcription factor family, which can no longer transactivate the genes necessary to proceed through the S phase of the cell cycle.

Moreover, since p21<sup>WAF1/Sdi1/CIP1</sup> was first described as a p53 inducible gene, it was expected that p53 would be up-regulated both at the level of mRNA and protein as well as activity. However p53 mRNA and protein are not always found to increase even though the activity is higher suggesting that another regulatory event may exist (Kulju and Lehman, 1995). Interestingly it has recently been found that p53 activity could be modulated by the p63 family of proteins, and it has been found that p63, a recently discovered member of the same family, accumulate upon replicative senescence (Djelloul *et al.*, 2002).

In senescent cells p53 is active and its activity has been recently shown

to be critical for the maintenance of senescence status since microinjection of anti-p53 antibodies into senescent cells induces DNA synthesis and reversion to the young phenotype (Gire and Wynford-Thomas, 1998).

Furthermore enzymes required for DNA replication, such as thymidine kinase (tk), thymidylate synthetase and dihydrofolate reductase (DHFR) are not expressed in senescent cells (Good *et al.*, 1995).

### **1.3 Differential expression techniques**

When the sequence of the genome of several organisms, including human, became available the scientific approach to study complex biological phenomena changed radically. It became a priority to exploit the massive amount of information generated by genome sequencing in order to understand the function of the sequenced genes, which remained largely unknown, as well as biological processes under physiological and pathological conditions. New methods for the simultaneous assessment of the gene expression of hundreds of thousand of genes in individual cells or tissues became available and they are now used instead of the gene-by-gene approach, which only allows the study of one or few genes at a time. These novel techniques are known as “post-genomic era techniques” and their goal is to acquire a global view on any biological phenomenon and to produce a list of genes whose expression changes in the examined samples.

Proteomics and microarrays are the two most widely used “post-genomic era techniques”. They offer a systematic way to survey changes in gene expression at the protein level, for proteomics, and the transcriptional level, for microarrays.

These techniques are particularly suitable to study multigenetic and complex processes such as replicative senescence.

### ***1.3.1 The proteome and proteomics***

The word proteome was introduced for the first time in late 1994 by Mark Wilkins during the Siena 2-D electrophoresis meeting to describe the total set of PROTEins expressed (and modified following expression) by a given genOME at a given time (Dove, 1999).

Proteomics is the science that studies proteomes and results in the complete description at the protein level of an organism or tissue under a given set of conditions.

Unlike the genome of an organism, which is basically a subset of fixed information, the proteome is highly dynamic; it is subjected to changes due to different developmental stages, environmental circumstances or disease states (Williams, 1999). Moreover the proteome of an organism is more complex than its genome: diverse proteins isoforms might be produced from the same gene by alternative splicing or alternative initiation codons, and a huge variety of post-translational modifications result in more than one protein being expressed by a single gene. In higher mammals as many as 15 or 20 proteins can be expressed by one single gene, observation which leads to the suggestion that the human proteome may contain as many as 500,000 proteins.

The functional molecules of biology are proteins, genes represent the information banks for protein specification (Williams, 1999) but ultimately organisms utilise proteins as their functional molecules and proteomics is the way to look at the ultimate products of the genome: the proteins.

The activity state of a protein often depends on its modification state; for example a gene might be equally expressed under two different conditions but its phosphorylation status might be different. The different phosphorylation status ultimately results in an active protein under one set of conditions and an inactive protein under the other. Proteomics also allows identification of different isoforms and post-translational modifications of the same protein. Many different possible post-translational modifications are possible: phosphorylation, glycosylation, methylation, acetylation, myristoylation,

palmitoylation, sulfation, ubiquitination etc. The variant forms can be distinguished from the primary translated products by 2D-polyacrylamide gel electrophoresis (2-D PAGE) because post-translational modifications may lead to changes in molecular weight (protein processing) and/or pI (chemical modifications) (Celis and Gromov, 1999), which ultimately result in different coordinates for the same protein within the 2-D separation.

### ***1.3.2 History of proteomics***

In the first applications 2-D gels were mainly used to compare the resulting pattern of spots from different tissues, or cells kept in different conditions; however with the introduction of mass spectrometry in conjunction with 2-D PAGE it became possible to determine the identity of the proteins represented by the spots (Dove, 1999).

The electrophoresis technology was introduced for the first time by Tiselius about 90 years ago. The first two-dimensional (2-D) separation for proteins was introduced by Smithies and Poulik (Smithies O., 1956) in 1956 and consisted in a combination of paper and starch gel electrophoresis. Improvements in the technology were subsequently achieved with the introduction of discontinuous buffer systems and gradient gel electrophoresis, which contributed to increased resolution of the separated proteins (Margolis and Kenrick, 1967). The isoelectric focusing technique (IEF) for 2-D separation was introduced few years later (Dale and Latner, 1969; Emes *et al.*, 1975; Macko and Stegemann, 1969). In 1975 the first 2-D PAGE system for separating the proteins according to their charge in the first dimension, using IEF, and according to their size, by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension became available. It was O'Farrel (O'Farrell, 1975) that in the late seventies optimised the 2-D separation setting the basis for the future development of the 2-D technique.

### 1.3.3 Two-dimensional gel electrophoresis technique (2-D PAGE)

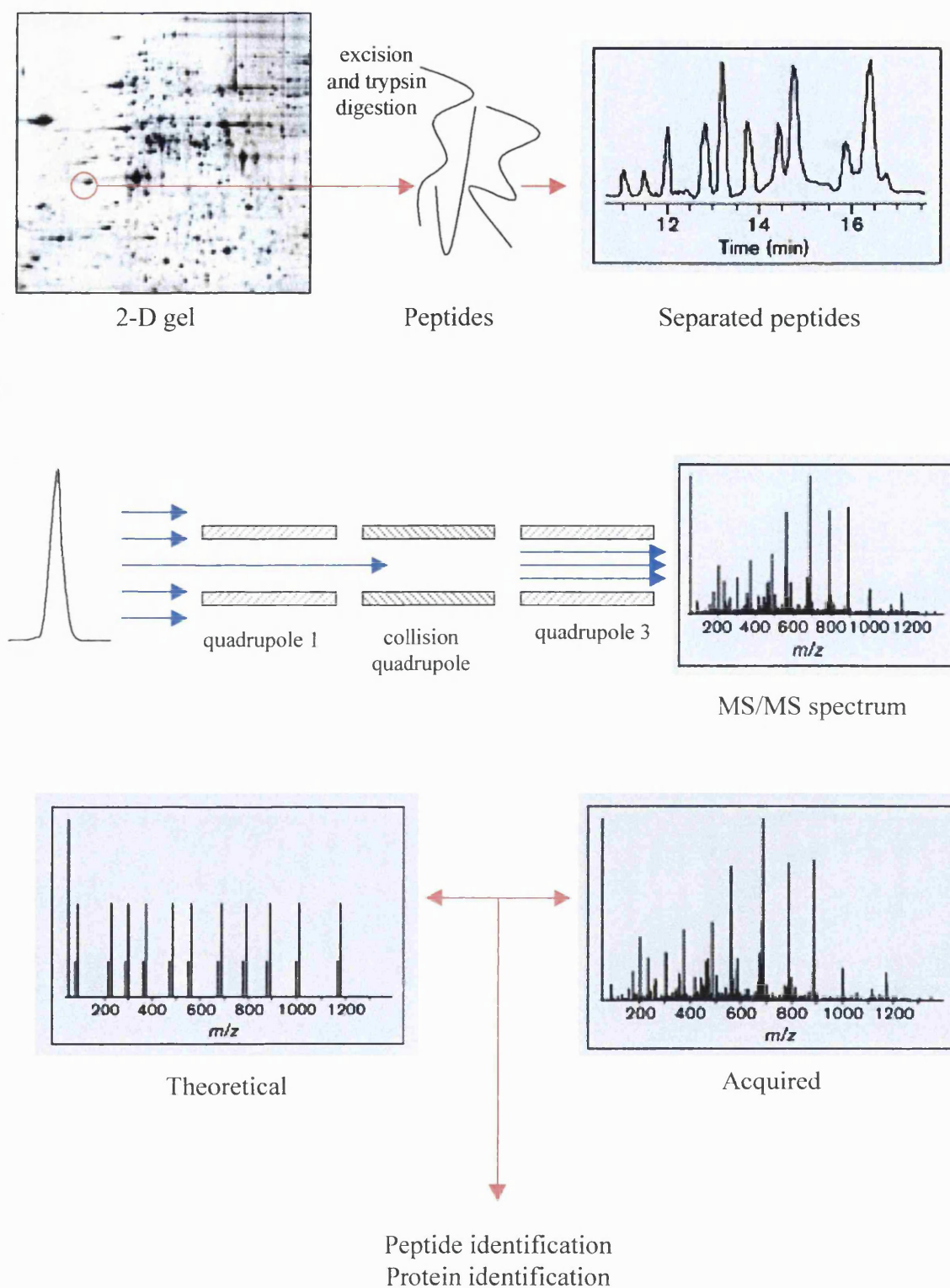
Any proteome study consists of the following steps: extraction of the proteins from selected cells or tissues, and sometimes enrichment of proteins; separation of individual proteins by 2D-PAGE; visualisation and quantitation of the separated proteins; identification of the selected proteins by mass spectrometry (Fig. 1.7) followed by validation of the data.

The core technology of proteomics is 2-D PAGE. Within 2-D PAGE the proteins extracted from any cell type or tissue are separated according to their isoelectric point (pI) by isoelectric focusing (IEF) in the first dimension and to their molecular weight (MW) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Since the two parameters are unrelated it is possible to obtain a uniform distribution of protein spots across a 2-D gel, and ultimately the separation results in a definite pattern of spots (O'Farrell, 1975).

Currently proteomics can separate simultaneously several thousand proteins. While this represents the major proportion of the proteome of relatively small organisms, such as *Saccharomyces cerevisiae*, with 5800 genes, or *Escherichia coli*, with 4000 genes, it is only the tip of the iceberg for more complex organisms. The 1500-2000 proteins separated by the average 2-D gel represent not more than 10% of the probable 15000 or more total polypeptides present within a mammalian cell (Celis *et al.*, 1992). Because it is likely that many of the less abundant protein have a very important regulatory effect in the cells, the detection of low copy number proteins is therefore a very important issue in any proteomic study.

Even though 2-D gel electrophoresis has been the technology most used during proteome analysis, this method is far from perfect. Several issues remain to be perfected: reproducibility, sensitivity and analytical power. Lack of sensitivity is considered one of the major low points in proteomic studies, particularly when compared to mRNA profiling methods.





**Fig. 1.7** Schematic illustration of a standard proteome analysis by 2-D PAGE in combination with mass spectrometry.

After separation on a 2-D gel, the protein of interest is excised and digested with trypsin. The resulting peptides are then submitted to mass spectrometry and the identity obtained.

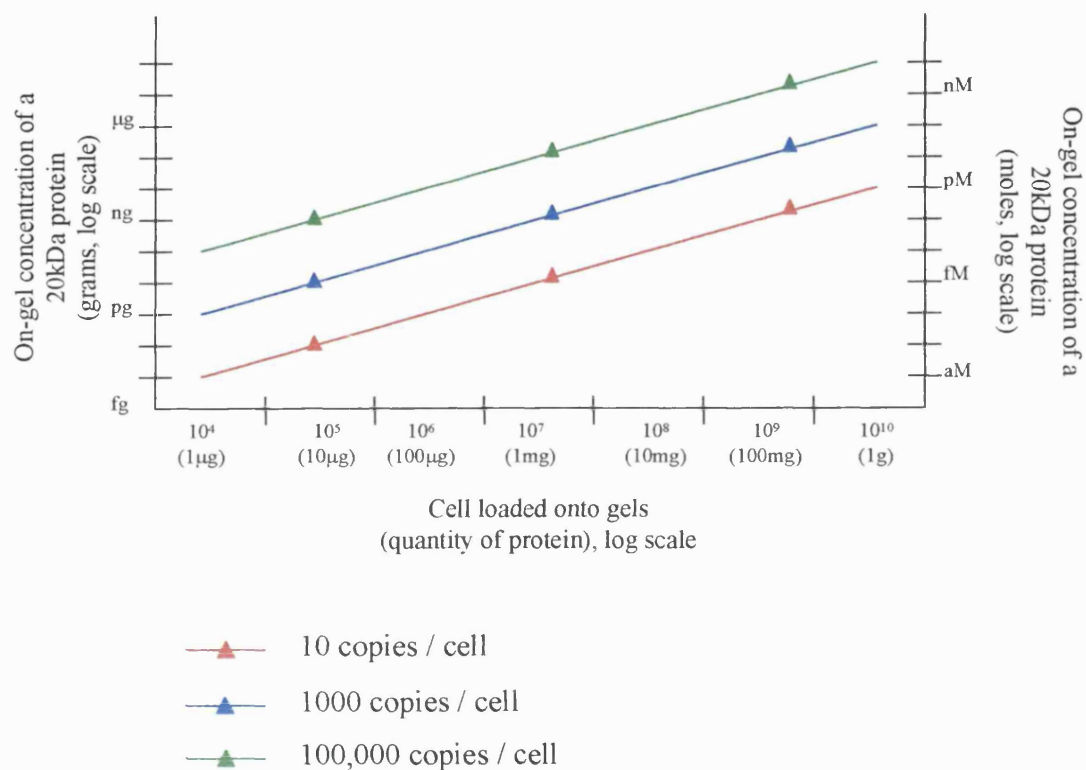
### 1.3.4 Considerations

It is relatively easy to study by proteomics the most abundant proteins within cells; that is the proteins present in cells in the order of 100 million molecules/cell and therefore representing several per cent of the total protein content of eukaryotic cells. However it is less straightforward to analyse the less abundant proteins. It is likely that many rare proteins are hidden underneath the more abundant ones within a 2-D separation or that simply may not be detected by the standard detection procedures.

Although not all proteins enter a 2-D gel and it is not yet possible to separate and detect on a single 2-D gel all proteins expressed by a genome, 2-D PAGE is the most powerful technique for the simultaneous study of protein expression.

Many spots separated on a wide pH range 2-D gel (pH 3 to 10) result not adequately separated. For prokaryotes it has been proposed that about 20% (Link *et al.*, 1997) of all the spots separated on an average 2-D gel contain two or more protein, and for eukaryotes the number approaches 40%. Parker *et al.* (Parker *et al.*, 1998) have shown in detail the extent and the nature of spot cross-contamination in 2-D gels of yeast extracts, where many of the spots were found to contain three or more proteins. The presence of multiple proteins per spot makes the interpretation of some experiments extremely difficult. However the mass spectrometry technique combined with the 2-D gels consents to resolve the protein mixtures found in a single spot (Arnott *et al.*, 1998).

It is still not completely clear how many copies of a certain protein are necessary within a cell to be detected on a gel. In Fig. 1.8, the relationship between the quantity of a protein loaded onto a 2-D gel and the final concentration of a 20kDa protein present at 10,  $10^3$ ,  $10^5$  copies per cell is shown. The proportion of the total protein that can be detected on a gel ultimately depends upon the copy number, the quantity of protein loaded onto the gel and also the detection procedure (Wilkins M.R., 1997).



**Fig. 1.8** Final concentration of a 20kDa protein after 2-D separation.

The final concentration of a 20kDa protein varies according to the quantity of extract loaded onto the gel and the number of copies of the protein per cell. This is based on  $10^9$  cells being 1g fresh weight, 200μg dry weight and containing 100μg protein (Wilkins *et al.*, 1997).

One way to increase the number of detectable low abundant proteins is by carrying out an enrichment of a specific sub-cellular fraction, a procedure known as pre-fractionation or purification of organelles prior to 2-D separation. The enrichment can be achieved by using sucrose density gradients or affinity or ion exchange chromatography (Jung *et al.*, 2000). This allows removal of the huge excess of the most abundant proteins and access to the rare ones. However when pre-fractionation methods are used, reproducibility of the gels becomes very difficult and the risk of introducing variability or artefacts to the results becomes very high. Moreover the more one manipulates a sample the higher becomes the chance of degradation and/or introduction of artificial protein modifications (Celis and Gromov, 1999).

There are other techniques that allow a gain in sensitivity while performing a proteomic study other than the pre-fractionation. It is possible for example, to raise the loading capacity of the gels by increasing the ionic strength of the gel from 2 to 10 mequiv/l (which allows a 4-fold increase in the loading capacity) (Righetti *et al.*, 1990). Alternatively it is possible to enhance the thickness of the gels from 1 to 5mm, which allows for an increase in the loading capacity by a factor 5 (Sanchez *et al.*, 1997). It is also possible to increase the size of the gels. Larger format gels may be used to yield better resolution. Klose reported that in a single large 2-D gel it is possible to separate and detect 9000 features (Klose, 1999a; Klose, 1999b), versus 2000 features detected on an average “normal size” gel. However large 2-D gels are technically very difficult to run.

Resolution and sensitivity can also be improved by using narrow pH gradients range gels, which give a linear increase of the loading capacity, and by running multiple gels that cover the narrow and overlapping pH ranges (for example 1 pH unit) (Hoving *et al.*, 2000; Wildgruber *et al.*, 2000). Better resolution is achieved because not only do the spots become further apart, but also the number of spots increases due to the fact that several apparently single spots divide into two or more protein spots (Wildgruber *et al.*, 2000). The combination of multiple narrow-range pH gradients used to stretch the samples

in the first-dimensional separation is known as “ultra-zoom gels”. This allows a higher sample load (up to 10mg) and ultimately gives a higher resolution (Hoving *et al.*, 2000; Wildgruber *et al.*, 2000). Hoving and colleagues expanded a wide range gel (pH 3-10) over six partially overlapping “ultra-zoom gels” (pH 3.5-5, pH 4.5-5.5, pH 5-6, pH 5.5-6.7, pH 6.2-6.8 and pH 7-10) and detected 5000 unique features (versus the 1000-3000 features that they detected in their standard wide pH range gel, pH 3 to 10) (Hoving *et al.*, 2000). However the use of the “ultra zoom gels” increases the number of gels that need to be run in each experiment and also the amount of sample required.

Metabolic labelling with specific isotopes is another way of increasing the detection of very low abundance proteins.

In addition some classes of proteins are particularly difficult to separate on 2-D gels, such as proteins with a very low or very large molecular weight or charges, and membrane-bound proteins. The hydrophobic membrane-bound proteins do not dissolve in the solvents used for isoelectric focusing, and therefore do not run on a 2-D gel. It has been estimated that the membrane proteins can represent 30% of the total cellular proteins (Paulsen *et al.*, 1998), and that just 1% of the integral membrane proteins can be resolved on a 2-D gel (even when thio-urea is used in the lysis buffer) (Garrels *et al.*, 1997). This can be a huge limiting factor for proteomics because many membrane proteins play key roles in signal transduction, cell adhesion, metabolite and ion transport, etc.

Also the separation of highly basic proteins with pI above pH 10, such as histones or ribosomal proteins, can be problematic; this is because at the extreme pH the buffering power of the water increases dramatically and causes high conductivity (Righetti *et al.*, 1990). The system more commonly used for IEF to separate basic proteins is a non-equilibrium pH gradient electrophoresis (NEPHGE), in which the proteins are focused to allow a separation but where the pattern never reaches a steady state. However the resolution of these gels is poor and reproducibility very difficult to achieve.

### 1.3.5 Applications

Proteomics has been used to detect changes in the proteome that occur in response to changes in the physiological conditions, and to measure these changes.

Proteome analysis is an extremely versatile technique, which can be used for innumerable different applications. It can be used to measure the changes in protein expression during development, through the cell cycle, during apoptosis, in diseases and in response to different external stimuli and drug treatments. Therefore proteomics has been used to: identify differences in the proteomes of pathogenic versus non-pathogenic organisms; cancer versus normal tissue (differential display proteomics); characterise all the post-translational modification (phosphorylation, acetylation, glycosylation etc.) of a single protein under different circumstances and in response to different stimuli; identify the components of multi-protein complexes and protein-protein interactions; describe the sub-cellular localisation and the potential changes in the sub-cellular localisation of proteins by pre-fractionation of the sample prior to separation; confirmation of the open reading frames in DNA sequences and obtain information on possible alternatively spliced mRNAs; look at the multiplicity of factors involved in diseases, very few of which are caused by a single gene; discover and develop drugs (pharmacogenomics) (Mullner *et al.*, 1998).

2-D gels have been used to compare the patterns of protein expression between normal and transformed cell lines. In studies where 400-1000 proteins were compared, it was reported that transformation caused a significant change in expression for 10-30% of the proteins (Garrels and Franza, 1989). In addition a number of marker proteins for this process such as the tropomyosin family of cytoskeletal proteins and the proliferating nuclear antigen (PCNA) were identified (Garrels and Franza, 1989; Morris and Mathews, 1989). These studies were extended by Garrels and his colleagues to generate a 2-D gel database for REF52 cells under a variety of growth states such as quiescent and

rapidly dividing, and upon transformation by DNA and RNA tumour viruses (Garrels and Franza, 1989). Moreover for a number of the spots separated on 2-D gels, they determined the identity, the sub-cellular localisation and the post-translational modifications.

However, as previously mentioned, in the first applications, 2-D PAGE was merely used to compare the resulting pattern of spots from different tissues, or cells kept in different conditions. The major limitation was that it was not possible to annotate the spots found to be differentially expressed. Only with the combination of 2-D PAGE with Edman sequencing and more recently with mass spectrometry it became possible to determine the identity of the proteins represented by the separated spots (Dove, 1999).

It was clear since the beginning that the proteomic technique was extremely useful in studying a complex and multigenetic processes like signal transduction pathways and several works have been published.

Godovac-Zimmermann *et al.* in 1999 (Godovac-Zimmermann *et al.*, 1999) developed and applied a functional proteomic method to investigate a signal transduction pathway involving platelet-derived growth factor (PDGF), endothelin and bradykinin receptors. Mouse fibroblast cells were stimulated with PDGF or endothelin and the phosphorylation and dephosphorylation status of several hundred proteins was studied as a function of time following stimulation using 2-D PAGE and anti-phosphotyrosine or anti-phosphoserine antibodies. Up to 100 proteins showed strong changes in phosphorylation within minutes of receptor stimulation, and some of them were further identified by mass spectrometry. It appeared that some of the differentially expressed proteins were previously known to be associated with PDGF signalling, whereas others have been shown to be involved in other signalling pathways, but not the PDGF pathway, and some others were not previously associated with any signal transduction pathway.

More recently Lewis *et al.* combined functional proteomics with selective activation and inhibition of MKK1/2 (mitogen-activated protein kinase kinases), in order to identify cellular targets regulated by the ERK

(extracellular signal-regulated kinase) cascade (Lewis *et al.*, 2000). They identified 25 targets of this signalling pathway, of which only 5 have been previously characterised as MKK/ERK effectors.

Several studies have been performed using proteomics as differential display technique to compare cancer versus normal tissue. Among these, Celis *et al.* adopted a proteomic strategy to identify metaplastic lesions in bladder squamous cell carcinoma as well as biomarkers in the urine for follow-up studies of squamous cell carcinoma (SCC)-bearing patients (Celis *et al.*, 2000). The comparison of the protein expression profiles of normal urotelium biopsies and SCCs yielded the identification of several differentially expressed proteins. Some polypeptides, comprising keratin 10, 14 and 16, psoriasin, migration inhibitory factor-related proteins (MRPs) 8 and 14, were expressed only by the tumour; others, comprising aldose reductase, keratin, 14-3-3s as well as several proteins of unknown function, were deregulated in the SCC. During this study a low molecular weight calcium-binding protein, called psoriasin, was also identified as a putative urinary marker for SCC lesions.

As previously mentioned very little work has been published in which a proteomic approach has been used to study replicative senescence.

DiPaolo *et al.* examined the differential protein expression profiles in quiescent early versus late passage of WI-38 human diploid fibroblasts (HDF) (DiPaolo *et al.*, 1995). They observed age-associated changes in gene expression of both secreted and cellular proteins by 2-D PAGE. During the analysis two proteins that were absent upon senescence were identified by western blot and N-terminal sequencing:  $\alpha$  I (type III) procollagen and EPC-1 (early population doubling level cDNA-1). The EPC-1 gene, also named pigment epithelial cell derived factor (PEDF) (Tombran-Tink *et al.*, 1996), encodes for a 50kDa secreted protein that belongs to the family of the serine protease inhibitors. Quiescent human fibroblast cells express high levels of the mRNA for the EPC-1 gene, and steady-state levels decrease on serum stimulation and entry into the cell cycle and it is strongly down-regulated in either growing young cells or non-growing senescent cells (Coljee *et al.*, 2000).



Moreover EPC-1 has been shown to inhibit growth of several endometrial carcinoma cell lines (Palmieri *et al.*, 1999) as well as glial cells (Sugita *et al.*, 1997); it has also shown to inhibit basic fibroblast growth factor induced neovascularisation *in vivo* suggesting a potential role for the protein as an inhibitor of angiogenesis (Dawson *et al.*, 1999).

More recently Tsugita *et al.* used 2-D gels to investigate changes in the expression of proteins in the mouse brain during the course of aging. The proteins separated by 2-D gels were isolated from 5 different regions (cerebellum, cerebral cortex, hippocampus, striatum and cervical spinal cord) and mice at 5 consecutive ages starting from the 10<sup>th</sup> week followed by the 3<sup>rd</sup>, 6<sup>th</sup>, 12<sup>th</sup> and 24<sup>th</sup> month (Tsugita *et al.*, 2000). This analysis identified 17 spots that were differentially expressed upon aging, which however have not yet been identified.

Kondo *et al.* have attempted to find alterations that are essential for cellular aging by comparing the proteomes of young and old fibroblasts. They showed that a spot representing a fragment of  $\alpha$ -2-macroglobulin (A2M) accumulated in fibroblasts of late passages (Kondo *et al.*, 2001) and showed that the amount of intracellular A2M in the immortalised cells was lower than their normal counterpart, in both early and late passages, reflecting the fact that immortalised cells lose the age-related phenotype. A2M is one of the major plasma proteins and functions as a panproteinase inhibitor. A possible contribution of A2M to the aetiology of the age-related disease, Alzheimer's disease, has been suggested (Blacker *et al.*, 1998)

Proteomic studies have also been performed to study senescence-related changes in response to stress. For example Liu *et al.* used the 2-D PAGE approach to study the heat shock response of HDFs during replicative cellular senescence (Liu *et al.*, 1989).

A very impressive application of proteomics in combination with mass spectrometry was performed by Page *et al.*. They described in a differential proteomic analysis the protein expression map of matched normal adult human luminal and myoepithelial breast cells, extracted from reduction

mammoplasties and separately purified (Page *et al.*, 1999). During this study 170 proteins that were expressed significantly differently between the two cell types were detected and quantitated. Furthermore using mass spectrometry they established the identity of 51 of these differentials. This is one of the most extensive differential proteomic studies to date, which demonstrates the capability of identifying novel proteins by 2-D PAGE used in combination with mass spectrometry.

### ***1.3.6 Protein identification by mass spectrometry***

In any proteomics project the identification of the proteins (all or just the ones of interest) is crucial. In the simplest meaning identification assigns a name and/or a database accession number to a feature on the gel, generally linking the amino acid sequence of the protein to DNA sequences of genes, and therefore ultimately linking proteomes to genomes.

Mass spectrometry (MS) is an analytical technique, which determines the mass, or to be more concise, the mass-to-charge ratio of molecules (Fig. 1.7) starting from very small amount (femtomoles) of material.

Although mass spectrometry can measure the molecular mass of a whole protein, this is usually not sufficient to identify the protein. Hence the general strategy for protein identification involves enzymatic digestion of proteins into peptides, which can then be analysed to identify the protein. The most commonly used enzyme is trypsin that cleaves only at the C-terminal side of lysine or arginine residues, producing peptides mostly in the range of 1000 - 3000Da.

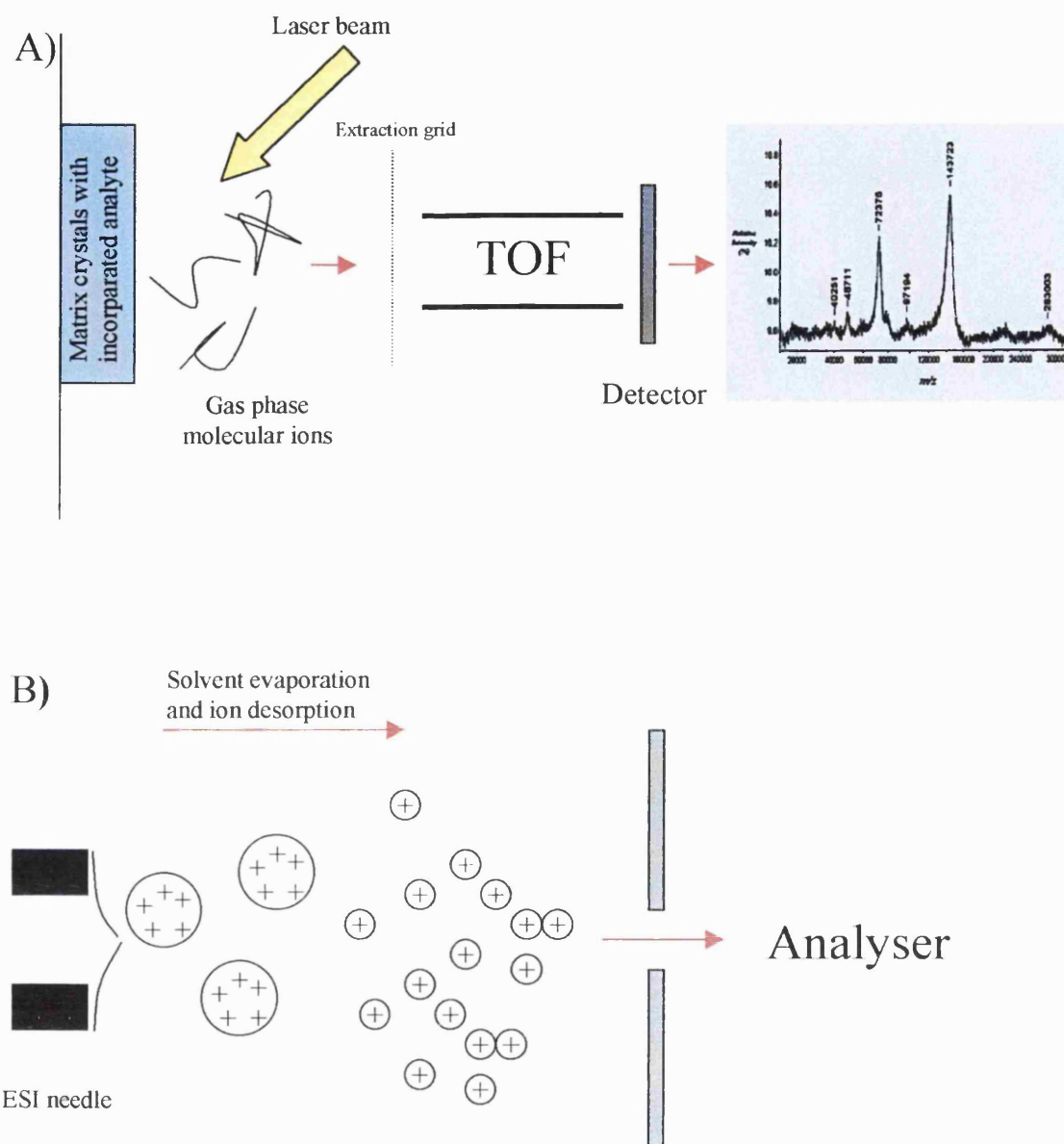
Having produced these digests two different MS techniques are commonly employed for identifying the protein: matrix assisted laser desorption/ionisation (MALDI) (Fig. 1.9 A) and electrospray ionisation (ESI) (Fig. 1.9 B). MALDI generates ions from a solid phase sample, which are usually analysed in time-of-flight mass spectrometers (TOF-MS) while ESI

generates ions from samples in a liquid phase, often directly from on-line coupled liquid chromatography (LC). Generally the mass spectrometers are used to acquire the mass spectra, are made of two different parts: an ion source, that introduces the sample into the machine, and an analyser that measures the mass (mass-to-charge ratio) of the introduced ions.

#### 1.3.6.1 Matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS)

In MALDI experiments nano- to microlitre quantities of sample are mixed with a solution of a matrix compound (such as  $\alpha$  cyano 4 hydroxy cinnamic acid). After drying a crystalline sample with a molar analyte-to-matrix ratio of typically 1:10000 or even higher is obtained. A pulsed laser (for example N<sub>2</sub> laser emitting at 337nm) is then used to irradiate the sample generating molecular ions that are accelerated by an electric field (between the sample and an extraction grid). After ion extraction from the ion source and some further ion manipulation, ions are then drifted through a field-free flight tube, in which their time of flight for reaching the detector depends on their mass-to-charge ratio (Fig. 1.9 A). To increase mass resolution some MALDI-TOF MS instruments use ion reflector and/or delayed reaction.

In this way the masses of all the peptides within a mixture are obtained. These peptide masses are then matched against theoretical masses of peptides from proteins present in databases that have been generated by cleaving proteins with the same enzyme, which has been used for generating peptides within the experiment (peptide mass mapping). The output of the search is a list of proteins ranked by the number of peptides shared with the unknown protein, where the correct identification for an unknown protein is likely to be that with the largest number of peptide “hits”. Confidence in the identification



**Fig. 1.9** Schematic diagram of MALDI and ESI techniques.

A) In a MALDI experiment the sample is mixed with an excess of a matrix component. Upon laser irradiation a plume of neutral molecules are desorbed. The ions are then guided to the mass analyser and the detector. B) In an ESI experiment a spray of fine droplets that contains the charged analyte and solvent molecules is created upon the application of an high electrical tension on a needle.

is achieved by looking for a large gap in the number of matching peptides between the top and the second ranked protein, and a good coverage of the top ranked protein with the experimentally determined peptides.

The identification procedure is based on the general concept that the set of peptide masses obtained by mass spectrometry analysis of a digested protein with a specific protease is unique to each protein and can be therefore used as a fingerprint for that protein. Moreover the identification of a protein is possible only if its sequence is recorded in databases, and a positive identification depends on the amount of sequence information available for the species studied. If a spot contains either a mixture of proteins, a small protein or a highly post-translationally modified protein MALDI MS might not lead to an unambiguous identification. In this case more information can be achieved by peptide sequencing and ESI.

#### 1.3.6.2 Electrospray ionisation quadrupole-time-of-flight tandem mass spectrometry (ESI-QTOF-MS/MS)

The alternative method for identifying proteins is to select individual peptides obtained by proteolysis and subject them to tandem mass spectrometry (MS/MS). In MS/MS analyses peptides are often fragmented by a process called CID (collision induced dissociation) to generate a tandem mass spectrum, which contains the sequence information for a single peptide.

When ESI is used the liquid sample is delivered from the end of a high potential capillary either under pressure from a pump or by electrostatic forces alone if via a nanospray capillary, where it forms a mixture of small solvent drops of high electrical charge, containing the analyte. Subsequently the solvent is induced to evaporate further reducing the droplet size and ultimately releasing charged analyte ions into the gas phase. When fragmenting tryptic peptides the charge is generally retained on the C-terminal basic residue, hence

a ladder of sequence ions extending from the C-terminus is observed (Fig. 1.9 B).

Three types of instruments can be used: ion traps, triple quadrupoles or Q-TOFs. When using a triple quadrupole, the first quadrupole measures the masses of the parent ion and selects it for further analysis; chosen molecules will then be transmitted to the second quadrupole, where they are fragmented by collision with an usually inert gas. The mass, i.e. mass-to-charge ratio, of the fragmentation products is finally measured in the third quadrupole (Fig. 1.9 B). A Q-TOF tandem mass spectrometer can be described, in the simplest way, as a triple quadrupole with the last quadrupole section replaced by a TOF analyser (Chernushevich *et al.*, 2001).

Often LC is coupled with MS; the analytes are first separated through a HPLC column and then analysed by MS.

Tandem mass spectrometry, which is usually undertaken using ESI, is more powerful than peptide mass mapping typically done by MALDI and generally provides protein identities when peptide mass mapping by MALDI MS fails. MALDI however, being fast and having a great propensity for automation, remains the MS technique routinely performed, while ESI-MS/MS generally is carried out only where peptide mass mapping by MALDI gives an ambiguous result. Recent development in MS technology allows peptide mass mapping and tandem mass spectrometry to be performed on one MALDI instrument.

### **1.3.7 Microarrays**

Expression profiling is the measurement of the expression level of mRNAs of thousands of different genes expressed by a given cell type at a given stage, at the same time, with a special interest in those changes in gene expression that correlate with changes in cell function.

When a gene is expressed in a cell its DNA sequence is first transcribed into mRNA, which is then translated into a protein. Therefore expression profiling (also known as transcriptomic) gives a snapshot of the genome's plans for proteins synthesis under a particular cellular condition at a particular time, but it does not represent the realisation of this plan.

Even if the central dogma states that the information flows from DNA via RNA to proteins, it is not possible to assume that mRNA expression reflects exactly the proteome. It is not always possible to predict when an increase in the expression of a particular species of mRNA will correspond to an analogous increase in its protein expression and activity. For example even if a protein cannot be synthesized without its mRNA being present, the same protein can be present in a cell when its mRNA is no longer present. Vice versa it is possible to have high concentration of a certain mRNA without its translation into proteins.

Several post-translational mechanisms are known to control gene expression. They include: control of the transcriptional rate, when mRNA is synthesised from the template DNA, and control of the half-lives of the mRNAs. An analogous regulation occurs at translation, when mRNAs are used to synthesise proteins. As for mRNA, the rate of protein synthesis together with the half-lives of the proteins is used to control protein abundance; also the intracellular localisation and molecular association of the protein products are used to modulate gene expression.

Several studies have shown the lack of correlation between mRNA and protein levels. In order to obtain an estimate of the overall level of correlation between mRNA and protein abundances Anderson and Seilhamer (Anderson and Seilhamer, 1997) have analysed human liver by quantitative 2-D PAGE (for protein abundances) and by transcript image methodology (for mRNA abundances). From this study the authors suggested a substantial lack of correlation between the mRNA and protein concentration in a cell at a given time; in particular they showed that human liver mRNA was enriched for

secreted proteins, while mRNAs for cellular proteins were under-represented (Anderson and Seilhamer, 1997).

In an analogous study Gygi *et al.* (Gygi *et al.*, 1999) analysed the correlation between mRNA and protein levels of a group of genes expressed in exponentially growing *S. cerevisiae* cells. Protein expression levels were quantified by metabolic labelling, while the corresponding mRNA transcript levels were calculated from SAGE frequency tables. In this study the authors showed that in yeast the correlation between mRNA and protein level was insufficient to predict protein expression levels from quantitative mRNA data. In fact they showed that the mRNA levels for some genes were the same, but the protein levels varied more than 20 fold; conversely invariant steady-state levels of certain proteins were observed with respective mRNA transcript levels that varied by as much as 30 fold. The authors also showed that the codon bias (a measure of the propensity of an organism to selectively utilise certain codons which result in the incorporation of the same amino acid residue in a growing polypeptide chain) (Gygi *et al.*, 2000) of a gene is not sufficient to predict its protein expression level.

However even though mRNA levels do not always correlate with protein levels, the hypothesis that there is a connection between the function of a gene product and its expression pattern is generally accepted. It has been proposed as a general rule that each gene is expressed in a cell at a certain time and under specific conditions to ensure that its product has a role (Brown and Botstein, 1999). Therefore quantitative analysis of global mRNA levels is often used to describe the state of cells and tissue. This technique is fast and very sensitive when compared with proteomics, which is more time consuming and limits the analysis to relatively highly expressed proteins.

Proteomics however gives types of information that are of critical importance for the description of a biological system, which cannot be discovered by studying the mRNA expression level. Ultimately proteins are the functional molecules within cells, and mRNA based approaches, measuring



mRNA abundance rather than that of the proteins, are necessary but are by themselves not sufficient for the quantitative description of biological systems.

In the last few years several different methods have been developed to screen cells or tissues for differentially expressed genes, analysing hundreds or even thousand of genes at a time. Amongst these methods there are differential cDNA library screening (plus/minus screening) (Dworkin and Dawid, 1980), subtractive cDNA library screening (Sargent, 1987), differential display (Liang and Pardee, 1992), sequencing of cDNA libraries (Adams *et al.*, 1991), and serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995).

Whilst these methods are able to detect five-fold or greater changes in gene expression (Han and Hilsenbeck, 2001), they are inadequate for detecting subtle changes (Han and Hilsenbeck, 2001).

Alternatively other methods can be used to achieve a better sensitivity such as northern blots (Alwine *et al.*, 1977), S1 nuclease protection assay (Berk and Sharp, 1977) and RT-PCR; however those methods allow investigation of only few genes at a time.

Microarray, together with proteomics, is one of the most exploited “post-genomic era technique”.

Microarrays, [also known as DNA hybridisation arrays, DNA arrays, DNA chips and/or high-density oligonucleotide arrays (Gene Chips)] bring gene expression analysis to a genomic scale by permitting investigators to simultaneously examine changes in the expression of thousands of genes simultaneously. They allow for rapid screening and quantification of differences in gene expression as little as 1.5-fold and are particularly good for studying multigenetic and complex processes, such as replicative senescence.

For hybridisation arrays, the general approach is to immobilise gene-specific sequences (probes) on a solid-state matrix. These sequences are then queried with labelled nucleic acids from biological samples (targets). Two mRNA samples are isolated from a pair of cell cultures to be compared, reverse transcribed into cDNA and labelled using two different fluorescent dyes (usually Cy3, red, and Cy5, green) and then hybridised simultaneously to a

microarray. The chip is then washed and scanned for fluorescent intensities (Fig. 1.10 and Fig. 1.11). The underlying theory is that the greater the expression of a gene, the greater the amount of labelled target, and hence, the greater output signal (De Benedetti *et al.*, 2000; Freeman *et al.*, 2000). In Fig. 1.12 is represented a subarray region obtained during an experiment in which sample 1 was labelled with Cy3 and sample 2 with Cy 5. Red spots on the composite image (such as spot A) represent clones whom mRNA was up-regulated in sample 1, while green spots on the composite image (such as spot B) represent clones whom mRNA was up-regulated in sample 2. Spots resulting yellow on the composite image (such as spot C) represent clones equally expressed in the two samples.

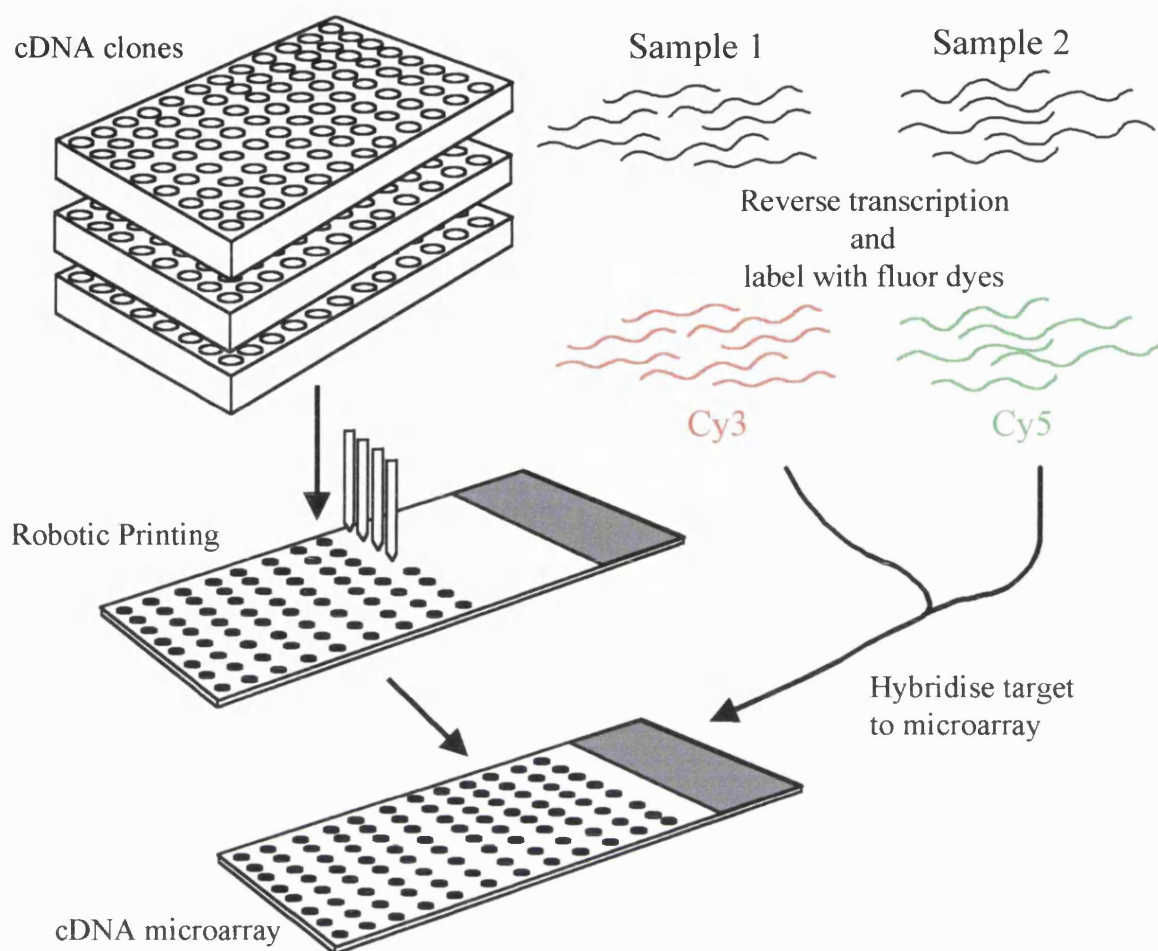
Arrays are flexible and universal (they can be used with any source of RNA). They are fast, user friendly and require an extremely low reaction volume per hybridisation (Brown and Botstein, 1999)

### ***1.3.8 Different types of microarrays***

DNA arrays can be defined according to two parameters: the length of the DNA (probes) attached to the solid support and the density of the different probes on a given matrix. High density is achieved in microarrays by representing thousand of genes on the same slide (Helmberg, 2001).

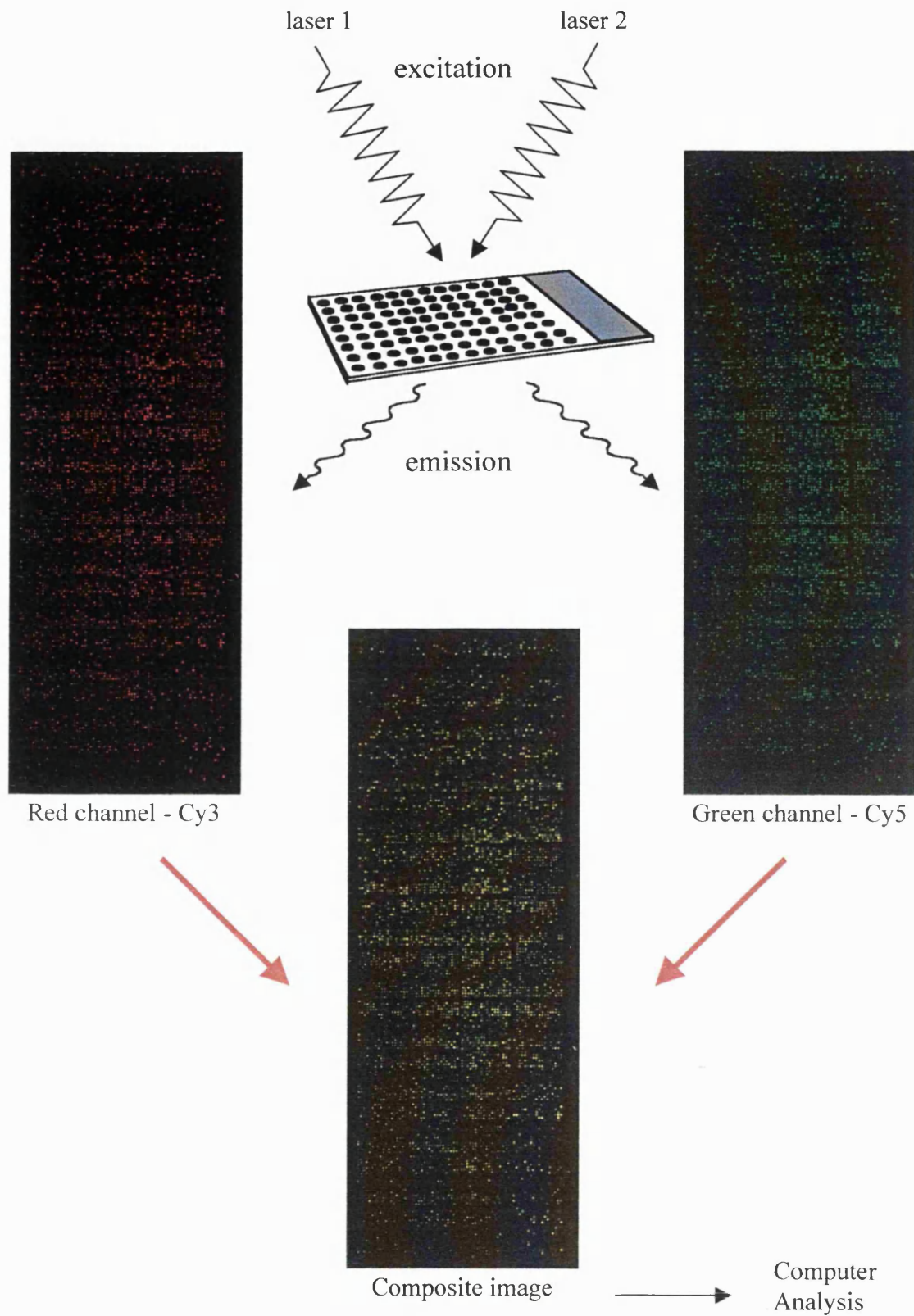
According to the length of the strands attached to the solid matrix, the DNA microarrays can be broadly divided into two classes: cDNA-based arrays and oligonucleotide-based arrays.

The probes in the cDNA-based arrays are several hundred bases long while in the oligonucleotide arrays short oligonucleotides usually 25 bases long are used.



**Fig. 1.10** Schematic representation of a microarray experiment.

Two samples are labelled, while being reverse transcribed, with two different fluor dyes, usually Cy3 and Cy5. The two samples are then mixed and hybridised to a cDNA microarray slide, previously obtained spotting cDNA clones with a robotic printer (arrayer).



**Fig. 1.11** Image acquisition.

After the hybridisation step, the array slide is scanned at the emission for the two dyes (usually red channel for Cy3 and green channel for Cy5) and a composite image obtained.

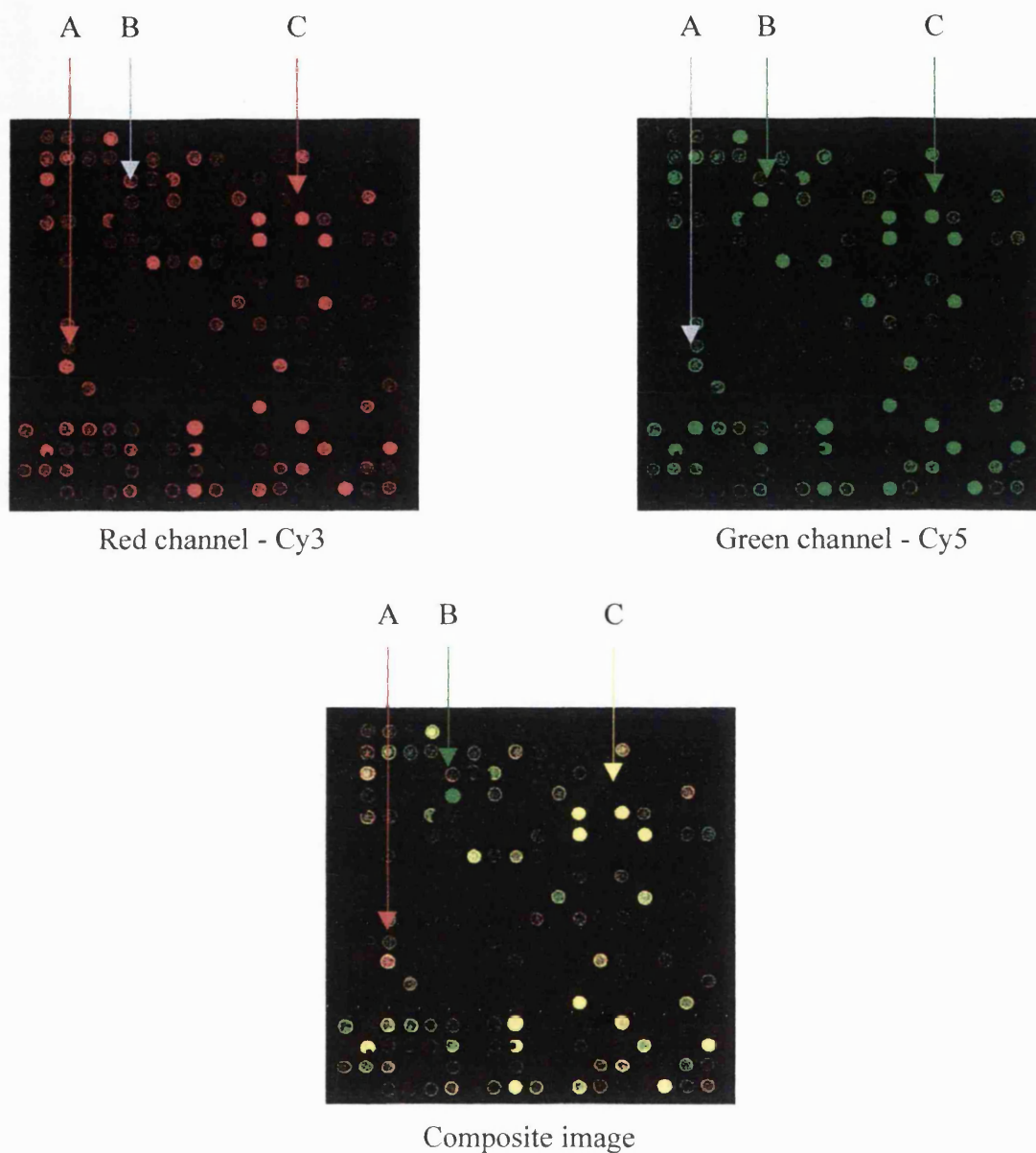
#### 1.3.8.1 cDNA arrays

cDNA arrays generally use as probes gene-specific polynucleotides derived from the 3' end of RNA transcripts. These transcripts are produced by spotting PCR products of approximately 0.6-2.4kb onto the array slides. The PCR products are purified by precipitation and/or gel-filtration to remove the unwanted salts, detergents, PCR primers and proteins present in the PCR reaction. In fact purity of RNA is a key factor in the hybridisation performance, particularly when using fluorescent detection systems because cellular proteins, lipids and other contaminants can promote a non-specific binding of labelled cDNAs to the slide surface.

Within cDNA microarrays each spot is obtained by the deposition of few nanolitres of purified PCR product (100-500ng) onto the slide using an arraying robot.

A potential problem that can occur using the cDNA arrays is cross-hybridisation of members of the same gene family with the same probe. As a large fraction of mammalian genes are members of gene families and share a high percentage of homology, it is possible to incur cross-hybridisation. To minimise this possible source of error the PCR fragments are usually derived from the 3' UTR because these are likely to be the least conserved within homologous genes.

The first cDNA arrays were nylon/plastic membrane based, however the most recent ones are glass based. Using glass as a support allows obtaining the high density as well as numerous other advantages. First glass is a rigid and durable material and sustains high temperatures and high ionic strength washes. It provides also a non-porous and impermeable support. Therefore the hybridisation liquid cannot penetrate the surface of the support and this enhances the rates of hybridisation and decrease the volume in which the hybridisation can be carried out. The kinetics of the interactions are not



**Fig. 1.12** A sub-array.

Proteins extracted from sample 1 were labelled with Cy3 (red) and proteins extracted from sample 2 were labelled with Cy5 (green). Clone A, red in the composite image, was a clear up-regulated spot in sample 1; clone B, green in the composite image, was up-regulated in sample 2, while clone C, yellow in the composite image, was equally expressed in the two samples.

complicated by the diffusion of solvents and solutes into and out of pores or by the interactions that can occur once the probe has entered a pore. The use of glass as support renders also the washing steps faster and more effective as the washes are not perturbed by diffusion. Another advantage of using glass as support is that glass has very low intrinsic fluorescence and therefore does not contribute to the background noise when using fluorescence detection systems. Additionally the glass spotted arrays benefit from a very well defined location of the probes compared to the arrays spotted on flexible membranes (Cheung *et al.*, 1999; Southern *et al.*, 1999).

Glass based slides are usually coated with poly-lysine, amino silanes or amino-reactive silanes to enhance the hydrophobicity of the slides and the adherence of the deposited DNA. The coating also limits the spread of the spotted DNA drop on the slide (Duggan *et al.*, 1999).

#### 1.3.8.2 Oligonucleotide arrays

Oligonucleotide-based arrays were introduced by Affimetrix (Santa Clara, CA). They are synthesised using photolithography. The synthesis of oligonucleotides involves light-directed removal of blocking groups (deprotection) that are initially attached to the end of the growing chain. The checkerboard can be imagined as hundreds of rows in both dimensions. If the next base to be added to the growing array is an adenosine (A) all the fields requiring an A in the next position are exposed to the light, whereas the fields requiring any other nucleotide are not. This is achieved by interposing a photo mask between the light source and the array. This process means that a different photo mask has to be made for every single nucleotide addition step during the array synthesis.

A core element of the Affymetrix array design is the Perfect Match/Mismatch probe strategy. For each probe designed to be perfectly complementary to a target sequence, a partner probe is generated that is

identical except for a single base mismatch in its centre. These probe pairs, called the Perfect Match probe (PM) and the Mismatch probe (MM), allow the quantitation and subtraction of signals caused by non-specific cross-hybridisation. The difference in hybridisation signals between the partners, as well as their intensity ratios, serve as indicators of specific target abundance (Weindruch *et al.*, 2002). This intrinsic structure of the oligonucleotide arrays cancels any possibility of cross-hybridisation. Moreover the short chains of oligonucleotides used as probes are a more accessible targets for the hybridisation when compared with the cDNA arrays that use as probes, long PCR products sometimes as big as 2.4kb. However using short-chain detectors the variation in the melting temperature due to AT-GC composition can become large and the hybridisation specificity can be greatly decreased by reducing the number of nucleotides from hundreds to as few as 25 (Duggan *et al.*, 1999).

### ***1.3.9 Experimental design***

When performing a microarray experiment it is important to consider the two main sources of variability: experimental variability and biological variability.

Multiple factors contribute to experimental variability: the responses to the microenvironment, such as the position of a tissue culture dish in the incubator, and the possible operator-related variations than can occur even when the same protocol has been performed. The biological variability reflects the differences between individual samples. The combination of the experimental plus the biological variability can lead to a incorrect interpretation of the data derived from a global expression profile, in which the operator is screening through 10,000 genes to find the small subgroup that varies (Lander, 1999).



It was commonly found that more variability comes from biological differences between samples rather than from technical discrepancies and replicate hybridisations of the sample are generally highly reproducible, and ultimately array to array differences are less pronounced when the same sample is tested by repeated hybridisation (Han and Hilsenbeck, 2001). It is therefore possible to conclude that differences due to experimental variability due to sample preparation (such as labelling, hybridisation and quantitation) are less relevant than the differences coming from biological variability between samples (Han and Hilsenbeck, 2001).

#### ***1.3.10 Applications***

A very impressive application of the cDNA arrays was carried out by DeRisi *et al.* in yeast (DeRisi *et al.*, 1997). In this study cDNA microarrays containing virtually every gene of *S. cerevisiae* were used to carry out a comprehensive investigation of the temporal program of gene expression that accompany the metabolic shift from fermentation to respiration. The expression profiles observed for genes with known metabolic functions identified features of the metabolic reprogramming that occur during the diauxic shift, and the expression patterns of many previously uncharacterised genes provided clues to their possible functions (DeRisi *et al.*, 1997).

Another pioneering microarray study was done by Iyer *et al.* at the beginning of 1999 (Iyer *et al.*, 1999). They used cDNA microarrays representing about 8600 different human genes, to study the temporal program of gene expression during the response of fibroblasts to serum. The authors demonstrated that many features of the transcriptional program appeared to be related to the physiology of wound healing, suggesting that fibroblasts play a major role in this complex response (Iyer *et al.*, 1999).

It was clear from the beginning that the microarray technique would be powerful for studying complex and multigenetic processes such as cellular

senescence and aging, because it would allow the analysis of large groups of functionally related genes in a systematic way.

One of the first groups to publish the gene expression profile of an aging process using microarrays was Lee *et al.* (Lee *et al.*, 1999a). They used high-density oligonucleotide arrays representing 6347 genes to study the gene expression profile in skeletal muscle from young and old mice, and old mice subjected to a caloric restriction (CR) regime. This study revealed that aging resulted in a differential gene expression pattern indicative of a marked stress response and lower expression of metabolic and biosynthetic genes, and furthermore that most alterations were either completely or partially prevented by CR, the only intervention known so far to retard aging in rodents. The transcriptional patterns of calorie-restricted animals suggested that CR retards the aging process by causing a metabolic shift toward increased protein turnover and decreased macromolecular damage (Lee *et al.*, 1999a).

The same group undertook a similar study to determine the changes in the expression profile of gastrocnemius muscle of male C57BL/6 mice during aging. In this study aging also resulted in a differential gene expression pattern indicative of a marked stress response and lower expression of metabolic and biosynthetic genes, alterations which were completely or partially prevented by CR (Lee *et al.*, 1999a).

More recently Weindruch *et al.* used high-density oligonucleotide microarrays to determine the gene-expression profile of the ageing neocortex and cerebellum in mice. Ageing resulted in a gene-expression profile indicative of an inflammatory response, oxidative stress and reduced neurotrophic support in both brain regions. At the transcriptional level, ageing in the mouse brain displayed parallels with human neurodegenerative disorders. Also from this study it appeared that CR selectively attenuates the age-associated induction of genes for inflammatory and stress responses (Weindruch *et al.*, 2001; Weindruch *et al.*, 2002).

Park *et al.* (Park *et al.*, 2001) used cDNA microarray containing 384 known genes to compare the expression profiles of three different types of

ageing models: replicative senescence, fibroblasts from progeria and fibroblasts from an elderly donor. They found that despite showing the typical senescent phenotypes, distinct sets of genes were altered in each group. Furthermore a cluster analysis revealed closer relationships between fibroblasts from progeria and from the old individual than replicative senescence (Park *et al.*, 2001).

Senescence-associated gene expression has also been assessed by Shelton *et al.* (Shelton *et al.*, 1999) in three different cell types: dermal fibroblasts, retinal pigment epithelial cells, and vascular endothelial cells. They found that fibroblasts demonstrated a strong inflammatory-type response, but a overlap in senescent gene expression patterns with the other two cell types was limited and concluded that the characteristics of the senescence response were highly cell-type specific. They also showed that several genes that are constitutively over-expressed in senescent fibroblasts are regulated during the cell cycle in early-passage cells, suggesting that senescent cells are locked in an activated state that mimics the early remodelling phase of wound repair (Shelton *et al.*, 1999).

More recently Schwarze *et al.* (Schwarze *et al.*, 2002) performed DNA microarray analysis to investigate the pathways that positively regulate senescence. During this study the expression of 20,000 genes in human prostate epithelial cells passaged to senescence was assessed and then compared to the expression pattern of HPECs immortalised with the human papilloma virus 16 E7 oncoprotein. It was found that senescent cells display gene expression patterns that reflect their non-proliferative, differentiated phenotype and express secretory proteases and extra-cellular matrix components (Schwarze *et al.*, 2002). Moreover a comparison of genes transcriptionally up-regulated upon senescence to those whose expression was significantly down-regulated in immortalised HPECs identified three genes: the chemokine BRAK, DOC1 and a member of the insulin-like growth factor, IGFBP-3. Thus, it was suggested that these genes might function in novel pathways that regulate senescence and are inactivated during immortalisation.

## **2 Materials and methods**

### **2.1 Mammalian cell culture**

#### **2.1.1 *Cell media***

Tissue culture media and the cell culture reagents were purchased from Invitrogen. Primary cells as well all the conditionally immortalised cell lines (tsa4, tsa8, tsa14, tsa129, and also SV2 and SV4), REF52 and BOSC23 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 2mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

#### **2.1.2 *Cell lines***

Conditionally immortalised cell lines, tsa4, tsa8, tsa14, tsa129 were initially maintained in a 5% CO<sub>2</sub> and 20% oxygen atmosphere at the permissive temperature (33°C), at which temperature they proliferate continuously due to the fact that LT-ag was expressed and active. Cells were then shifted up to the non-permissive temperature (39.5°C) at which temperature, upon inactivation and degradation of LT-ag, they become senescent after 72 hours. The same growth conditions were used for the two control cell lines SV2 and SV4, which were however immortal at both temperatures (33°C and 39.5°C).

REF52 cell line (a kind gift from S. Lowe, Cold Spring Harbor Laboratories, New York) and BOSC23 cell line were maintained in a 5% CO<sub>2</sub> and 20% oxygen atmosphere at 37°C in DMEM supplemented with 10% FCS, glutamine and antibiotics as above.

### 2.1.3 *Primary cells*

Rat embryo fibroblasts (REFs) and mouse embryo fibroblasts (MEFs) were primary cell cultures; they were maintained in a 5% CO<sub>2</sub> and 20% oxygen atmosphere at 37°C in DMEM supplemented with 10% FCS, glutamine and antibiotics as above.

#### 2.1.3.1 Preparation of REFs

REFs were prepared from 13-day-old Sprague-Dawley rat embryos. A 13-day-old day pregnant rat was sacrificed by cervical dislocation and the uterus was removed. The embryos were then isolated from the uterus under sterile conditions and immediately placed into Leibbovitz's L-15 medium (Invitrogen). Head and tails were removed and the remaining tissues were disaggregated by fine mincing followed by treatment with trypsin-EDTA (ethylenediaminetetraacetic acid) (BDH) to produce a cell suspension. The cells were subsequently plated in 15 cm dishes (one dish was used for each embryo prepared). This was defined as passage 1 (P1). REFs were maintained in a 5% CO<sub>2</sub> and 20% oxygen atmosphere at 37°C in DMEM supplemented with 10% FCS, glutamine and antibiotics as above.

#### 2.1.3.2 Serial passaging of REFs

Once REFs were plated, they were passaged following the 3T3-passaging regime of Todaro and Green (Todaro, 1963).

According to the protocol  $2.6 \times 10^6$  cells were plated in each 15 cm dish. The cells were plated on day 1, the media was changed on day 2, and the cells lysed on day 3 or alternatively left to grow and then detached and plated on

fresh dishes (at the same concentration stated before) on day 4. Cells were continuously monitored microscopically to determine when they had ceased dividing and when the cell number no longer increased. When cells reached passage 5 (P5), they were detached and counted to make sure that the population was growth arrested by determining whether the cell number had increased. It was also checked that the number did not decrease, and therefore that the cells were not undergoing apoptosis (from each 15 cm dish it was possible to re-plate another 15 cm dish).

#### 2.1.3.3 Preparation of MEFs

MEFs were prepared from 13-day-old mice embryos. A 13-day-old pregnant mouse was sacrificed by cervical dislocation and the uterus was removed. The embryos were then isolated from the uterus under sterile conditions and immediately placed into Leibbovitz's L-15 medium (Invitrogen). Head and tails were removed and the remaining tissues were disaggregated by fine mincing followed by treatment with trypsin-EDTA to produce a cell suspension. The cells were subsequently plated into 15 cm dishes (one for each embryo prepared). This was defined as passage 1 (P1). MEFs were maintained at 37°C in 5% CO<sub>2</sub> and 20% oxygen atmosphere in DMEM supplemented with 10% FCS, glutamine and antibiotics as above.

#### 2.1.3.4 Serial passaging of MEFs

Once the MEFs were plated, they were also passaged following the 3T3-passaging regime of Todaro and Green (Todaro, 1963). According to the protocol  $2.6 \times 10^6$  cells were plated in each 15 cm dish. The cells were plated on day 1, the media was changed on day 2, and the cells lysed on day 3 or alternatively let to grow and then detached and plated on fresh dishes (at the

same concentration stated before) on day 4. Using this passaging regime it takes MEFs until about passage 8 (P8) to stop dividing. When cells reached P8, they were detached and counted to make sure that the population was growth arrested and no events of spontaneous immortalisation occurred, and therefore the cells' number no longer increased. This is very important because MEFs undergo spontaneous immortalisation very readily. It was also checked that the number did not decrease, and therefore that the cells were not undergoing apoptosis (from each 15 cm dish it was possible to re-plate another 15 cm dish).

#### ***2.1.4 Sub-culturing of cells***

Cells were grown in a 15 cm dish until day 4 (when dealing with primary cells) or until a sub-confluent (80% confluence) state when cell lines were cultured. The media was then removed and the monolayer of cells was washed once with 1X trypsin-EDTA (0.25% v/v trypsin, 0.03% w/v EDTA). The monolayer was subsequently detached using 1X trypsin-EDTA (1ml was used each 10 cm dish, for 5 min at 37°C) and the trypsin-EDTA inactivated by adding 10ml of complete media. The cells were then transferred into a 50ml falcon tube, spin down for 5 min at 1000rpm, the supernatant discharged and the cells resuspended in 10ml of fresh media. The cells were then counted and plated at the suggested density and maintained at 37°C (or other temperature where stated) in a 5% CO<sub>2</sub> and 20% oxygen atmosphere.

#### ***2.1.5 Preservation of cells***

Cells from a confluent 15 cm dish were trypsinised, washed once in complete media to remove any traces of trypsin and resuspended in complete media containing 10% DMSO (dimethyl sulphoxide) (BDH) (4ml each 15 cm

dish). One ml aliquots were transferred to cryotubes and frozen at -70°C in a polystyrene box lined with cotton wool. The tubes were transferred into liquid nitrogen after 24 hours.

#### ***2.1.6 Recovery of frozen cells***

Cells were removed from liquid nitrogen storage and thawed rapidly at 37°C. The cell were first diluted with 9ml of complete media in a 15ml falcon tube and then pelleted at 1000rpm for 2 min to remove the DMSO containing media. The pellet was then resuspended in 10ml of complete media and the cells split into two 10 cm dishes and incubated at 37°C in a 5% CO<sub>2</sub> and 20% oxygen atmosphere until sub-confluence was reached and then expanded into 15 cm dishes.

#### ***2.1.7 Serum starvation***

Normal media (DMEM supplemented with 10% (v/v) heat inactivated FCS, 2mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin) from semi-confluent dishes was removed and replaced with DMEM supplemented with 0% FCS, glutamine and antibiotics as above. Cells were then incubated at 37°C in a 5% CO<sub>2</sub> and 20% oxygen atmosphere for 4 days and then lysed.

#### ***2.1.8 DNA transfections***

Primary cells at passage 2 (P2) were transfected using FuGENE 6 Transfection reagent (ROCHE) according to manufacturer's instructions. The



DNA:transfection reagent ratio was 1:2. Cells were plated at a density of  $8 \times 10^6$  cells per 10 cm dish the day prior to transfection. Cells were then transfected with 5µg of the vector containing the cDNA of interest or the empty vector. Where co-transfections with Ha-ras were performed 2.5µg of the cDNA of interest together with 2.5µg of Ha-ras containing plasmid were used; if the co-transfections were also performed together with E7, 1µg of E7 containing plasmid was also added.

#### 2.1.8.1 Selection of stable transfectants

Forty-eight hours post-transfection cells from each plate were split 1:4 onto 15 cm dishes. At the time of the split, 0.25 µg/µl of G418 (Invitrogen) was added to each 15 cm dish. The media was then changed every 3-4 days adding fresh G418 each time. After 14 days, the media was removed and the cells were fixed and stained with a solution containing 2% (w/v) methylen blue and 60% (v/v) ethanol. After an incubation of a few minutes the stain was washed away with H<sub>2</sub>O and the colonies were counted.

#### 2.1.8.2 Isolation of clonal cell lines

After transfection and selection with 0.25 µg/µl of G418, single clones were expanded into cell lines. Single clones were picked using sterile cloning cylinders (Sigma). The growth medium was removed from each dish and a cloning cylinder (diameter 0.25 cm) was applied around each selected clone by pressing it lightly against the Petri dish after the edge of the cloning cylinder have been dip into sterile silicone grease. Then two drops of trypsin were added and the cells incubated at 37°C for 5 min. The detached cells were then collected with a Pasteur pipette and the trypsin inactivated with complete

medium. The cells were then plated into a 24 well plate and allowed to adhere and grow. When 80% confluence was reached (that took from a couple of days to a week, according to the growth rate that was different for each clone) the cells were transferred into 3 cm dishes. When 80% confluence was reached again the cells were transferred to 6 cm dishes and eventually to 10 cm dishes and subsequently passaged to new 10 cm dishes after 1:6 dilutions.

### **2.1.9 Premature senescence of REF52 cells**

BOSC23 cells ( $1.75 \times 10^6$ ) were plated in a 6 cm dish and transfected 24 hours later with 3  $\mu$ g of either pBabePuroEJras or empty pBabePuro using FuGENE (Roche). Forty-eight hours after transfection, the virus-containing medium was removed, filtered (through a 0.45  $\mu$ M filter) and used to infect REF52 cells in the presence of 8  $\mu$ g/ml polybrene (Aldrich). REF52 were plated at  $8 \times 10^5$  cells per 10 cm dish and incubated overnight prior to infection. For the infection, the culture medium was replaced by 2ml of virus containing medium for two hours. The infection was repeated a second time 6 hours later. Sixteen hours after, the infected cultures were purified using 2  $\mu$ g/ml puromycin (Sigma) containing medium. Transduced cells were incubated at 37°C in a 5 % CO<sub>2</sub> and 20% oxygen atmosphere. The medium was changed on the third day and the infected cells lysed with RIPA buffer after 7 days.

## **2.2 Bacterial manipulations**

### **2.2.1 Bacterial strains**

The JS4 *Escherichia Coli* strain (kindly provided by J. Sedivy, Brown University) was used for plasmid manipulation and preparation. JS4, a *recA1*

derivative of MC1061 has the following genotype: F<sup>-</sup>*araD*139,  $\Delta$ (*ara*, *leu*)7697,  $\Delta$ (*lac*) $\chi$ 74, *galU*, *galK*, *hsdR*2 (*rk*<sup>-</sup> *mk*<sup>-</sup>), *mcrA*, *mcrBC*, *rpsL* (Str<sup>r</sup>) *thi*, *recA*1.

### **2.2.2 Media and maintenance**

*E. coli* cells were grown Luria Broth, (LB medium) containing: bacto-tryptone (Oxoid or Difco) 10 g/l, yeast extract (Oxoid or Difco) 5 g/l and NaCl 10 g/l. Bacto-agar (Oxoid or Difco) 20 g/l was added when plates were prepared. To make up the media all the components were dissolved in ddH<sub>2</sub>O and autoclaved for 20 min at 37°C. When required, ampicillin (Sigma) was added to 100 µg/ml final concentration.

### **2.2.3 Preparation of competent bacteria**

500ml of LB were inoculated with a single colony and incubated shaking at 37°C overnight. 5ml of the overnight culture were then diluted into 500ml of LB and grown at 37°C with aeration for 2 to 2.5 hours until the OD<sub>600</sub> reached 1.0-1.2 (mid-exponential phase). The bacteria were harvested by centrifugation at 2500rpm for 20 min, resuspended in 5ml ice cold 0.1M CaCl<sub>2</sub> and incubated in ice for 20 min. The bacteria were then centrifuged again and resuspended in 5ml of ice-cold 85:15 solution 0.1M CaCl<sub>2</sub> and glycerol. 50µl aliquots were frozen in pre-chilled 1.5ml tubes using dry ice, and then stored at -70°C.

### **2.2.4 Bacterial transformations**

A single 50µl aliquot of frozen competent bacteria was thawed on ice. 950µl ice cold 0.1M CaCl<sub>2</sub> was added; 100µl of this mixture were then added to

each DNA to be transformed. The DNA was incubated with the bacteria on ice for 30 min followed by heat shock at 42°C for 90 sec. The transformations were returned to ice for 2 min, after which 1ml of LB medium (without ampicillin) was added and the transformations incubated at 37°C with shaking for 30 min. The cells were then concentrated by centrifugation (2500rpm for 10 min) and resuspended in 100µl LB medium. The transformations were finally plated onto 10 cm pre-warmed LB plates containing ampicillin and incubated overnight at 37°C.

## **2.3 DNA manipulation**

### ***2.3.1 Plasmid DNA preparation***

All the plasmid preparations (both small scale and large scale preparations) were done using QUIAGEN kits, and following the manufacturer's instructions.

#### **2.3.1.1 Small scale preparation**

Bacterial stocks were kept at -70°C in LB containing 80% glycerol. Liquid cultures of bacteria picked from single colonies were grown in a bacterial shaker (vigorous shaking) overnight at 37°C in 5ml of LB medium with 100 µl/ml ampicillin. 1.5ml of culture was then transferred to an eppendorf tube and microfuged at 13000rpm for 5 min. The pellet was resuspended in 250µl of solution P1 (50mM Tris/HCl, pH 8.0, 10mM EDTA, 100 mg/ml RNase A). Then 250µl of solution P2 [200mM NaOH, 1% sodium dodecyl sulphate (SDS)] were added and gently mixed by inverting the tube 4-6 times. To the same tube, 350µl of solution N3 (3.0M sodium acetate, pH 5.5) were added and immediately mixed by inverting the tubes 4-6 times. The

mixture was then centrifuged in a microfuge for 10 min at 13000rpm and the supernatant (450µl) transferred to a QIAprep column. The column was then centrifuged for 30 sec at 13000rpm and the flow through discarded. The columns were first washed with 0.5ml of PB buffer (QIAprep Miniprep kit, QUIAGEN) and then 0.75ml of PE buffer (QIAprep Miniprep kit, QUIAGEN). The DNA was then eluted with 30µl of EB (10mM TrisCl, pH 8.5) or H<sub>2</sub>O.

#### 2.3.1.2 Large scale preparation

250ml of LB containing 100 µl/ml ampicillin were inoculated with an overnight culture of bacteria and grown overnight at 37°C with vigorous shaking. Bacteria were harvested at 2500rpm for 15 min and the pellet resuspended in 10ml of resuspension buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA, 100 µg/ml RNase A, stored at 4°C). 10ml of lysis buffer P2 (200mM NaOH, 1% SDS) were added. After 5 min incubation at room temperature, 10ml of ice-cold neutralisation buffer P3 (3mM potassium acetate pH 5.5) were added and the mixture further incubated on ice for 20 min with occasional inversion. The cell debris was pelleted by centrifugation at 15000rpm in Beckman JA-17 rotor for 30 min. The recovered supernatant was applied to a previously equilibrated QIAGEN-tip 500 column (equilibration buffer QBT: 750mM NaCl, 50mM MOPS [3 - (N-morpholino) propanesulphonic acid] pH 7.0, 15% Ethanol v/v, 0.15% Triton X-100) and allowed to enter the resin by gravity. The column was washed twice with 30ml of wash buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% Ethanol). The DNA was then eluted with 15ml of elution buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% Ethanol) and precipitated in 10.5ml of isopropanol at room temperature. Centrifugation was performed for 30 min at 15000rpm at 4°C in the same rotor previously used and the DNA pellet was then washed with 70% ethanol. The pellet was air dried for 5 min and resuspended in ddH<sub>2</sub>O or TE (Tris-EDTA 10mM and

0.5mM respectively) pH 8.0. The DNA obtained was then checked by appropriate restriction digests and run on an agarose gel.

#### 2.3.1.3 DNA quantification

To determine DNA concentration, the optical density of the solution was measured at 260nm (OD<sub>260</sub>) using a BioRad spectrophotometer (Bio-Rad Smart Spec™ 3000 Spectrophotometer). DNA concentration was calculated using the relationship:

$$1\text{OD unit at }260\text{nm} = 50\text{ }\mu\text{g/ml DNA.}$$

The DNA was then checked by running 50ng on an agarose gel.

#### 2.3.2 *Restriction digests*

Restriction digestions of DNA plasmids were performed using restriction enzymes from New England Biolabs (NEB) and Boehringer Mannheim (BM) according to the specified conditions. The 10X buffer provided for each enzyme was diluted to 1X in the final reaction mixture. Bovine serum albumin (BSA, New England Bio Lab) was used at 1X dilution when required. Other conditions such as incubation time and temperature were performed according to the manufacturer's guidelines for the specific enzyme.

#### 2.3.3 *DNA agarose gel electrophoresis*

DNA fragments were loaded with 1X DNA loading buffer (2.5% Ficoll, 0.04% (w/v) bromophenol blue and 0.04% Xylene) and fractionated by electrophoresis on 1% (w/v) agarose (Invitrogen) gels, prepared in 1X TAE (40mM Tris-acetate and 2mM EDTA) with 1  $\mu\text{g/ml}$  ethidium bromide (BDH).

Electrophoresis in 1X TAE was carried out in electrophoresis tanks and DNA fragments were separated at a constant voltage of 80V for 2 or more hours. Samples were loaded alongside double stranded DNA molecular weight markers, 1kb-ladder (GibcoBRL). Ethidium bromide stained DNA fragments were then visualized on a UVP (Dual intensity ultraviolet trans-illuminator) and an image was produced and printed with a Sony video graphic printer.

#### ***2.3.4 Extraction of DNA from agarose gels***

DNA restriction fragments separated on an agarose gels were purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. After agarose separation DNA was viewed with a long-wave length ultraviolet emission (UV) and the desired band was cut out using a razor blade and transferred to an eppendorf tube. The gel slice was dissolved by adding 3 volumes of QG buffer (QIAquick spin kit, QUIAGEN) and incubation at 50°C for 10 min. The sample was then placed in a QIAquick spin column and centrifuged for 1 min after which the DNA in the column was washed with 0.75ml of PE buffer (QIAquick spin kit, QUIAGEN). Traces of wash buffer were removed by centrifugation for 1 min. Finally, the DNA was eluted by adding 50µl of buffer EB (10mM Tris-Cl, pH 8.5) and centrifugation for 1 min at full speed in microcentrifuge. The DNA was collected in a 1.5ml microfuge tube.

#### ***2.3.5 Dephosphorylation***

DNA vectors were digested with the appropriate enzymes. The restriction enzymes were then inactivated by incubating the restriction mixture at 65°C for 10 min. After that Tris pH 9.0 was added to reach a 50mM final

concentration, together with 1 unit of calf intestinal alkaline phosphatase (CIP) (New England Biolabs) each  $\mu\text{g}$  of DNA used. The mixture was incubated at 37°C for 20-60 min and then at 65°C for 10 min to inactivate the CIP. The DNA was then run on an agarose gel and purified as described above.

### **2.3.6 Ligation**

Ligation reactions were performed using 1 $\mu\text{l}$  of T4-Ligase (New England Biolabs) in 1X ligation buffer (50mM Tris-HCl pH 7.5, 10mM  $\text{MgCl}_2$ , 10mM dithiothreitol, 1mM ATP, 25  $\mu\text{g}/\text{ml}$  BSA). The ratio of DNA:vector to insert was dependent on the sizes of the two DNA fragments and on whether it was a blunt or cohesive-end ligation. The ligation mixture was incubated at 16°C overnight or at room temperature for at least 2 hours before transforming competent *E. coli* cells.

### **2.3.7 cDNA constructs**

TUC-2, TUC-4 and TUC-4 $\beta$  (a kind gift of C.C. Quinn and S. Hockfield, Yale University School of Medicine) were cloned into pcDNA3.1 V5His vector (Invitrogen), between HindIII and XbaI sites.

Gelsolin cDNA (a kind gift from Dr. Kwiatkowski, Hematology Division Brigham and Women's hospital, Boston) originally cloned into pBluescript SK<sup>+</sup> into the EcoRI site, was sub-cloned into pcDNA 3.1 V5His vector (Invitrogen).

p53 GSE-56 was kindly provided by A. Gudkov (Ossovskaya *et al.*, 1996); pMoE7 plasmid encoding HPV E7 and pEJ6.6 plasmid encoding activated human Ha-ras (Tarunina *et al.*, 1996) were kindly provided by M. Tarunina.



## **2.4 Protein analysis**

### ***2.4.1 Preparation of total protein extracts***

Cells were washed twice with cold 1X PBS (Phosphate Buffered Saline), and a 0.5ml of 1X RIPA Lysis Buffer [150mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate (NaDOC), 0.1% SDS and 50mM Tris pH 8.0] was added to each 15 cm dish. The cells were incubated for 30 min on ice and then scraped and transferred to a 1.5ml microfuge tube. Finally the cell extracts were centrifuged for 30 min at 13000rpm and the supernatant was transferred to a fresh microfuge tube. The cell lysates were aliquoted and stored at -20°C.

### ***2.4.2 Determination of protein concentration***

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories), a protein assay based on the Bradford assay (Bradford, 1976). The dye reagent was diluted 1:5 in PBS. A BSA standard curve was established with protein dilutions ranging from 0.2 to 8.0 mg/ml. 10µl of each sample were mixed with 1ml of freshly diluted dye and incubated at room temperature for 5 min. OD<sub>595</sub> was measured (Bio-Rad Smart Spec<sup>TM</sup> 3000 Spectrophotometer) and plotted against protein concentration of standards. Regression coefficient was calculated and the unknown sample concentrations determined.

### ***2.4.3 One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1-D SDS-PAGE)***

Sodium dodecyl sulphate-polyacrylamide gels electrophoresis (SDS-PAGE) were prepared from a 30% (w/v) acrylamide stock solution (containing

a ratio of 29.2 acrylamide:0.8 NN'-methylenebisacrylamide; Genomic Solutions) in 373mM Tris-HCl pH 8.8 and 1% (w/v) SDS. Gels were polymerised by addition of ammonium persulphate (APS) [0.3% (w/v) final; Bio-Rad] and TEMED [N,N,N',N' tetraethylenemethyldiamine, 0.0006% (w/v) final; BDH Laboratory].

For direct immunoblot 30µg of each cell lysate was boiled for 5 min with Laemmli sample buffer [80mM Tris-HCl pH 6.8, 2% (w/v) SDS, 15% (w/v) glycerol, 100mM dithiothreitol, 2mM EDTA and 0.001% (w/v) bromophenol blue] and fractionated by SDS-PAGE on 6%, 8% or 15% polyacrylamide gels, as more convenient. Electrophoresis was carried out at a constant current of 30-40mA in running buffer containing 30 g/l Tris base, 144.2 g/l glycine and 1% (w/v) SDS. Proteins were stacked through 2 cm of stacking gel [4% polyacrylamide, 125mM Tris-HCl pH 6.8 and 0.1% (w/v) SDS]. Proteins were fractionated alongside broad range prestained SDS-PAGE standards (Bio-Rad Laboratories) and full range individually coloured Rainbow markers (Amersham Life Science).

#### **2.4.4 Immunoblotting**

Following electrophoresis, proteins were transferred to a nitrocellulose membrane Hybond-c extra (Amersham Life Science) by electrophoretic transfer in a wet tank blotting system (Bio-Rad Laboratories Trans-Blot cell). The transfer was carried out in transfer buffer [25mM Tris, 190mM glycine and 20% (v/v) methanol] for 4 hours at a constant current of 60mA at 4°C or alternatively overnight at a constant current of 20mA (4°C).

The nitrocellulose membrane was then blocked by incubation in 5% (w/v) skimmed milk powder (Marvel, Premier Brands) and 0.05% (v/v) Tween-20 (BDH Laboratory) in 0.5X PBS (PBS/Marvel) at room temperature for 1 hour. The filter was then incubated overnight with the primary antibody diluted

in PBS/Marvel at the indicated dilutions (see Paragraph 2.4.5). After which the filters were then washed three times (15 min each at room temperature) in 0.05% (v/v) Tween 20 and 0.5X PBS (PBS/Tween) prior to incubation with horseradish peroxidase (HRP) conjugated secondary antibody (Amersham Life Sciences ECL<sup>TM</sup> western blotting analysis system) diluted 1:2000 (or 1:5000) in PBS/Tween for 45 min. Following three further washes (15 min each at room temperature) in PBS/Tween, the filters were developed in the HRP detection reagents according to manufacturer's instructions ECL<sup>TM</sup> (Amersham Pharmacia Biotech). Briefly, 3ml of solution 1 were added to 3ml of solution 2 and immediately poured onto the drained membrane. Incubation was performed for 1 min and the membrane was wrapped with Saran-wrap and exposed to an auto-radiographic film (Kodak Biomax<sup>TM</sup> MR). The films were developed with an AGFA X-ray film processor.

#### **2.4.5 Antibodies**

Anti  $\alpha$ -glucosidase ( $\alpha$  subunit) rabbit polyclonal antibody (VAP-PT034) was from Stressgene; HSP27 mouse monoclonal antibody (Ab-1 G3.1) was from NeoMarkers; HSP70 (W7) mouse monoclonal and p21<sup>WAF1/Sdi1/CIP1</sup> (C-19) rabbit polyclonal were from Santa Cruz Biotechnology; cyclin A mouse monoclonal (Ab-4) was from CALBIOCHEM; anti CLP-36 (AB 5754) rabbit polyclonal was from CHEMICON International;  $\beta$  actin (A 5316) mouse monoclonal was from Sigma.

p19<sup>ARF</sup> rabbit polyclonal was kindly provided by A. Llyod (University College of London). The gelsolin mouse monoclonal antibody was kindly provided by H. Yin (University of Texas); anti TUC-2 (C4G) was a gift from Y. Hiara (University of Tokyo), while the antibodies anti TUC-1 (C1), anti TUC-3 (C3), anti TUC-4 (Ab25) and anti TUC4- $\beta$  (C4V) were a gift from C. Quinn and S. Hockfield (Yale University School of Medicine).

The antibodies used for western blots were diluted as follow:  $\alpha$ -glucosidase (VAP-PT034) 1:500; HSP27 (Ab-1 G3.1) 1:400; HSP70 (W7) 1:600; p21<sup>WAF1/Sdi1/CIP1</sup> (C-19) 1:500; cyclin A (Ab-4) 1:200; CLP-36 (AB 5754) 1:400;  $\beta$  actin (A 5316) 1:15000; p19<sup>ARF</sup> 1:1000; gelsolin 1:500; TUC-2 (C4G) 1:10000; TUC-1 (C1) 1:500, TUC-3 (C3) 1:1000, TUC-4 (Ab25) 1:2000 and TUC4- $\beta$  (C4V) 1:200.

## **2.5 2-D gel electrophoresis**

### **2.5.1 Sample preparation**

Lysis was undertaken with a solution containing 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 9M urea, 2M thiourea, 65mM dithiothreitol (DTT), 0.8% w/v Resolytes 3-10 (Bio-Rad), and trace bromophenol blue to a frozen cell pellet containing approximately 150 $\mu$ g of proteins, to bring the final volume to 925 $\mu$ l. This was vortexed, left to stand for 5 min, vortexed again then centrifuged at 13000rpm for 5 min at 15°C. 370 $\mu$ l of the solution were added to a channel in the re-hydration chamber and then the immobilised pH gradient gels (IPG) (Immobiline DryStrip 3-10 NL, Amersham Pharmacia Biotech) was layed on top of the lysis mix and let to rehydrate overnight covered with mineral oil.

### **2.5.2 First dimension, isoelectric focusing (IEF)**

The following day the sample was focused overnight 70kVh, 20°C according to the protocol described by Sanchez *et al.* (Sanchez *et al.*, 1997).

### **2.5.3 Second dimension, SDS-PAGE**

Immediately after being focused, IPG gels were equilibrated in 6M urea, 2% w/v SDS, 2% w/v DTT, 50mM Tris pH 6.8 and 30% v/v glycerol for 15 min before running in the second dimension on gradient polyacrylamide gels (9-16%), with the gel bound to one of the glass plates. The gels were run in an electrophoresis tank similar to that described by Amess *et al.* (Amess and Tolkovsky, 1995) at 30mA per gel and 16°C.

### **2.5.4 Staining and scanning**

Immediately after electrophoresis, gels were fixed in 40% v/v ethanol: 10% v/v acetic acid and stained with the fluorescent dye OGT 1238 (proprietary of Oxford GlycoSciences), and 16-bit monochrome fluorescence images at 200µm resolution were obtained by scanning gels with an Apollo II linear fluorescence scanner (Oxford GlycoSciences, Oxfordshire, U.K.).

## **2.6 Curation and analysis**

### **2.6.1 MELANIE II**

Primary images were processed with a customised version of MELANIE II (GeneBio, Geneva, Switzerland). Individually resolved protein features were enumerated and then quantified on the basis of fluorescence signal intensity. The pI and molecular weight of each feature were calculated by bilinear interpolation between landmark features on each image previously calibrated with respect to *E. coli* proteins. Intensity was measured by summing pixels within each feature boundary and recorded as a percentage of the total feature intensity on the image. The index [pI, relative molecular weight (RMM),

sample of origin], and percentage intensity data for each gel feature were entered into a database table.

The resulting definition of protein features found in each given gel was referred to as the PEM (protein expression map). For purposes of comparison between PEMs from different samples, it was necessary to assign to each individual protein a master index [referred to as the molecular cluster index (MCI)], which established the correspondence (on the basis of pI and RMM) between equivalent proteins across the entire set of PEMs. This process was achieved by using software (MELANIE II) to match the images and was manually checked. The algorithms underlying this process have been described by Wilkins *et al.* (Wilkins *et al.*, 1996). The purpose was to normalise individual PEMs into a single and coherent geometry, thereby allowing the precise construction of a protein expression database for all samples under investigation.

### **2.6.2 CHIMAP (change in intensity of multiply associated PEMs)**

CHIMAP (change in intensity of multiply associated PEMs) is a new program developed by M. Zvelebil (Ludwig Institute for Cancer Research, London, UK) to identify features that are interesting for identification by mass spectrometry. The program enables to track changes in protein expression over a set of defined parameters such as substrate-activation or time (Harris *et al.*, 2002). CHIMAP calculates percentage differentials across a set of matched spots in many gels with respect to a reference gel (Reference Gel). The differentials are given both as numbers and in a graphical format. The graphical format allows the easy identification of spots that have either increased expression or decreased expression with respect to the reference gel (Fig. 3.4). Reference gels can be changed at will and new differentials calculated interactively. Spots above or below a chosen cut-off can be selected for further analysis by mass spectrometry methods (Harris *et al.*, 2002).

## **2.7 Mass spectrometry**

The differentially regulated spots were then excised (using a customised OGS cutter, Apollo) and analysed by mass spectrometry to obtain their identity. To ensure that there was enough protein for the MS analysis, the features were excised from the gel in which they were most abundant.

The mass spectrometry methods are presented here for completion but they were carried out by the Analytical Biochemistry Laboratory of the Royal Free University College Branch of the Ludwig Institute for Cancer Research.

### ***2.7.1 Protein identification by mass spectrometry***

All identifications of differentially expressed proteins followed a typical approach using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and, if found necessary, electrospray ionisation tandem mass spectrometry (ESI-MS/MS).

After 2-D gel electrophoresis, single gel spots were excised from the gel inside each triplicate that showed the highest expression level for that particular spot. The 2-D gel spots were deposited in SlickSeal tubes (Bioquote, York, United Kingdom) to decrease protein/peptide adhesion to the tube walls.

### ***2.7.2 In-gel digestion***

Prior to mass spectrometry tryptic in-gel digests were obtained from all samples. The 2D-gel pieces were destained and dehydrated by two washes with a volume of 25mM ammonium bicarbonate (ABC) and 50% acetonitrile (ACN) sufficient to cover the gel pieces for 5 min. After each wash the supernatant was removed and discarded. The gel pieces were vacuum-dried in a centrifuge and then rehydrated with 20µl of a 10mM DTT and 25mM ABC

solution and incubated at 50°C for 45 min. Following removal of the supernatant, 20µl of a 50mM iodoacetic acid (IAA) and 25mM ABC solution were added and the samples incubated in the dark at room temperature for 1 hour. The supernatant was then removed and the gel pieces washed twice with a solution of 25mM ABC and 50% ACN and vacuum-dried in a centrifuge. The gel pieces were then rehydrated with 3µl of a 25mM ABC solution containing 30ng modified trypsin (Promega); 15µl of a 25mM ABC solution were added to sufficiently cover the gel pieces and incubated overnight at 37°C. 20µl of a 50% ACN and 5% trifluoroacetic acid (TFA) solution were added and the supernatant extracted and stored in a second SlickSeal tube. One additional extraction of the digest mixture was performed with 20µl of a 50% ACN and 5% TFA solution. The second extraction was added to the first. The recovered digest mixture was then concentrated by vacuum-drying in a centrifuge and then resuspended in 5µl of H<sub>2</sub>O. The samples were stored at -40°C for subsequent mass spectrometric analysis.

### ***2.7.3 Peptide mass mapping by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS)***

For MALDI-MS analysis 0.5µl of the digest peptide mixture was directly spotted on the target plate together with 1µl of a saturated aqueous 2,5-dihydroxybenzoic acid solution (DHB). Sample spots on the target plate were subsequently dried under a stream of warm air. All samples were analysed on a Reflex III reflector time-of-flight (TOF) mass spectrometer (Bruker-Daltonik, Bremen, Germany) equipped with delayed extraction and a Scout384 ion source. Spectra were obtained by averaging 32-128 single-shot acquisitions with a pulsed nitrogen laser (VSL-337ND; Laser Science, Franklin, MA) at a sampling rate of 2GS/s. No numerical data processing such as smoothing or baseline correction was performed on any spectrum. In general, spectra were



internally calibrated using two protonated trypsin autolysis products at the monoisotopic masses of 842.5100 and 2211.1046. If these two ions were absent, the spectra were externally calibrated using the calibration from a former acquisition or a known peptide mixture of an adjacent sample spot. For all peptide mass fingerprints peak masses were assigned by labelling the first monoisotope with a signal-to-noise ratio of at least 2:1.

Applying this criterion the peak mass list usually comprised up to 100 masses. Obvious contamination peaks other than keratin were usually excluded. If keratin contamination was excessive, i.e. keratin was primarily identified, the corresponding keratin peak masses were excluded in subsequent data base searches. The peak mass lists obtained under this protocol were submitted to the database search routine MS-Fit to search the non-redundant protein database compiled by the National Centre for Biotechnology Information (NCBI), USA. The main search parameters generally used were: 1000-150000Da molecular weight range (only exception was feature with ID 15, from the REFs gels, for which 1000-200000Da range was used); full pI range; all species; at least five peptides with a given match;  $\pm 100$ ppm peptide mass tolerance; monoisotopic peptide masses; proteolysis by trypsin; only allowed 2 missed cleavages; cysteine modified by carboxymethylation; N-terminus of peptides changed from glutamine to pyro-glutamic acid; oxidation of methionine; acetylation of N-terminus of protein, and acrylamide modification of cysteine were considered modifications.

#### ***2.7.4 Protein identification by electrospray ionisation tandem mass spectrometry (ESI-MS/MS)***

ESI-MS/MS analysis was usually performed in cases where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous. Additionally, a few samples with sufficient MALDI-MS data for protein identification were analysed by ESI-MS/MS to check on the criteria developed for the

identification by MALDI-MS peptide mass mapping. Generally, the remainder of the digest peptide mixture was run on an ABI 140B microcapillary HPLC (Applied Biosystems, Foster City, USA) using an ABI 112A injector (Applied Biosystems, Foster City, USA) with a pre-injection split of 1:50. After loading the sample on a 0.3 x 1 mm silica C18 (5 $\mu$ m particle size)  $\mu$ -Guard™ column (LC Packings, Amsterdam, The Netherlands) it was washed with two injections of 25 $\mu$ l aqueous 0.1% formic acid solution. Peptide separation was performed on a 0.075 x 150 mm silica C18 (5 $\mu$ m particle size) PepMap™ column (LC Packings, Amsterdam, The Netherlands) with a flow rate of approximately 200 nl/min using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. After equilibration with 10% solvent B for 30 min the concentration of solvent B was linearly increased to 40% within 30 min followed by an increase to 60% within 10 min and a further increase to 80% within 5 min. There it was held for another 1 hour; the PepMap™ column was coupled to a 50 $\mu$ m I.D. fused silica capillary via a zero dead volume connector, which was coupled to the standard nano-LC interface of a Q-TOF hybrid quadrupole orthogonal time-of-flight mass spectrometer (Micromass, Wythenshawe, United Kingdom). Mass spectra were acquired using the MassLynx 3.3 software package with automatic precursor ion selection. Survey scans were performed from 400-2000Da. In MS/MS mode the mass window was set to 50-2000Da. The collision energy in MS/MS mode was alternated between 28 and 32eV every two sec. Mass spectral data was separately integrated for each collision energy over 4 cycles, 8 sec. in total. Precursor ion selection criterion was 4 counts/sec. The collision gas was Argon, whereas nitrogen was used as desolvation gas at 50 l/h and as nebulising gas at 0.5bar. Of all the automatically acquired MS/MS spectra only those obtained from an unknown doubly charged precursor ion were considered for further protein identification. Within this subset all spectra belonging to the same precursor ion were combined and subsequently smoothed and ion signals were centroid-centred. Wherever it was possible to assign at least ten fragment ions with

more than ten counts per ion signal in a processed spectrum, database searches were undertaken using the MS-Tag search routine. All other MS/MS data was classified as insufficient for database searches. The fragment ion mass list for MS-Tag searches usually comprised ten masses starting with all masses of fragment ion signals with a mass-to-charge value higher than the precursor ion's and an ion signal count of more than five. In cases where ten or more fragment ion signals fulfilled the above criteria, the ten strongest ions were chosen. In all other cases the strongest fragment ion signals with a mass-to-charge value below the precursor ion's and an ion signal count of more than ten were selected to complement the fragment ion mass list. The fragment ion mass lists obtained under this protocol were submitted to the database search routine MS-Tag to search the non-redundant protein database compiled by the NCBI. The main search parameters generally used were: search mode by identity; no molecular weight restrictions; full pI range; all species; maximum of 2 unmatched ion;  $\pm 200$ ppm parent and fragment ion mass tolerance; monoisotopic peptide masses; only allowed 2 missed cleavages; cysteine modified by carboxymethylation; allowed fragment ion types: a, a-NH<sub>3</sub>, b, b+H<sub>2</sub>O, b-H<sub>2</sub>O, b-NH<sub>3</sub>, y, y-H<sub>2</sub>O, y-NH<sub>3</sub>, internal.

## **2.8 RNA manipulations**

### **2.8.1 RNA isolation**

RNA was extracted by lysing the cells directly on the culture dish. The media was removed and the cells washed once with 1X PBS. 4ml of TRIzol (Life Technologies) were then added to each 15 cm diameter dish and the cells were left to lyse for 15 min at room temperature. The cell lysates were then passed several times through a 5ml pipette, after which the homogenised samples were incubate for 5 min at room temperature. 0.2ml of chloroform, per ml of TRIzol used, were then added, and the samples vigorously shaken by hand for 15 sec and incubated at room temperature for 3 min. The samples

were centrifuged at 12000rpm for 15 min at 4°C. Following centrifugation, the aqueous phase of the mixture was transferred to a fresh tube and the RNA extracted with isopropyl alcohol (0.5ml of isopropyl alcohol for each ml of TRIzol used). The samples were incubated at room temperature for 10 min and then centrifuged at 10000rpm for 10 min at 4°C. The supernatant was removed and the pellet washed once with Ethanol 75% (1ml of Ethanol for each ml of TRIzol used). The RNA pellet was then briefly air-dried and, once dried, resuspended in 50-100µl of DEPC treated H<sub>2</sub>O (0.1% diethyl pyrocarbonate dissolved in ddH<sub>2</sub>O) and incubated at 55-60°C until pellet was completely dissolved.

### ***2.8.2 RNA quantification***

After the extraction, in order to determine the RNA concentration, the optical density of the solution was measured at 260nm (OD<sub>260</sub>) using a Bio-Rad spectrophotometer (Bio-Rad Smart Spec™ 3000 Spectrophotometer). RNA concentration was calculated using the relationship:

$$1\text{OD unit at } 260\text{nm} = 40 \mu\text{g/ml RNA}$$

The RNA was then checked on agarose/formaldehyde gel electrophoresis.

### ***2.8.3 Agarose/formaldehyde RNA gel electrophoresis***

RNA fragments were loaded with 1.5X RNA sample loading buffer (0.06% bromophenol blue, 0.06% xylene cyanol, 1.5X MOPS, 9% formaldehyde, 60% deionised formamide) and fractionated by electrophoresis on 1% (w/v) formaldehyde gels. The gel was made by melting 1g of agarose in 74ml of water and 10X MOPS (0.2M MOPS, 0.05M sodium acetate, 1mM EDTA pH 7.0) and cooled for few min at room temperature. 16ml of 37%

formaldehyde (w/v) were then added while stirring, and a 0.5-1.0 cm thick gel was then poured and allowed to solidify. Before loading on the gel the RNA samples were denatured in the presence of 2 volumes of 1.5X RNA sample buffer for 15 min at 65°C. To each sample 1µl of 1 mg/ml ethidium bromide was added after which they were chilled on ice for 2 min.

Electrophoresis in 1X MOPS was carried out in electrophoresis tanks and RNA was separated at a constant voltage of 100V for 2 or more hours. Ethidium bromide stained RNA fragments were visualized on a UVP (Dual intensity ultraviolet trans-illuminator) and an image was reproduced and printed with a Sony video graphic printer.

#### ***2.8.4 Reverse transcription (RT) of RNA***

cDNA was reverse transcribed from equal amounts (2µg) of total RNA using Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT). The SuperScript<sup>TM</sup>II kit from GibcoBRL was used according to manufacturer's instructions. The RNA samples were heat-denatured at 65°C for 10 min in the presence of 0.5µg of oligo dT primers (Promega), and 0.5mM dNTPs (Promega) and quickly chilled on ice. Then ribonuclease inhibitor (rRnasin, Promega) and DTT (0.1M) were added to the mixture together with the First-Strand buffer and incubated at 42°C for 2 min. Then M-MLV RT enzyme (200units) was added and incubated at 42°C for 50 min. The reaction was subsequently heat inactivated at 70°C for 15 min to denature the RNA-DNA duplex and inactivate the reverse transcriptase, and then chilled on ice. The resulting cDNA was stored at -20°C.

### 2.8.5 *Semi-quantitative Polymerase Chain Reaction (PCR)*

PCR is a technique used to amplify specific regions of DNA. The process uses oligonucleotides that hybridise to opposite strands of the template and flank the region to be amplified. The primers are orientated towards each other, such that repeated cycles of heat denaturation, primer annealing and extension using a thermostable DNA polymerase, result in an exponential increase of DNA product corresponding to the target region.

For each gene studied an optimal cycle number was established that enabled the bands to be visible on a gel and did not result in saturation of the amplification procedure. This was partially done by confirming that the same relative intensities were obtained when the cycle number was increased by 2 cycles.

The sequences of the primers used for PCRs are given in Supplementary Information I, along with annealing temperatures, numbers of cycles and MgCl<sub>2</sub> concentrations.

All PCR reactions were carried out in a volume of 50µl, which contained 1µl of the resulting cDNA mixture from the RT reaction. The PCR reaction also contained 0.5µg of each oligonucleotide primer, 2.5units *Thermus aquaticus* (Taq) DNA polymerase (Promega) in 10mM Tris HCl pH 9, 50mM KCl and 0.1% (w/v) Triton X-100 (10X PCR buffer from Promega), 0.5mM dNTPs (Promega) with 1.5-2.5mM MgCl<sub>2</sub> (Promega). The optimum MgCl<sub>2</sub> concentration was titrated for each primer set. The PCR reactions were performed in the PTC-200 Peltier Thermal cycler thermocycler.

A 5 min, 95°C denaturation step was used before amplification; the following amplification parameters were: denaturation at 94°C for 1 min; annealing at the specific temperature for each primer pair (see Supplementary Information I) for 1 min; extension at 72°C for 1 min, and a final extension of 5 min at 72°C after the last cycle. For each PCR reaction control amplifications were carried out which did not contain respectively any cDNA, left primer,

right primer or Taq DNA polymerase to check for contamination of the reaction mixture. The PCR reactions were resolved alongside molecular weight markers, Low DNA Mass<sup>TM</sup> ladder (GibcoBRL) on agarose gels; ethidium bromide stained DNA fragments were visualised on a UVP (Dual intensity ultraviolet trans-illuminator) and images reproduced and printed with a Sony video graphic printer.

All the PCR primers were designed using Primer3 version software ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)).

## **2.9 Microarrays**

### **2.9.1 *Generation of fluorescent-labelled cDNA targets***

25µg of total RNA were used for each hybridisation reaction. Where necessary the RNA was precipitated by adding 1/40 volume of 3M sodium acetate pH 5.2 to the RNA mixture and the precipitation was allowed to occur at -70°C for 20-30 min. The mixture was then spun for 10 min at 12000g to pellet the RNA. The pellet was subsequently washed in 100µl of 70% ethanol, air-dried and then resuspended in the following mixture: 2.9µl DEPC ddH<sub>2</sub>O and 2.5µl anchored oligo-dT<sub>17</sub> (2 µg/µl). The RNA/oligo mixture was then heated to 70°C for 10 min and snap-chilled on ice.

A reaction mix (15.4µl RNA/oligo mix; 6.0µl 5X first strand buffer [GibcoBRL]; 3.0µl 0.1M DDT [Invitrogen]; 0.6µl dNTP mix [25mM dATP, dTTP, dGTP and 10mM dCTP]; 3.0µl dCTP-Cy3 or dCTP-Cy5 [1mM stock] [Amersham] and 2.0µl Superscript II [200 unit/µl] [Invitrogen], in which the final volume was brought to 30.0µl with DEPC ddH<sub>2</sub>O) was added and the samples incubated at 42°C for 2 hours. After the addition of 1.5µl of 1M NaOH the samples were further incubated at 70°C for 20 min to hydrolyse the RNA. The reaction was then neutralised by adding 1.5µl of 1M HCl.

The nucleotides and the short oligomers were then removed using the AutoSeq<sup>TM</sup> G-50 columns (Amersham Pharmacia, BioTech). Once labelled the ss (single-stranded) cDNA purified through the G-50 columns was approximately 33µl in volume and faintly coloured in pink or blue depending on which Cy dye has been incorporated.

### ***2.9.2 Competitive hybridisation of labelled cDNAs onto microarrays***

The two cDNA samples were then combined as follow: 33µl cDNA sample 1 (Cy3-labelled); 33µl cDNA sample 2 (Cy5-labelled); 4µl polyA DNA (2 µg/µl) (Sigma); 4µl mice C<sub>0</sub>t1 DNA (2 µg/µl) (Invitrogen); 0.7µl 3M sodium acetate pH 5.2 and 218µl 100% ethanol (the final volume was approximately 293µl). cDNAs, polyA and C<sub>0</sub>t1 DNA were then precipitated at -70°C for 20 min. The sample was then pelleted, washed briefly in 70% ethanol, and then dried thoroughly. The pellet was then resuspended in 40µl of the hybridisation buffer (5X SSC, 6X Denhardt's solution, 60mM TrisHCl pH 7.6, 0.12% sarkosyl and 48% formamide) previously filter sterilised. 8µl of ddH<sub>2</sub>O were then added to bring the final volume to 48µl. The cDNA mixture was placed in 100°C water bath for 5 min, then removed and left cool to room temperature for 10 min, after which the samples were spun briefly to remove the evaporated liquid from the lid of tube. The samples were then applied with the hybridisation buffer onto the centre of a cover slip (25 x 60 mm) (BDH) placed on a flat surface. The microarray slide, DNA side up, was then placed in a humid hybridisation chamber (2 x 7 cm 3 mm paper moistened with 2ml of 40% formamide and 2X SSC in a Petri dish sealed with autoclave tape) to prevent the buffer from evaporating during the hybridisation. The samples were then incubated at 47°C for 12-24 hours.

After the hybridisation the microarray slides were removed from the humid chamber and quickly placed in slide rack submerged in 100-200ml of



room temperature wash solution 1 (2X SSC, filter sterilised). The cover slips were expected to slide off the array within 10-15 sec of being placed in the wash solution, at which point it could be carefully removed without scraping against the microarray slide. The slides were then washed at room temperature for 5 min with gentle shaking and then transferred into a slide rack containing wash solution 2 (0.1X SSC and 0.1% SDS, filter sterilised) at room temperature for 30 min with gentle shaking. The last wash was repeated two times. The microarray slides were then transferred to a slide rack containing the wash solution 3 (0.1X SSC, filter sterilised) and then washed at room temperature for 5 min with gentle shaking. Finally the slides were centrifuged at 1000rpm for 1-2 min to dry.

### ***2.9.3 Microarray image acquisition***

Laser scanning of the chips was performed after the hybridisation and post-hybridisation washes, to achieve a quantitative evaluation of each individual complementary sequence present on the sample. To acquire images a laser-based scanner was used, the ScanArray 4000 Microarray Autoloader from Packard BioChip Technologies (for more information see: <http://www.packardbioscience.com/products/446.asp>). The pixel intensity of each spot results proportional to the number of dye molecules and hence the number of probes hybridised with the spotted PCR product. The scanning was performed at the two wavelengths compatible with efficient excitation for Cy3 and Cy5 and the two resulting images acquired. The scan was performed as soon as possible after the hybridisation/washing, since fluorophore emission levels decreases with time once the slides have been dried. This is particularly a problem for Cy5, which decreases its emission levels by approximately 50% per week.

### **3 Differential proteomic analysis of REFs**

#### **3.1 Objectives**

Even though a variety of traditional approaches have been utilised to try to identify the underlying changes that are the causes of replicative senescence, this process is still not fully understood, probably because all the methods adopted so far have been insufficient to comprehensively analyse such a complex biological process.

However new approaches directed to address complex biological systems are becoming available; these novel techniques are known as “post-genomic era techniques” and they have the potential to monitor global changes in gene expression.

Proteomics is one of the “post-genomic era techniques”. It allows all the proteins expressed by a certain cell type under a particular condition (“proteome”) to be studied. The proteins are first separated by 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and then identified by mass spectrometry (MS).

This thesis describes one of the first proteomic applications for studying replicative senescence. The changes in the protein expression profiles of a given cell population upon replicative cellular senescence were analysed by 2D-PAGE and the differentially expressed proteins identified by MS.

The proteomic approach was chosen because it has the potential not only for identifying changes in protein expression, but also post-translational modification, stability and even changes in cellular localisation.

Since proteomic studies do occasionally identify false positives, it is necessary to validate any 2-D work. In the present study the validation has been done by one-dimensional (1-D) western blots using antisera where available. Furthermore some of the candidates were also investigated in two

other models commonly used to study replicative senescence: ras-induced premature senescence of REF52 cells and replicative senescence of mouse embryo fibroblasts (MEFs).

Some of the differentially expressed proteins were also investigated at the RNA level to check whether there was any correlation between protein and mRNA amount.

As a further step of this study it was necessary check whether any of the differentially expressed proteins were actively involved in replicative senescence, if they were actively inducing/suppressing replicative senescence or if they were markers for this biological process. To address these questions, ectopic expression of the best candidates in primary rat embryo fibroblasts was undertaken, and their effects analysed.

### **3.2 Experimental procedures**

Serially passaged rat embryo fibroblasts (REFs) have been chosen as a model system to study replicative cellular senescence. REFs, rather than primary human fibroblasts, have been preferred in order to minimise differences due to epigenetic variation between cells obtained from different donors. The issue of epigenetic variation was critical because in such a study it was necessary to be able to repeat the passaging with freshly isolated identical cells, to prepare protein extracts for validation of the proteome analysis and also to extract RNA for determining whether changes at the protein level correlate with changes at the RNA level. It was not recommended to freeze down primary cells for passaging at a later stage, because it has been shown that when cells are serially cultivated after freezing they exhibit an altered finite mitotic life span (Ikram *et al.*, 1994).

REFs were also preferred to human fibroblasts because human fibroblasts have a much longer finite proliferative life span *in vitro*. Human fibroblasts are capable of undergoing 50-60 divisions before undergoing

replicative senescence in contrast to 20-30 divisions for rodent embryo fibroblasts. This renders the REFs model easier to handle.

Changes in protein profiles upon cellular senescence were monitored by high-resolution 2-D PAGE and differentially expressed protein spots were identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and nano-HPLC electrospray ionisation tandem mass spectrometry (ESI-MS/MS).

### **3.3 Sample preparation**

#### **3.3.1 *Primary cells***

The primary REFs were passaged according to the Todaro and Green 3T3 cell regime (Todaro, 1963), until they ceased to divide, which took about 5 passages for REFs. Cultures were also continually examined microscopically to ensure that there were no mitotic cells visible when passaging had ceased. In addition cells that reached passage 5 (P5) were detached and re-plated to ensure that their numbers no longer increased.

The 3T3 protocol involved plating  $2 \times 10^6$  cells per 15 cm dish. Cells were plated on day 0 and allowed to adhere. The media was then changed on day 1 and the cells passaged on day 3. For preparation of cell lysates, cells were fed with fresh medium on day 2. In this way all lysates were prepared from cultures that were kept in fresh medium and sub-confluent. This was to ensure that cells were not in the quiescent state ( $G_0$  phase of the cell cycle). Total protein extracts were therefore prepared from serially passaged REFs at P2 (proliferating cells) and P5 (senescent cells), and also from P3 and P4 intermediate passages.

### ***3.3.2 Total protein extraction***

Because there were no previously published studies with the use of proteomics to study replicative senescence, it was decided to begin the analysis of replicative senescence using a global proteome approach.

This approach was used to gain an overall picture of replicative senescence, a complex and poorly understood phenomenon, even though it was documented that the detection and analysis of very low abundance proteins while using total cellular extracts could be challenging.

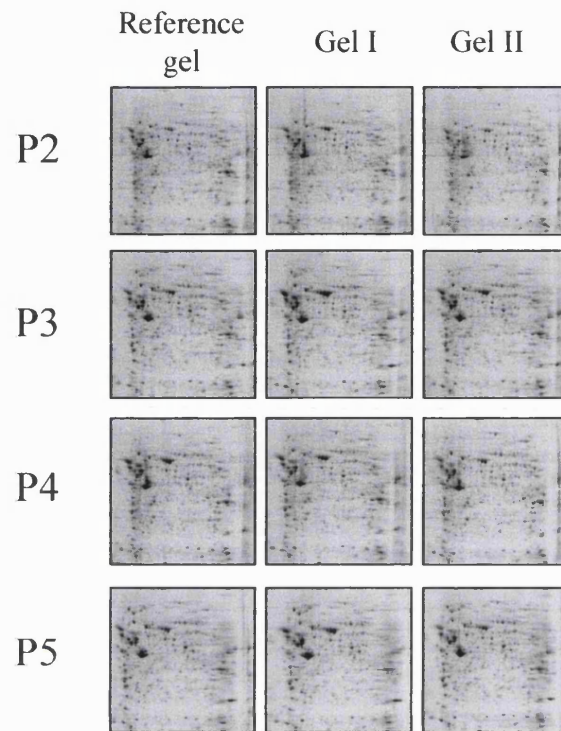
Total protein extracts were therefore prepared and separated on 2-D gels, to study the differential protein profiles upon cellular senescence.

The next step would be to enrich for rare or less abundant proteins prior to 2-D PAGE separation by sub-cellular fractionation or protein pre-fractionation (see Chapter 1).

### ***3.3.3 Independent triplicates***

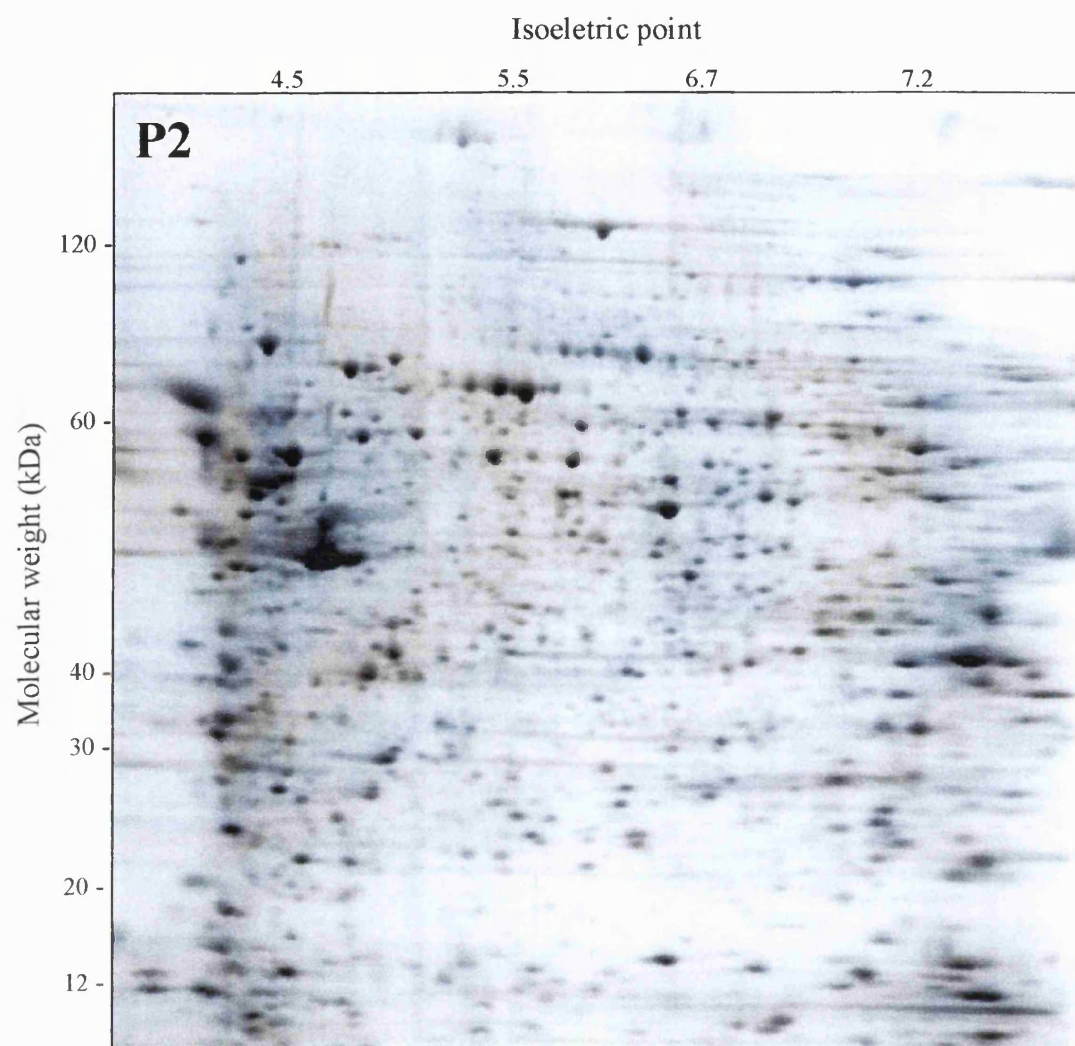
To minimise as much as possible all the sources of variability (experimental and operator related variability, as well as the variability caused by the micro-environment), three independent cellular extracts were prepared for each cellular condition. The extracts were then kept separate all through the process, and they were fractionated on different 2-D gels.

This procedure yielded three independent 2-D gels for each cellular condition studied. An image of all the 2-D gels utilised in the study is represented in Fig. 3.1.



**Fig. 3.1** Triplicate gels.

For each cellular condition three independent extracts were prepared and separated on three independent gels. P2, represents proliferating cells, P5 senescent cells, while P3 and P4 correspond to intermediate passages.



**Fig. 3.2** Representative REFs 2-D gel.

A representative 2-D gel image of primary proliferating cells obtained upon fractionation of 150 $\mu$ g of total cellular extract.

### **3.4 2-D gels: separation and detection**

The total protein extracts prepared from serially passaged REFs at P2, P3, P4 and P5 were then fractionated on 2-D gels, to visualise changes in their proteome upon replicative senescence.

The protein separation was performed using broad pH range gels in the first dimension (pH 3 to 10), and gradient SDS-PAGE in the second dimension. A representative 2-D gel image of primary proliferating cells obtained upon fractionation of 150µg of total cellular extract is represented in Fig. 3.2.

The fractionated proteins were then detected by staining the 2-D gels with the fluorescent dye OGT 1238 (proprietary of Oxford GlycoSciences), followed by scanning at a detection level of less than 1ng of protein.

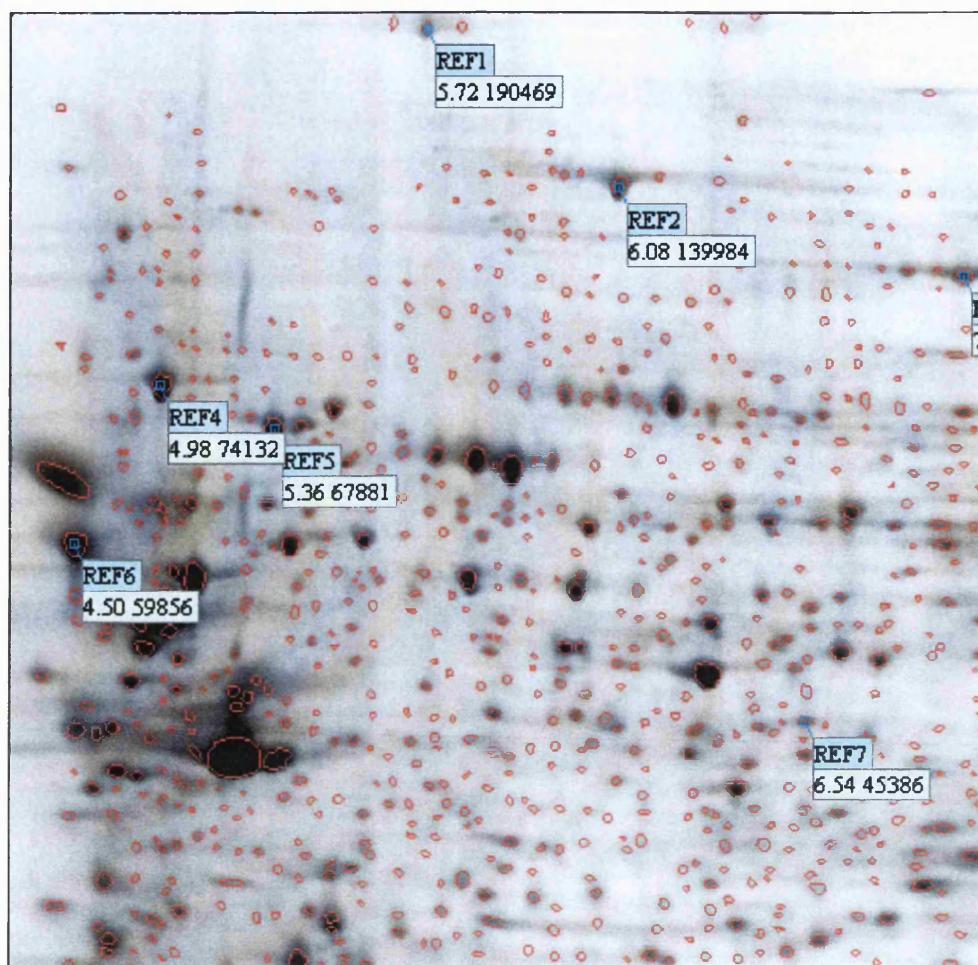
### **3.5 Curation (MELANIE II)**

The primary images were processed with a customised version of MELANIE II (GeneBio, Geneva, Switzerland). The protein spots were detected and quantified on the basis of fluorescence signal intensity.

The MELANIE II software was used to detect the proteins separated on the 2-D gels (Fig. 3.3) (see Paragraph 2.6.1). The proteins separated on the 2-D gels will be referred to here as features or spots, because the same protein can run as different spots on the 2-D gel (different isoforms, same protein but different post-translational modifications, different phosphorylation state etc.) and also the same spot can contain more than one protein, often in different relative amounts.

MELANIE II software was able to detect correctly most of the features, however it was necessary to edit its detection. Therefore after detection of the features separated by a 2-D gel a step of curation of the 2-D gel images was performed before proceeding to a further analysis of the samples.





**Fig. 3.3** Spot detection with MELANIE II software.

The spots were detected and highlighted on a 2-D image by the MELANIE II, a 2-D PAGE analysis software. In the image are also highlighted some of the landmark spots previously calibrated with respect to *E. Coli* proteins.

The curation step was crucial while conducting this proteomics study. The software (MELANIE II) detected as features some stains present on the gels that were not real proteins, or failed to detect others (this last error however was rare and could be overcome by setting the detection levels so as not to miss even the most faint features on the gel). It was therefore necessary to correct the software detection.

It was also possible that when two or more features were very intense or physically very near to one another the software only detected them as one big feature and it was not able to tell them apart. It was thus necessary to manually separate those distinct features detected as single features by the software.

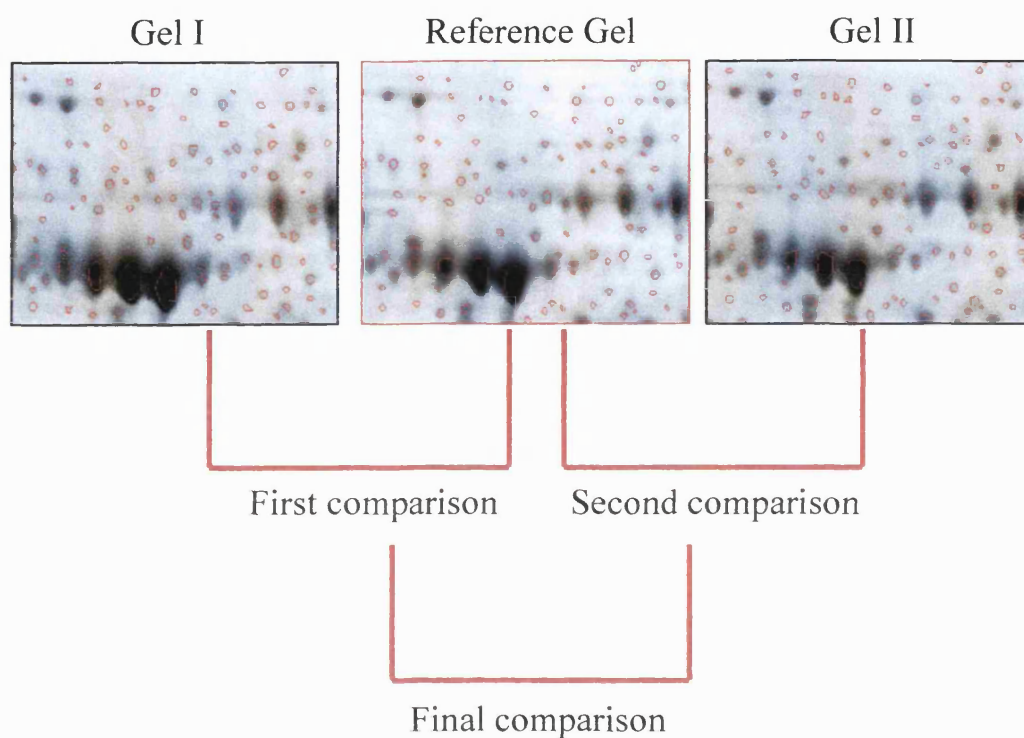
Another error occasionally committed by the software was to modify the real edges of the features making them square while they were not or adding tails or extensions that were not real. It therefore became necessary to edit the edges of the features detected with MELANIE II so as not to compromise the evaluation of the amount of protein present in each spot by those added extensions.

The isoelectric point (pI) and molecular weight of each spot was calculated by bilinear interpolation between landmark spots on each image that had previously been calibrated with respect to *E. coli* proteins. This allowed matching features from different gels.

### **3.5.1 Triplicate curation**

Triplicate gels were scanned and a triplicate curation was performed (Fig. 3.4). Triplicate curation means that the gel with the most spots in each triplicate detected using the MELANIE II software was chosen as the Reference gel. The two other gels in each triplicate were called gel I and II respectively.

First gel I and gel II were curated against the Reference gel. Gel I and



**Fig. 3.4** Triplicate curation.

A triplicate curation was performed to curate the three gels corresponding to the same cellular condition. The gel with the most spots (detected using the MELANIE II software) was chosen as the Reference gel and the two other gels were called gel I and II respectively. First, gel I was curated against the Reference gel and then, gel II was curated against it. Only in the last comparison gel I and gel II were compared to each other.

gel II were then compared to each other, and expected to be identical. The regions and the spots that were found to be different in gel I and gel II were checked in the Reference gel and in this way ambiguities clarified.

Although triplicate curation was an extremely time consuming procedure, it allowed identification of differential features in the first instance using only the Reference gels. However it was necessary, as a follow up, to manually check that all the features differentially expressed in the Reference gel were not only present in the other two gels within the triplicate, but were also differential in the same direction (up- or down-regulation) and that the magnitude of the change in gel I and gel II was at least as big as the magnitude detected in the Reference gel.

After the curation step each gel was found to comprise over 1200 spots.

### 3.5.2 Gel Similarity

The percentage homology (defined below) can be a useful measure of the overall gel quality and similarity in addition to providing a test of a matching algorithm.

The percentage homology (%H) has to be standardised for a particular gel analysis program (in this case MELANIE II) and scanning techniques.

Equation 1 describes %H.

$$\text{Eq. 1:} \quad \% H = \frac{\left( \frac{1}{N} \sum_{i=1}^N F_i \right) / M}{r} \times 100$$

where F is the number of features in a gel, M is the number of matched features between gels N, and r is the reproducibility factor.

Using MELANIE II r has been previously calculated from a subset of

**Table 3.1** Percent homology (%H) between the gels within each triplicate.

	Reference gel	Gel I	Gel II
<b>P2</b>	100	98.6	97.1
<b>P3</b>	100	98.0	99.0
<b>P4</b>	100	95.0	93.0
<b>P5</b>	100	96.0	96.1

Triplicate 2D-gels were run for each cellular condition. The images were then curated and the percentage of homology (%H) among the gels within each triplicate calculated.

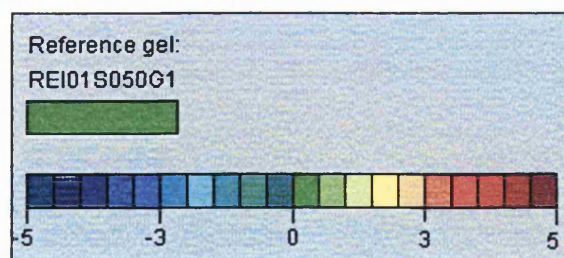
sibling gels to be 0.9555 (Harris *et al.*, 2002). This indicated that about 4% of features in the examined gels were not matched mainly due to problems in the matching algorithms and partially due to curation errors (Harris *et al.*, 2002).

After curation the similarity among the gels inside each triplicate was calculated as percentage of homology of the gels within each triplicate. The %H between the gels was very high, and therefore the gels were considered to be very reproducible within each triplicate. The %H was above 90% in all the sets of triplicate gels (P2, P3, P4 and P5) (Table 3.1). The P2 set gave a score above 97%, P3 above 98% and P5 above 96%. For the P4 set the percentage of homology values were slightly lower, but they were not below 93%.

### 3.6 Analysis of the raw 2-D data

The detected spots were then submitted to CHIMAP to identify those spots that were differentially regulated. CHIMAP is a newly developed program that calculates a differential value between a large series of matched features either as a percentage change or a fold change and represents them graphically (Fig. 3.5) (see Paragraph 2.6.2). CHIMAP generally uses the Ward's minimum variance method although other agglomeration methods can be selected within the program (Harris *et al.*, 2002).

Only the features that changed two-fold or more in magnitude (and in either direction: up or down) when P2 was compared with P5, were considered. The analysis was begun using only the Reference gels, but then it was necessary to check that the changes found in the Reference gels were not only present in the other two gels within each triplicate (gel I and gel II), but were also differential in the same direction (up- or down-regulated) and that the magnitude of the change in gel I and gel II was at least as big as the magnitude detected in the Reference gel. The fold change of the differentials P2 versus P5 (P2vP5) is represented in Table 3.3 A for the up-regulated spots and in Table 3.3 B for the down-regulated features. In the table each spot is represented by



A)

Group ID	S052G1	S056G1	S060G1
103	4.15	2.31	19.84
105	2.95	1.51	4.73
359	2.3	2.03	3.23
710	2.19	1.27	2.16
713	1.08	1.07	1.36
883	1.48	1.69	1.45
1076	1.77	2.43	1.91

B)

Group ID	S052G1	S056G1	S060G1
460	-1.17	-1.09	-2.03
736	-2.27	-2.2	-2.54
863	-2.95	-2.08	-4.67

**Fig. 3.5** CHIMAP.

In (A) are represented some up-regulated spots identified and visualised by CHIMAP, and in (B) some down-regulated spots. The differentials are calculated as differences in % volume in respect to a reference gel. REI01S050G1, S052G1, S056G1 and S060G1 are the reference gels for, respectively, P2, P3, P4 and P5.

its identity number (ID); to unequivocally identify each spot MW and pI are also indicated.

### **3.7 Changes in P3 and P4**

After the comparison of P2 with P5 (and vice versa), the differentials were also analysed in P3 (represented by the column P3vP5 in Table 3.2) and P4 (represented by the column P4vP5 in Table 3.2) to determine whether the changes occurred gradually or in a stepwise manner. Both types of change were found. Most of the changes appeared to occur upon passage 5 (P5); the amount of protein started to increase slightly in P3 and P4 but the major change was detected only when cells underwent senescence (P5).

The up-regulated spot 104 showed a minor increase in P3 and P4 (+1.2 and +1.0 respectively), but the major change occurred in P5 (+2.3). Some other spots showed a more dramatic up-regulation in P5; spots 108, 110, 122, 127, 223, 369, 1093 and 1264 were up-regulated more than 10 fold in P5.

A smaller set of proteins appeared to be regulated from passage P3 and in a stepwise manner; spots 102, 1095 and 1319 showed a consistent increase as early as P3, with the increase enlarging in accordance with the passage number.

A last subclass of changes included spots 185, 190, 248, 426, 677 and 725. These spots were up-regulated more than 2 fold when P2 was compared with P5, and they were also up-regulated more than 2 fold in P3 but the magnitude of those changes was below the threshold in P4 (lower than 2) (Table 3.2 A). Spots 1124 and 1302 in contrast showed the major increase in the protein level at P4, which diminished again in P5 but remained above the threshold.

An analogous pattern of changes was found when the down-regulated spots were studied in the intermediate passages (P4 and P5). Most of the down-regulations occurred in P4 (spots 278, 304, 736, 1054, 1295 and 1299); three



**Table 3.2** Fold change of the differentials.**A) Up-regulated features**

<b>ID</b>	<b>MW</b>	<b>pI</b>	<b>P3vP2</b>	<b>P4vP2</b>	<b>P5vP2</b>
<b>102</b>	125386	5.48	+ 2.3	+ 2.7	+ 3.2
<b>104</b>	125386	6.37	+ 1.2	+ 1.0	+ 2.3
<b>108</b>	124240	6.53	+ 1.3	+ 1.6	> 10
<b>110</b>	123105	5.53	+ 1.4	+ 1.0	> 10
<b>122</b>	114390	5.57	+ 2.0	+ 1.3	> 10
<b>127</b>	113345	5.82	+ 1.7	+ 1.4	> 10
<b>158</b>	98305	5.01	+ 1.5	+ 0.9	+ 2.3
<b>177</b>	933348	6.52	+ 1.3	+ 1.3	+ 2.2
<b>185</b>	91608	4.96	+ 8.6	+ 4.3	> 10
<b>190</b>	91608	5.66	+ 3.1	+ 1.6	+ 4.8
<b>223</b>	83776	5.31	+ 1.4	+ 1.6	> 10
<b>248</b>	81062	7.08	+ 3.6	+ 2.8	> 10
<b>290</b>	74833	6.64	+ 0.8	+ 0.9	+ 2.0
<b>369</b>	67881	5.90	+ 1.9	+ 1.4	> 10
<b>426</b>	64712	6.19	+ 2.2	+ 1.3	+ 2.9
<b>493</b>	60767	5.76	+ 0.9	+ 0.8	+ 2.0
<b>495</b>	60767	6.47	+ 1.6	+ 1.4	+ 3.3
<b>497</b>	60462	6.06	+ 1.6	+ 1.5	+ 2.0
<b>504</b>	60158	6.94	+ 1.0	+ 1.3	+ 2.2
<b>677</b>	47888	5.57	+ 3.0	+ 1.4	+ 3.0
<b>713</b>	45110	5.14	+ 2.4	+ 1.7	+ 3.0
<b>773</b>	45386	6.54	+ 1.9	+ 0.9	+ 3.1
<b>902</b>	40126	6.58	+ 1.3	+ 1.2	+ 2.0
<b>907</b>	40010	5.71	+ 1.7	+ 1.0	+ 2.1
<b>1075</b>	47888	5.79	+ 0.9	+ 1.0	+ 2.0
<b>1076</b>	33986	7.22	+ 1.3	+ 1.3	+ 2.0
<b>1093</b>	23343	5.84	+ 1.8	+ 0.9	> 10
<b>1095</b>	33385	6.42	+ 1.9	+ 1.9	+ 2.0
<b>1124</b>	32329	5.34	+ 1.1	>10	+ 2.0
<b>1264</b>	25789	6.05	+ 0.8	+ 1.1	> 10
<b>1302</b>	24177	5.58	+ 4.3	>10	+ 3.0
<b>1319</b>	23370	5.57	+ 2.3	+ 2.7	> 10

B) Down-regulated features

<b>ID</b>	<b>MW</b>	<b>pI</b>	<b>P2vP5</b>	<b>P3vP5</b>	<b>P4vP5</b>
<b>15</b>	158986	6.38	+ 2.0	+ 1.7	+ 1.4
<b>153</b>	79874	4.76	+ 2.5	+ 1.7	+ 2.2
<b>278</b>	64858	5.09	+ 3.2	+ 1.2	+ 1.3
<b>304</b>	63329	6.09	+ 2.4	+ 1.5	+ 1.5
<b>460</b>	53263	7.39	+ 2.0	+ 1.7	+ 1.9
<b>736</b>	39212	6.13	+ 2.6	+ 1.1	+ 1.1
<b>863</b>	34304	5.41	+ 4.7	+ 1.6	+ 2.2
<b>882</b>	34172	5.57	+ 3.9	+ 2.3	+ 2.8
<b>1054</b>	25301	5.01	+ 2.0	+ 1.2	+ 1.4
<b>1239</b>	14189	5.66	+ 2.2	+ 2.5	+ 1.3
<b>1295</b>	24425	5.87	+ 2.0	+ 1.7	+ 1.6
<b>1299</b>	24549	5.78	+ 2.1	+ 1.4	+ 1.5

A) Representation of the magnitude of the changes for the up-regulated features at different passages; B) down-regulated features.

changes occurred in a stepwise manner in accordance with the passage in culture (spots 15, 882 and 1239), while for other changes it was possible to detect an overall down-regulation from P2 to P5 but without a smooth trend in the intermediate passages (Table 3.2 B).

### **3.8 Protein identification by mass spectrometry**

The spots found to be differentially expressed upon replicative senescence were then excised from the 2-D gels (using a customised OGS cutter, Apollo), and analysed by mass spectrometry to determine their identity. To ensure that there was enough protein for the mass spectrometry analysis, the features were excised from the gel in which they were most abundant.

The differentially regulated spots were first subjected to MALDI-MS peptide mass mapping. The results are presented in Table 3.3 and Supplementary Information II, Table 1. For all spots where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous, ESI-MS/MS was performed. In addition, a few samples with sufficient MALDI-MS data for protein identification were also analysed by ESI-MS/MS to verify the MALDI-MS peptide mass mapping. These results are summarised in Table 3.3 and Supplementary Information II, Tables 2 and 3. Positive protein identification by MALDI-MS was confirmed in all cases where ESI-MS/MS was employed and all putatively identified proteins were confirmed. For the five samples, which were not identified by MALDI-MS, positive protein identification was obtained by ESI-MS/MS.

#### **3.8.1 *Peptide mass mapping by MALDI-MS***

Analysis of the differentially regulated spots by MALDI-MS yielded 24

**Table 3.3** Identification of the differentially expressed features by mass spectrometry.

**A) Up-regulated features**

ID	MW	pI	F.C. P5vP2	MALDI-MS SPOT IDENTIFICATION	ESI SPOT IDENTIFICATION
102	125386	5.48	+ 3.2	Alanyl-tRNA synthetase (h)	
104	125386	6.37	+ 2.3		2-Oxoglutarate dehydrogenase precursor (h)
108	124240	6.53	> 10	*	O-GlcNAc Transferase p110 subunit (r)
110	123105	5.53	> 10	105kDa heat shock protein (m)	
122	114390	5.57	> 10	$\alpha$ -glucosidase II, $\alpha$ subunit (m)	
127	113345	5.82	> 10	$\alpha$ -glucosidase II, $\alpha$ subunit (m)	$\alpha$ -glucosidase II, $\alpha$ subunit (m)
158	98305	5.01	+ 2.3		
177	933348	6.52	+ 2.2	Lysyl hydroxylase isoform 2 (m)	
185	91608	4.96	> 10	Heat shock protein 90- $\alpha$ (h)	
190	91608	5.66	+ 4.8		Gelsolin (h and m) + myosin heavy chain (c)
223	83776	5.31	> 10		
248	81062	7.08	> 10		
290	74833	6.64	+ 2.0	Transferrin (b)	
369	67881	5.90	> 10		
426	64712	6.19	+ 2.9	Vesicle transport-related protein (RA410) (r)	
493	60767	5.76	+ 2.0	$\gamma$ -butyrobetaine, 2-oxoglutarate dioxygenase (r)	
495	60767	6.47	+ 3.3	Chaperonin containing TCP-1, $\gamma$ -subunit (m) + TUC-4 (m)	
497	60462	6.06	+ 2.0	Seryl-tRNA synthetase (h)	Seryl-tRNA synthetase (h) + T-complex polypeptide 1 (r)
504	60158	6.94	+ 2.2	MPAST1 (m)	MPAST (h) + Lamin A or C (r)
677	47888	5.57	+ 3.0	ERF1 (h) + probable ATP-dependent RNA helicase p47 (r)	
713	45110	5.14	+ 3.0	Similar to cdc37 (r)	
773	45386	6.54	+ 3.1	Elongation factor-1- $\gamma$ (h)	Elongation factor-1- $\gamma$ (h)
902	40126	6.58	+ 2.0	Arp2 (h)	Arp2 (h) + hnRNP-E2 (h)
907	40010	5.71	+ 2.1	26S proteasome subunit p40.5 (m)	26S proteasome subunit p40.5 (h)
1075	47888	5.79	+ 2.0	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (r)	
1076	33986	7.22	+ 2.0	CLP36 (r)	
1093	23343	5.84	> 10	Thiol-specific antioxidant protein (r)	
1095	33385	6.42	+ 2.0		
1124	32329	5.34	+ 2.0	*	
1264	25789	6.05	> 10	*	
1302	24177	5.58	+ 3.0	HSP27 (r)	
1319	23370	5.57	> 10	*	

## B) Down-regulated features

ID	MW	pI	F.C. P2vP5	MALDI-MS SPOT IDENTIFICATION	ESI SPOT IDENTIFICATION
15	158986	6.38	+ 2.0	*	
153	79874	4.76	+ 2.5	Sec23 protein (h) + ischemia responsive 94kDa protein (r)	Sec23 protein (h) + ischemia responsive 94kDa protein (r) + HSP70 (h)
278	64858	5.09	+ 3.2	Preimmunoglobulin heavy chain binding protein (r)	
304	63329	6.09	+ 2.4	TUC-2 (r)	TUC-2 (r)
460	53263	7.39	+ 2.0	IMP dehydrogenase (m)	
736	39212	6.13	+ 2.6	30kDa protein (h)	
863	34304	5.41	+ 4.7	Tubulin $\beta$ chain 15 (r)	Tubulin $\beta$ chain 15 (r and ch)
882	34172	5.57	+ 3.9	Transitional endoplasmic reticulum ATPase (r)	
1054	25301	5.01	+ 2.0	$\rho$ GDP dissociation inhibitor (b)	$\rho$ GDP dissociation inhibitor (h and b)
1239	14189	5.66	+ 2.2	Proteasome subunit RC10-II (r)	Proteasome subunit RC10-II (r) + PRx III (r)
1295	24425	5.87	+ 2.0	PRx IV (r)	PRx IV (r) + HSP27 (r)
1299	24549	5.78	+ 2.1		Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (r)

## C) Features which shift in their migration

ID	MW	pI	MALDI-MS SPOT IDENTIFICATION	ESI SPOT IDENTIFICATION
331	71199	6.67	Moesin (r)	Moesin (r)
332	71199	6.56	Moesin (r) + guanosine 5'-monophosphate synthetase (h)	Moesin (r) + guanosine 5'-monophosphate synthetase (h)
1117	22014	6.19		
1348	22134	6.20	Proteasome subunit RC10-II (r)	Proteasome subunit RC10-II (r)
1351	22134	6.10		PRx III (r)

ID: identity number; FC: fold changes; \* Only contaminants such as human keratin were identified

(h): *Homo sapiens*; (m): *Mus musculus*; (r): *Rattus norvegicus*; (c): *Gallus gallus*; (b): *Bos taurus*; (ch): *Chinese hamster*

A) Identity of the features found to be up-regulated on the 2-D gels upon replicative senescence; B) down-regulated features C) features with a shift in their migration.

single positive identifications, 2 double positive identifications, 6 single putative identifications, 1 double putative identification and 1 double mixed (positive and putative) identification (Tables 3.3 and in greater detail as Supplementary Information II, Table 1). Five spots yielded only keratin and in 4 cases more than 20 peptide ion signals were obtained, but database searches with these peptide mass lists did not identify any proteins. For the remaining 6 out of the 49 differential spots, the mass spectra recorded exhibited not more than 10 peptides; these data were classified as insufficient for peptide mass mapping. From all identified proteins only 18 were identified as rat proteins (*Rattus norvegicus*), 9 were mouse (*M. musculus*), 9 were human (*Homo sapiens*) and 2 were bovine (*Bos Taurus*). Interestingly, five proteins identified as non-rat proteins have rat homologues in the NCBI protein database suggesting either extensive nucleotide polymorphisms or incorrect database entries for the rat protein sequences.

### **3.8.2 Protein identification by ESI-MS/MS**

After MALDI-MS was performed for all the excised spots, nano-HPLC ESI-MS/MS was used for almost two thirds of the excised spots (in all cases where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous and for a few other spots as positive controls). The results for the protein identification by ESI-MS/MS (false positives excluded) are also summarised in Tables 3.3 and presented in greater detail as Supplementary Information II, Tables 2 and 3. From all the proteins identified by searching the NCBI protein database only 15 proteins were identified as rat proteins, whereas 3 were from mouse (*M. Musculus*), 1 from chicken (*Gallus gallus*), 1 from *Chinese hamster* and 10 were human (*H. sapiens*). MS/MS data from two spots identified human proteins with some peptides giving matches to the mouse (spot 190) and bovine (*B. Taurus*) (spot 1054) homologues that did not match the human protein. Similarly, MS/MS data from one other spot (spot 863) resulted in

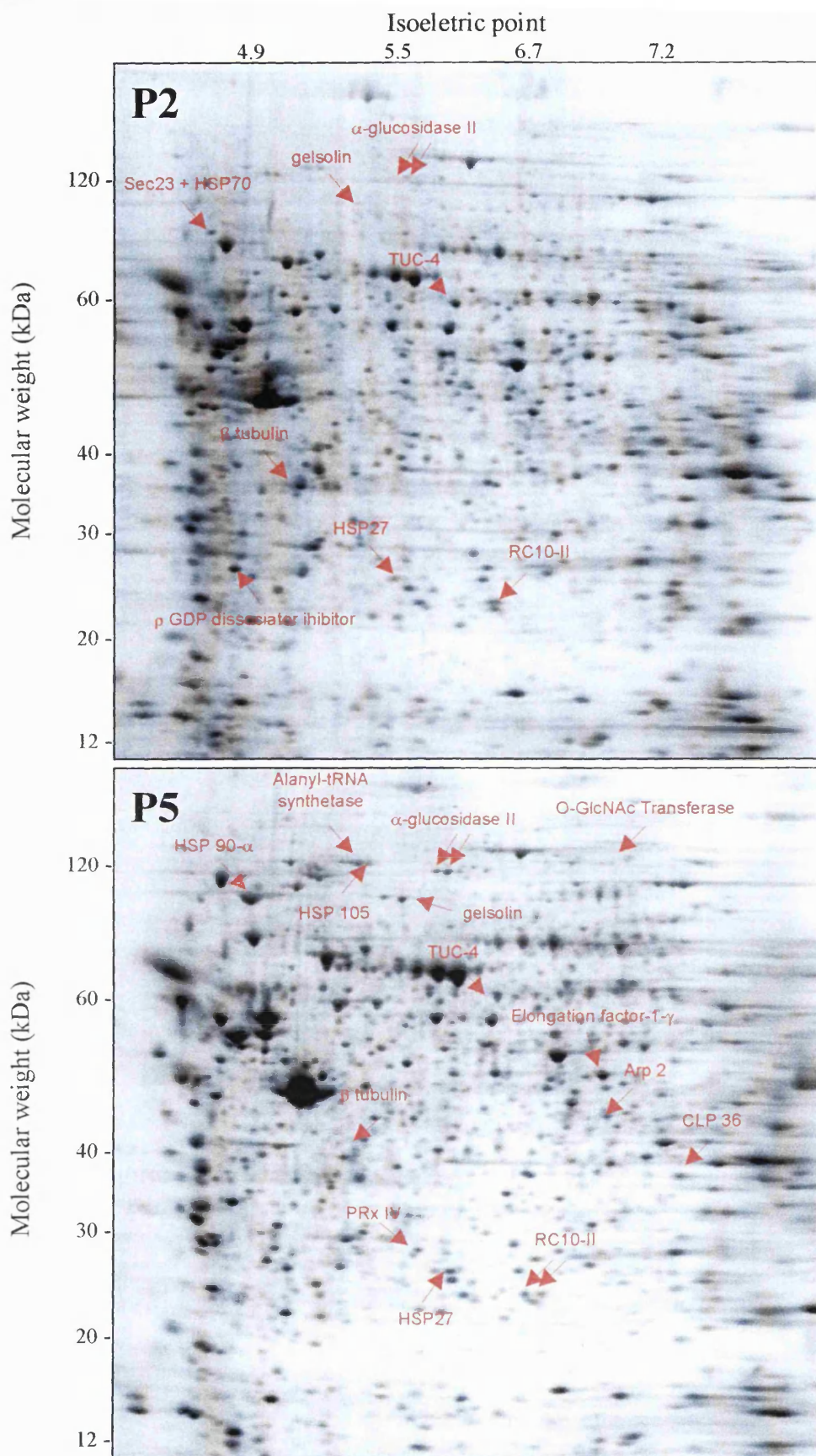
peptide matches not matching the rat protein but the *C. hamster* homologue. Positive protein identifications obtained by MALDI-MS peptide mass mapping were confirmed in all cases where ESI-MS/MS was employed. All the putatively identified proteins by MALDI-MS peptide mass mapping were also confirmed by ESI-MS/MS whenever sufficient data was obtained. In addition to these confirmations three more proteins were identified (t-complex polypeptide for spot 497, lamin A or C2 for spot 504 and heat shock protein 27 for spot 1295). For five previously unidentified spots, positive protein identification was obtained. The comparison of the results from the MALDI-MS and ESI-MS/MS data demonstrated that both techniques show comparable sensitivity in protein identification. Furthermore, this comparison showed that the criteria applied for protein identification was reliable and gave virtually no false identifications, with a minimal loss in analytical sensitivity.

### **3.9 Differentially expressed proteins**

During the global 2-D differential proteome analysis of replicative senescence in serially passaged REFs, 49 spots (~4% of the total features separated and detected on 2-D gels) whose expression was altered more than two-fold were identified; 32 of these spots were up-regulated (three representative up-regulated features are shown in Fig. 3.7 A), 12 were down-regulated (a representative down-regulated feature is shown in Fig. 3.7 B), and 5 displayed an altered migration pattern in the 2-D gel coordinates (two representative features shifting in their migration are shown in Fig. 3.7 C).

Two representative gels for proliferating (P2) and senescent (P5) primary fibroblasts are represented in Fig. 3.6; with highlighted the identities of some of the differential spots.

Mass spectrometry yielded positive protein identification for 39 of these spots. During the mass spectrometry analysis, 82% of the excised spots gave a

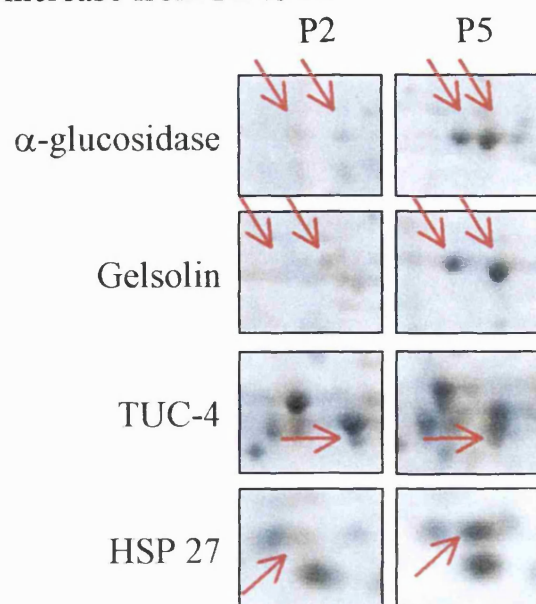


**Fig. 3.6** Representative REFs 2-D gels with highlighted some differentially expressed features.

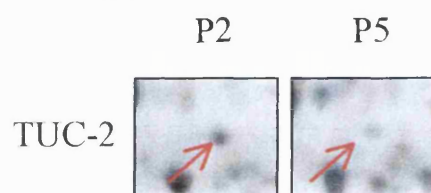
Representative 2-D gels of proliferating (P2) and senescent (P5) rat embryo primary fibroblasts (150 $\mu$ g of total cell extract) highlighting the identities of some of the differential spots.



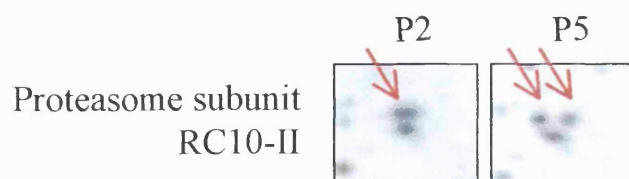
A) Features that increase from P2 to P5



B) Feature that decreases from P2 to P5



C) Feature that shifts in migration from P2 to P5



**Fig. 3.7** Representative features.

2-D gels images showing selected up-regulated features (panel A), a down-regulated feature (panel B) and a feature with an altered migration (panel C).

positive identification and only 5 of them appeared to be contaminated by human keratin.

The differentially expressed proteins comprised a variety of cytoskeletal, heat shock and metabolic proteins as well as proteins involved in trafficking, differentiation and protein synthesis, turnover and modification. Nine spots were shown to contain more than one protein, and they will be referred to here as multiple spots. For the purposes of the discussion below it has been assumed that all the proteins identified by mass spectrometry for each spot were differentially expressed. However this may clearly not be the case for spots that yielded multiple proteins; for these spots western blot analysis using specific antibodies will be required to determine the identity of the differential protein.

### **3.10 Validation of the system: p19<sup>ARF</sup>, p21<sup>WAF1/Sdi1/CIP1</sup> and cyclin A**

To validate the 2-D proteome analysis, REFs derived from freshly prepared embryos were serially passaged using the 3T3 passaging regime previously used to prepare the cell extracts separated on the 2-D gels (Todaro, 1963). Extracts from P2, P3, P4 and P5 were made and analysed for expression by one-dimensional (1-D) western blots.

To ensure that the extracts exhibited changes in expression of proteins known to be involved in replicative senescence, extracts were first analysed for expression of p19<sup>ARF</sup> and p21<sup>WAF1/Sdi1/CIP1</sup>, two markers of cellular senescence known to be up-regulated upon replicative senescence, and for expression of cyclin A, known to be down-regulated upon replicative senescence.

As expected p19<sup>ARF</sup> and p21<sup>WAF1/Sdi1/CIP1</sup> were up-regulated and cyclin A was down-regulated as REFs underwent replicative senescence (Fig. 3.8).

### 3.11 Validation of the 2-D proteome analysis

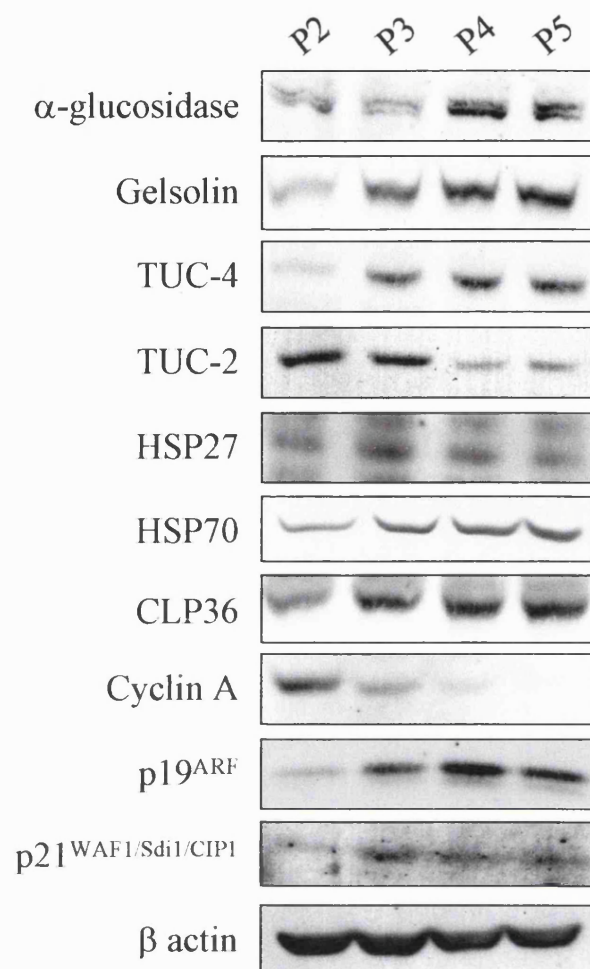
Even though the power of proteomics for identifying potentially important and significant changes in protein expression occurring under any biological condition is enormous, it is possible for false positives to occur. However these can be eliminated with an accurate validation process.

The validation process for any proteomic study can be done at different levels; 1-D western blots can be used to validate both the differential proteome *per se* and the mass spectrometry analysis. However the mere use of 1-D western blots to validate a proteome study can be challenging, and not complete. Most mammalian proteins undergo post-translational modifications and/or are present in cells as multiple isoforms, which could be separated on 2-D gels as multiple spots. It is possible that only one or few of these spots is differentially expressed. Given that in a 1-D western blot all the different post-translational modifications or isoforms of a protein (or at least the ones with very similar molecular weight) run compressed in one single band, it is not likely to detect the differential expression if only one isoform is differential and the overall amount of protein remains unchanged. The perfect validation for a differential proteome study therefore would be to perform western blots of 2-D gels, however this would be rather difficult and very time consuming. Therefore the most commonly used validation process for proteome studies is to perform 1-D western blots for the differentially expressed features.

In the present study, to validate the results obtained from the 2-D gel analysis, 1-D western blots were carried out, wherever possible.

The 1-D western blots were performed using protein extracts prepared from a different batch of primary REFs that had been passaged by the standard 3T3 protocol, since the lysis procedures for preparing extracts for 2-D gels was different than that for the 1-D gels which were carried out in RIPA buffer.

However when this study was performed it was not possible to obtain rat specific antisera for all the candidates, hence the validation was conducted whenever antisera were available and it remains to be completed.



**Fig. 3.8** Validation of 2-D results by one-dimensional western blots.

One-dimensional western blots were performed using 30µg of total protein extracts per sample. α-glucosidase, gelsolin, TUC-4, TUC-2 and CLP36 showed correlation between 2-D and 1-D gels. No correlation was found for HSP27 and HSP70. p19<sup>ARF</sup>, p21<sup>WAF1/Sdi1/CIP1</sup> and cyclin A known to be modulated upon replicative senescence, were used as a positive control; β actin was used as control for equal loading.

The 1-D western blot validation experiments were very robust. Each blot was repeated several times using different batches of extracts prepared from REFs cultured according to the standard 3T3 passaging protocol.

Expression analysis, in REFs by 1-D western blots, of  $\alpha$ -glucosidase, gelsolin, CLP36 and TUC-4 showed that these four proteins were up-regulated from P2 to P5 whereas TUC-2 was down-regulated, in accordance with the proteome analysis (Fig. 3.8).

In contrast, even though HSP27 was identified as an up-regulated feature, during the proteome analysis, it was not found to be differential when analysed by 1-D western blots (Fig. 3.7 A and Fig. 3.8). The most likely explanation for this contradictory result is that HSP27 could be present in cells as multiple isoforms and therefore could be represented on a 2-D gel by multiple features, and only one of these features might be differential. Since in a 1-D all the different isoforms of a protein (or at least the ones with very similar molecular weight) run compressed as a single band it was unlikely that the differential expression of one isoform would be detected if the overall amount of protein remained unchanged. Thus the identity of the form of HSP27 that is differentially expressed upon replicative senescence remains to be determined.

HSP70 was found to be down-regulated in the 2-D gels while it appeared to be up-regulated in the 1-D western blots (Fig. 3.8). The explanation could be similar to the one for HSP27; in this particular case it must be noted that HSP70 was detected as one of the proteins present in a multiple spot (a single spot containing at least three proteins detected by mass spectrometry). Therefore it would be intriguing to check the behaviour of the other proteins in the same spot.

Once the validation had been done using extracts prepared from REFs, a further validation step was carried out. Some of the differentials were tested by 1-D westerns using extracts made from two other commonly used models of senescence: oncogene induced premature senescence of REF52 cells (Serrano *et al.*, 1997) and serially passaged mouse embryo fibroblasts (MEFs) (described

in Paragraph 3.23).

### ***3.11.1 Western blots of 2-D gels***

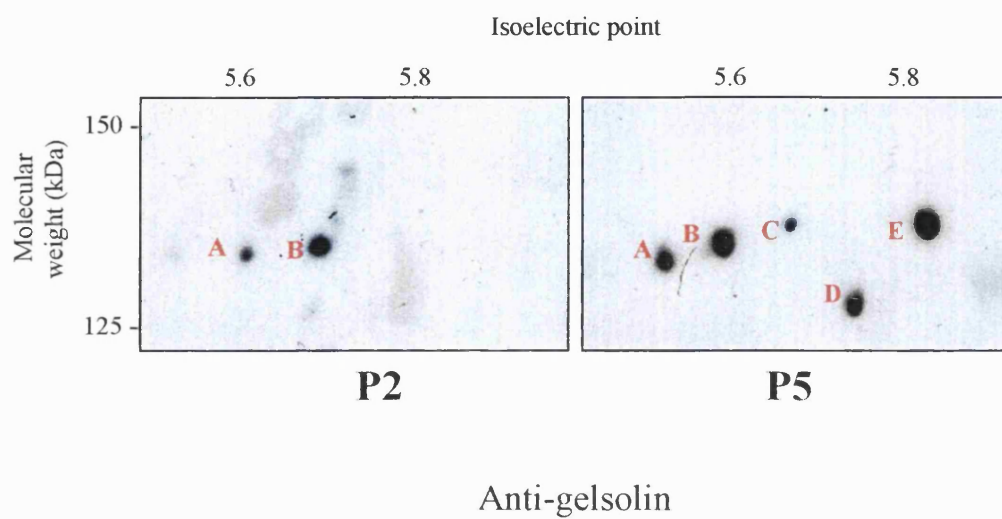
As previously stated, a crucial step in any proteome analysis is to match it with an accurate validation process. The perfect validation for a differential proteome study would be to perform western blots of 2-D gels, to take into account all the post translational modifications and/or different isoforms of the same protein.

Some of the spots found to be differential during the present proteomic study were validated using 2-D western blots. This step of validation was performed for those candidates for whom a clean validation could not be reached using only western blots, and particularly those candidates that appeared to be present on the 2-D gels as different features and therefore present in the cells as different post-translational modifications or multiple isoforms of the same protein.

Therefore HSP27 and HSP70 were investigated by 2-D westerns. Gelsolin, clearly found to be up-regulated upon replicative senescence in both 2-D gels and 1-D westerns, and against which a very clean monoclonal antibody was available, was used as a positive control.

Freshly prepared protein extracts corresponding to P2 and P5 REFs were separated on 2-D gels, two gels corresponding to proliferating and senescent primary REFs were then blotted with anti-HSP27, anti-HSP70 and anti-gelsolin antibody.

The westerns of proliferating and senescent REFs made with HSP27 and HSP70 did not give any positive result (data not shown). HSP27 antibody gave an extremely high background that did not allow the detection of any feature, while HSP70 antibody, even though it was a monoclonal antibody, identified



**Fig. 3.9** Immunoblotting of 2-D gels.

2-D gels from P2 (growing) and P5 (senescent) cells were run and then probed with the monoclonal anti-gelsolin antibody. Spot A and B appeared to be up-regulated upon senescence. Moreover 3 new spots (C, D and E) appeared in P5.

on the 2-D gel many proteins, some comprised in the expected MW range (around 70kDa) and many more with an unexpected MW. Therefore it was not possible to tell whether the identified spots were different post-translational modification of HSP70 or they were cross-reacting spots, possibly other HSP family members.

However the gelsolin 2-D western blot gave positive results, as shown in Fig. 3.9. The two spots previously detected as differentials, and here called A and B, were confirmed to be up-regulated upon senescence. Moreover three spots called C, D and E appeared in P5. Spots C and E appeared to have approximately the same molecular weight but different pI, with spot C more acidic; spot D also showed a slightly different molecular weight, running faster on the gel, when compared with the original spots A and B.

The presence of these three new spots upon senescence showed that not only the total amount of protein was increasing upon senescence in primary REFs but also that 3 new isoforms appeared in P5; they might correspond to different post-translational modifications of the same protein or particular isoforms expressed only upon senescence. The identity of these newly identified spots and their post-translational modification remains to be confirmed.

Subsequent analyses were focused on those proteins that may be particularly relevant for replicative senescence. They will be described in greater detail in the following sections.

### **3.12 Metabolic proteins**

Several metabolic proteins were differentially expressed upon replicative senescence. Among those 2-oxoglutarate dehydrogenase precursor (spot 104),  $\gamma$ -butyrobetaine, 2-oxoglutarate dioxygenase (spot 493) and isopentenyl diphosphate: dimethylallyl diphosphate isomerase (spot 1075) were up-regulated whereas GDP dissociator inhibitor of the Rho protein (spot 1054) was



down-regulated.

PRx III, an enzyme with antioxidant function was identified in a down-regulated multiple spot together with proteasome subunit RC10-II (spot 1239). PRx III was also found in another single protein spot (spot 1351), which was not differentially expressed but shifted in mobility. PRx IV, another protein with antioxidant function and a member of the same family of proteins as PRx III, was down-regulated (spot 1295). Guanosine 5'-monophosphate synthetase (spot 332) was identified in a multiple spot, together with moesin, and did not appear to be differentially expressed but underwent a shift in migration.

### **3.12.1 *α-glucosidase***

Two nearby up-regulated spots (spots 122 and 127) were identified to be the  $\alpha$  subunit of the  $\alpha$ -glucosidase II complex (Fig. 3.7 A). The 2-D gel separation showed that the two spots were approximately the same molecular weight (MW) (114390 and 113345kDa respectively) but spot 127 was slightly more acidic when compared with spot 122, with pI 5.82 versus 5.57 of spot 122, which suggested that the two features were the same protein with a different post-translational modification. It was likely that the post-translational modification observed was phosphorylation, which could result on 2-D gel spots having an almost identical MW, but a different pI, and in particular a pI shift towards the acidic region for each phosphate group added.

$\alpha$ -glucosidase II is the soluble form of the enzyme that removes the  $\alpha$ -1,2-glucose and  $\alpha$ -1,3-glucose residues following transfer of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> to nascent polypeptides (Herscovics, 1999).  $\alpha$ -glucosidase participate in glycoprotein folding mediated by calnexin and calreticulin by forming the monoglucosylated high mannose oligosaccharides required for interaction with chaperones. In addition to their role in N-glycan processing, these enzymes are intimately involved in quality control in the endoplasmic

reticulum (ER), a process that ensures proper folding of newly formed polypeptide chains leading to retention and/or degradation of incorrectly folded proteins (Herscovics, 1999). Interestingly it was recently shown that a 3'-UTR (un-translated region) of a  $\alpha$ -glucosidase-related mRNA was able to promote colony formation and immortalisation in REFs and cooperate with an immortalisation-defective mutant of SV40 LT-ag to immortalise REFs (Powell *et al.*, 1998). Moreover, a gene named *klotho* (Kuro-o *et al.*, 1997), that shares sequence homology to the  $\beta$ -glucosidase family of enzymes and encodes a secreted protein that appears to function outside cells, has recently been identified. A defect in *klotho* expression in mice results in a syndrome that resembles human ageing (short life span, infertility, arteriosclerosis, skin atrophy, osteoporosis and emphysema) (Kuro-o *et al.*, 1997).

### 3.13 Proteins involved in differentiation

Two differentially regulated spots were identified as proteins belonging to the TUC (TOAD-64/Ulip/CRMP) family of intracellular phosphoproteins implicated in axon guidance and outgrowth and thereby regulation of neuronal differentiation (Byk *et al.*, 1998; Quinn *et al.*, 1999). TUC-4 was identified in a multiple up-regulated spot (spot 495), which contained also the chaperonin TPC-1,  $\gamma$ -subunit, while TUC-2 was identified in a down-regulated spot (spot 304) (Fig. 3.7 A and B).

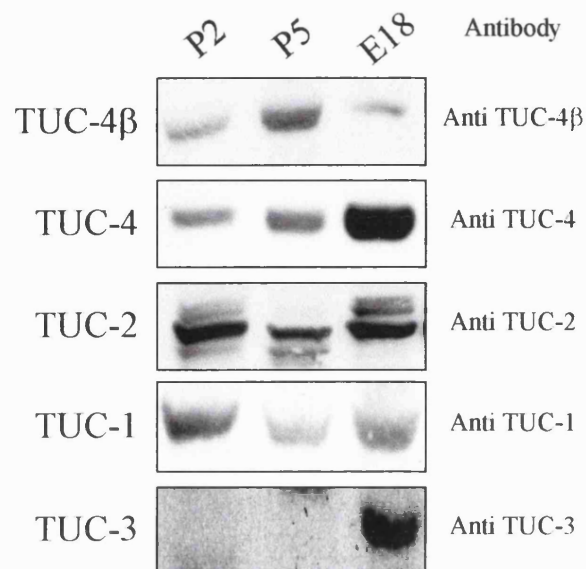
The TUC family consists of four 64kDa isoforms known as TUC-1 (CRMP-1/Ulip-3), TUC-2 (CRMP-2/Ulip-2), TUC-3 (CRMP-3/Ulip4), and TUC-4 (CRMP-4/Ulip-1). In addition, TUC-4 $\beta$  has recently been identified as a 75kDa variant of TUC-4 (C.C. Quinn, personal communication). The TUC proteins are up-regulated in the rat brain after embryonic day 12, a time that corresponds to the beginning of neurogenesis.

### 3.13.1 TUC-2 and TUC-4

TUC-4 is the most abundant member of the TUC family. It is first detected in new post-mitotic neurons after neuronal birth (Minturn *et al.*, 1995), and reaches peak expression during neurogenesis but is not expressed by neural progenitor cells. It is down-regulated in the adult brain but is expressed again if axonal re-growth is triggered. It is also increased upon differentiation of PC12 cells by nerve growth factor (NGF) and neuroblastoma cells by retinoic acid (RA). In contrast to TUC-4, much less is known about the expression of other TUC proteins. TUC-2 is expressed by both neurons and their progenitors. It is also slightly up-regulated upon treatment of PC12 cells with NGF. Recently TUC-2 was found to be associated with the microtubule bundles at the mitotic spindle and proposed to be involved in regulating microtubule dynamics (Gu and Ihara, 2000). The TUC proteins are also required for the growth cone collapsing activity of semaphorin-3A, an extracellular guidance cue for axonal outgrowth, suggesting a role for the TUC proteins in the signal transduction pathway initiated by extracellular stimuli. The importance of the TUC proteins in neuronal differentiation is further suggested by their homology to *unc-33*, a *Caenorhabditis elegans* gene that is required for normal axon outgrowth and guidance. Mutations in *unc-33* produce nematodes with a severely uncoordinated phenotype in which the axons show errors in path finding or terminate early (Quinn *et al.*, 1999), which suggests a double role in both axonal guidance and outgrowth for this protein.

The TUCs are also highly conserved across species; the rat TUC-2 protein shares 98% identity with its chicken ortholog and 89% identity with its *Xenopus laevis* ortholog.

TUC-4, identified in the proteomic study, was up-regulated when REFs underwent senescence (Fig. 3.7 A and Fig. 3.8), which is in accordance with the idea that TUC-4 is an early marker of post-mitotic neurons (Wang and Strittmatter, 1996). Interestingly TUC-2 showed an opposite regulation to



**Fig. 3.10** Protein expression analysis of the TUC family of proteins.

Levels of expression of the TUC family of proteins were analysed in primary cells using specific antisera. An E18 rat brain extract was used as a positive control.

TUC-4; it was down-regulated in the post-mitotic senescent cells (Fig. 3.7 B and Fig. 3.8). Rat brain extracts from embryonic day (E) 18 were used as positive control for the TUC's 1-D western blots.

### **3.13.2 *TUC-4 $\beta$ , TUC-1 and TUC-3***

Since TUC-4 and TUC-2 are two members of the same family of proteins but showed opposite regulation at the protein level, it was interesting to analyse the expression of other members of this family. The results presented in Fig. 3.10 show that TUC-4 $\beta$ , a variant of TUC-4, was up-regulated and TUC-1 was down-regulated upon cellular senescence, while it was not possible to detect TUC-3, suggesting that this member was missing in REFs. TUC-4 $\beta$  was previously found to be expressed only in neural cells and cell lines of neural origin such as PC12, N1E-115, and neuro-2a cells. Interestingly both TUC-4 and TUC-4 $\beta$  were detected in all three of these cell lines as well as brain and REF extracts.

The significance of the changes in expression of this family of proteins is not yet understood, neither is it clear what is their function and whether they have overlapping functions. Nevertheless it is highly intriguing that the different family members show divergent but identical regulation in both neural differentiation and in fibroblasts upon replicative senescence. It is likely that they play a role in concert, and that to trigger a biological effect some of the TUCs have to be expressed while others have to be switched off.

## **3.14 Heat shock proteins**

Four heat shock proteins (HSP) were identified as differentially expressed upon senescence during this study. HSP105 (spot 110), HSP90- $\alpha$

(spot 185) and HSP27 (spot 1302) were up-regulated and HSP70, identified in spot 153 together with Sec23 and ischemia responsive 94kDa protein, was down-regulated.

HSPs are a group of proteins that are highly conserved from bacteria to mammals and classified into different families according to their size: HSP110/105, 90, 70, 60, 40 and 27. They were initially identified by virtue of their rapid induction under stress conditions and proposed to play a critical role in protection from hypothermia and other types of stress (Hendrick and Hartl, 1993; Lindquist, 1986; Verbeke *et al.*, 2001). However they are now known to play essential roles under normal physiological conditions such as assisting the folding of newly synthesised proteins, protein translocation across organelle membranes, promoting assembly or disassembly of oligomeric proteins and facilitating protein degradation of incorrectly folded or denatured proteins. They have also been shown to localise to the centrosome but the significance of this localisation is not clear (Rattner, 1991).

### **3.14.1 HSP27**

The finding that HSP27 was up-regulated upon senescence (spot 1302) (Fig. 3.7 A), even though it was not possible to confirm the differential expression by 1-D western blot analysis (Fig. 3.8) (see Paragraph 3.11), was in accordance with the observation that over-expression of HSP27 in bovine pulmonary endothelial cells stimulates their growth rate and accelerates the rate at which the cultures reach senescence (Piotrowicz *et al.*, 1995). HSP27 has also been found to be differentially expressed between human breast luminal and myoepithelial cells (Page *et al.*, 1999), however the significance of this differential regulation remains unknown.

### 3.14.2 HSP70, 110/105

HSP70 is the most abundant and most evolutionarily conserved among the heat shock proteins. It is able to transiently bind the hydrophobic peptides of unfolded or newly synthesised proteins and prevent misfolding by masking their hydrophobic regions. The mammalian HSP110/105 may exist as two forms,  $\alpha$  and  $\beta$ . Even though both forms are induced upon stress; the  $\alpha$  form is also expressed constitutively (Hatayama *et al.*, 1986). Both forms associate with HSP70 and have been proposed to negatively regulate its chaperone activity. They are also induced upon adipocyte differentiation (Imagawa *et al.*, 1999).

Increases in resistance to stress have been shown to increase chronological life span in *Drosophila melanogaster* and *C. elegans* by acting on post-mitotic cells rather than affecting replicative potential (Harris *et al.*, 2001). Stress resistance could be increased by reducing expression of HSP90 which can down-regulate heat shock transcription factor HSF1 (Harris *et al.*, 2001). Interestingly, induction of HSP70 by stress is significantly lower in late passage senescent fibroblasts, probably due to reduced levels of HSF1 that is required for transcriptional up-regulation of HSP70 (Gutsmann-Conrad *et al.*, 1998). Recently two mortalin genes (Mot1 & 2), members of the HSP70 family that are derived from two distinct genes were identified. They encode proteins that differ by only two amino acids but exhibit different sub-cellular localisation and have contrasting activities. Mot1 induces senescence in NIH 3T3 cells whereas Mot 2 results in transformation. Mot2 has also been shown to increase proliferative life span and block induction of SA- $\beta$ -gal activity in normal human diploid fibroblasts by interfering with p53 activity by blocking nuclear translocation of p53 and down-regulating p53 responsive genes (Kaula *et al.*, 2000; Wadhwa *et al.*, 1998). Since up-regulation of HSP90 could account for down-regulation of HSP70 via HSF1, it suggests that it would be very important to determine what is the cause of the up-regulation of HSP90 and if it

is due to stress of *in vitro* tissue culture, or alternatively to determine the nature of the inducing stress. It will also be interesting to determine whether HSP105/110 and 27 are up-regulated by the same mechanism. One possible mechanism would be via a common transcription factor. It would be also important to determine the exact form of HSP70 that has been found to be down-regulated and investigate whether it corresponds to one of the mortalin proteins, particularly Mot2.

### 3.15 Protein turnover, synthesis and modification

Some differential spots were identified as subunits of the proteasome complex: the 26S proteasome sub-unit p40.5 and RC10-II. The level of subunit p40.5 (spot 907) increases upon senescence whereas RC10-II both decreased in level in a multiple spot (spot 1239, together with PRx III) and shifted in its migration (spot 1348) (Fig. 3.7 C).

The p40.5 subunit has been previously found to be differentially expressed between human luminal and myoepithelial cells (Page *et al.*, 1999), however the significance of this differential regulation remains unknown.

In contrast, changes in the migration pattern of the RC10-II have not been observed previously. The shift is most likely to be due to changes in post-translational modification such as phosphorylation, and it would therefore be very interesting to determine the site of phosphorylation, the kinase responsible and how the regulatory events.

The category of proteins involved in protein turnover, synthesis and modification are also included alanyl-tRNA synthetase (spot 102), O-GLCNAC transferase p110 subunit (spot 108), seryl-tRNA synthetase, found in a multiple spot together with T-complex polypeptide 1 (spot 497) and an ATP-dependent RNA helicase p47 in a multiple spot with ERF1 (spot 677). All of these proteins were found to be up-regulated.



### **3.15.1 O-GlcNAc transferase p110 subunit**

O-GlcNAc transferase p110 subunit was identified as an up-regulated protein (spot 108). O-GlcNAc is a highly conserved, dynamic and inducible enzyme capable of glycosylating both serine and threonine residues on nuclear and cytosolic proteins. On several proteins the O-GlcNAc and O-phosphorylation post-translational modifications occur on the same or adjacent sites, and thus it is possible that one function of the addition of O-GlcNAc residues is to block phosphorylation at this site (Wells *et al.*, 2001). Therefore the O-GlcNAc modification may have an important role controlling intracellular signalling. Disruption of O-GlcNAc transferase in mouse embryonic stem cells is lethal, demonstrating the importance of this modification.

### **3.16 Cytoskeletal proteins**

A number of identified proteins, including gelsolin,  $\beta$ -tubulin and actin related protein 2 (Arp2), have previously been shown to be involved in cytoskeletal scaffolding. Most of these proteins were up-regulated during senescence, however  $\beta$ -tubulin chain 15 (spot 863) was down-regulated (Kumazaki, 1992; Kumazaki *et al.*, 1997).

It is perhaps not surprising that proteins involved in the cytoskeleton organisation showed changes in expression levels upon replicative senescence because senescent cells are morphologically very different from growing cells. They have the flat and enlarged morphology, known as “fried egg” morphology, typical of senescent cells. They are much larger, have lots of stress fibres and over-express many extracellular matrix components such as collagenase, collagen, stromolysin and fibronectin.

### 3.16.1 Gelsolin

Gelsolin is involved in nucleation of actin filaments and is responsible for regulating the growth rate of these filaments, and therefore has a role in cell motility (Kwiatkowski, 1999). It mediates the rapid remodelling of cortical actin filaments and has a part in stress fibre dependent cell functions (Arora *et al.*, 1999). Gelsolin is also a caspase 3 substrate and is cleaved during Fas-mediated apoptosis (Kothakota *et al.*, 1997) which generates a fragment that can depolymerise actin filaments and either promote or inhibit apoptosis depending on the cell type (Kusano *et al.*, 2000).

Gelsolin (spot 190), identified in a multiple spot with myosin heavy chain, was found to be up-regulated upon replicative senescence (Fig. 3.7 A and Fig. 3.8); growing cells expressed lower levels of gelsolin when compared with post-mitotic senescent cells. This was consistent with previous observations that gelsolin was down-regulated in several types of transformed cells and tumours (Dong *et al.*, 1999; Lee *et al.*, 1999b; Vandekerckhove *et al.*, 1990). The partial or total loss of gelsolin expression in the majority of breast cancers of diverse aetiologies has led to the proposal that gelsolin is a candidate tumour suppressor gene for breast cancer (Asch *et al.*, 1996). Both gelsolin protein and its mRNA have been shown to be down-regulated in cancer cells, suggesting that an alteration in the rate of transcription underlies the dysfunction of this gene (Asch *et al.*, 1996). The decrease at the transcriptional level is most likely to be due to loss of p53 since it has recently been found that gelsolin is transcriptionally up-regulated by p53 (Kannan *et al.*, 2001).

### 3.16.2 ERM family

Moesin, radixin and ezrin, members of the ERM family of proteins, are general cross-linkers between cortical actin filaments and plasma membranes. They are involved in the formation of microvilli, cell adhesion sites, cleavage

furrows and membrane ruffling (Tsukita and Yonemura, 1999). It is interesting to note that moesin was identified as an up-regulated (spot 331) as well as a down-regulated (spot 332) protein and thus represents a feature that shifts in its migration. Since these spots migrate at the same molecular weight but different pI, can be suggested that it is not the level of the protein that changes but the post-translational modification. Since the feature that is up-regulated has a higher pI, it was most likely that moesin underwent changes in its phosphorylation level upon senescence.

### **3.16.3 Arp2/3**

Spot 902 that was up-regulated in senescent REFs was found to comprise Arp2 and hnRNP-E2. The Arp2/3 protein is ubiquitous and represents an essential component of the actin cytoskeleton in eukaryotic cells. It nucleates actin filaments, caps their pointed ends and cross-links them into orthogonal networks (Mullins, 2000; Mullins and Pollard, 1999). Arp2/3 proteins are activated by the GTPase Cdc42, together with the WASP family of proteins (Welch, 1999). They have been previously implicated in cancer and have also been found differentially expressed between human breast myoepithelial and luminal epithelial cells (Page *et al.*, 1999), however the meaning of this differential regulation remains unknown.

### **3.17 Factors affecting protein synthesis**

Two proteins, ERF1 (Eukaryotic Releasing Factor) found in a multiple spot with probable ATP-dependent RNA helicase p47 (spot 677) and Elongation factor-1- $\gamma$ , (spot 773), that may play a role in protein synthesis, were up-regulated upon senescence.

ERF1 has been implicated in translation termination in eukaryotes (Frolova *et al.*, 1994). Although its function in translation termination remains obscure, it has been shown to promote a stop codon and ribosome dependent hydrolysis of aminoacyl-tRNAs (Frolova *et al.*, 1999) and thus it has been proposed to catalyse the termination of protein synthesis at all three stop codons. ERF1 has been remarkably conserved during evolution suggesting that it may have an essential role in the termination of translation (Guenet *et al.*, 1999).

Elongation factor-1- $\gamma$  a subunit of EF1, one of the G proteins that mediate transport of aminoacyl-tRNA to the 80S ribosomes during translation, has been found to be over-expressed in colorectal carcinomas and adenomas (Mimori *et al.*, 1996; Mimori *et al.*, 1995). Therefore it was rather surprising that we found elongation factor-1- $\gamma$  be up-regulated upon replicative senescence.

### **3.18 Protein trafficking**

SEC23 identified in the down-regulated spot 153 (in a multiple spot together with ischemia responsive 94kDa protein and HSP70) in senescent cells has been proposed to be involved in protein trafficking. It is a component of COPII, a cytosolic complex that is responsible for the formation of vesicles within the ER and involved in the anterograde transport of proteins to the Golgi (Barlowe, 1995; Nickel *et al.*, 1998). In contrast to SEC23, transferrin (spot 290) and RA410, a vesicle transport-related protein (spot 426) both appeared to be up-regulated upon senescence. None of these proteins have previously been implicated in senescence or tumourigenesis. Nevertheless, RA410 has been proposed to have a role in post-Golgi transport, and participate in the ischemia-related stress response in astrocytes (Matsuo *et al.*, 1997). Another transport related protein, transitional ER ATPase was found to be down-regulated in spot

### 3.19 mRNA level

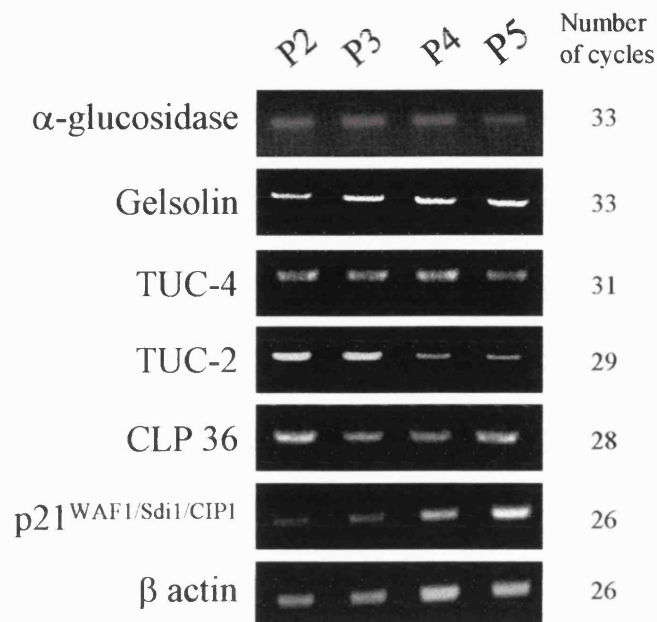
Some of the genes found to be differentially expressed at the protein level were further investigated at the mRNA level to check whether the changes in the protein expression detected in the proteomic study correlated with changes in mRNA. This was done in attempt to understand at which level the expression for these proteins was regulated. A transcriptional regulation could be suggested in the case in which the mRNA showed to undergo the same regulation found for the protein, or alternatively, translational regulation, or stability of the protein in the case in which the mRNA level did not show any change.

Semi-quantitative RT-PCRs were therefore performed to investigate the mRNA level for some of the differentials (Fig. 3.11).

p21<sup>WAF1/Sdi1/CIP1</sup>, a transcript that is known to be up-regulated upon replicative senescence (Bringold and Serrano, 2000), was used as a positive control for the RT-PCR experiments. As expected p21<sup>WAF1/Sdi1/CIP1</sup> mRNA was up-regulated in primary REFs from P2 to P5.  $\beta$  actin was used as a control for the RT-PCR procedure.

The mRNA corresponding to the TUC-2 protein was found to decrease in accordance with the decrease shown during the differential proteomics study and the validation steps. The mRNAs corresponding to TUC-4,  $\alpha$ -glucosidase and CLP36 did not change while the gelsolin mRNA showed a minor increase in accordance with senescence.

These results were consistent with the studies in *S. cerevisiae* and human liver which have shown that mRNA levels correlate poorly with corresponding protein levels (Anderson and Seilhamer, 1997; Gygi *et al.*, 1999).



**Fig. 3.11** RT-PCR analysis of differentially expressed candidate genes.

A direct correlation between protein and mRNA levels was found for TUC-2 (both down-regulated upon senescence); gelsolin showed a slightly correlation while no correlation was shown for  $\alpha$ -glucosidase and TUC-4. p21<sup>WAF1/Sdi1/CIP1</sup>, a gene known to be up-regulated upon replicative senescence, was used as a positive control;  $\beta$  actin was used to control for the PCR procedure.

### 3.20 Immortalisation assay

After having identified proteins that were up and down-regulated in primary rat embryo fibroblasts upon replicative senescence, it was interesting to check whether any of the differentially expressed proteins were playing an active role in the process of replicative senescence.

An immortalisation assay was utilised to speculate whether any of the proteins found to be differentially expressed during the proteomic study were up-stream of the cellular senescence process, and therefore they were causes of this complex biological phenomenon or, alternatively, they were not actively inducing this phenomenon and were therefore down-stream targets of the process.

For the immortalisation assay the cDNAs corresponding to the proteins of interest were ectopically expressed in primary REFs. The transfected cells were then grown in selective media and stable transfectants obtained. One read-out of such experiments is to measure the number of colonies present in dishes of cells transfected with the DNA of interest, at the time in which not-transfected control cells are undergoing senescence.

The cDNAs of interest were cloned into the expression vector pcDNA 3.1 (see also Chapter 2), a vector containing an expression cassette for the selectable marker G418. After cloning in the expression vectors, the chosen cDNAs were transfected into freshly prepared REFs at passage 2 (P2), corresponding to an actively dividing population of cells. After transfection the addition to the media of the indicated concentration of G418 drug allowed the selection of stable colonies. All the experiments were repeated twice, and in each experiment four 15 cm dishes were transfected with each different construct. Afterwards the dishes were stained and the number of colonies in each dish counted. The graph Fig. 3.12 shows the average number of colonies obtained in the different dishes for each transfection.

TUC-2 cDNA, one of the more promising candidates identified during the proteomic analysis, was cloned into pcDNA 3.1 and its ability to induce

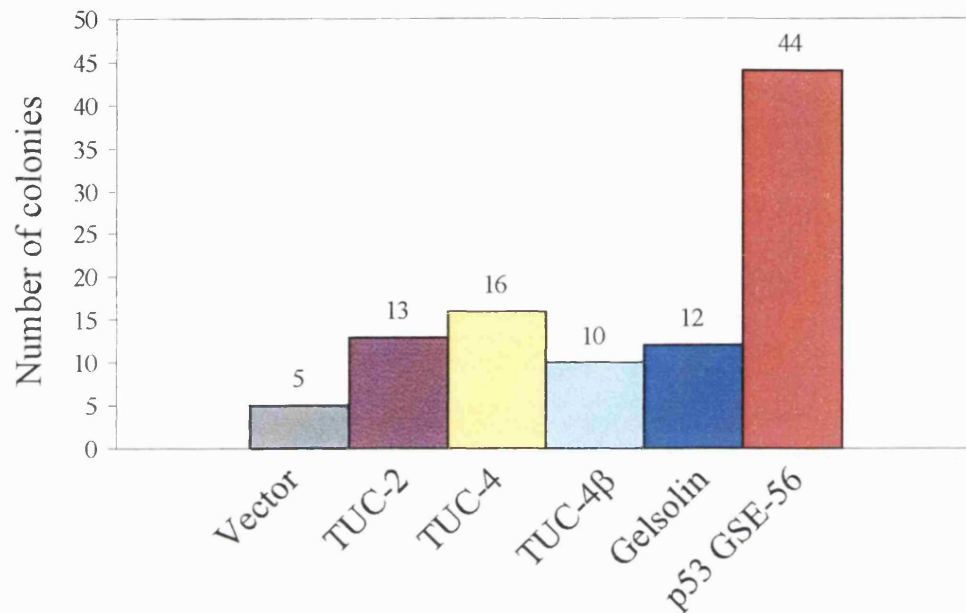
replicative senescence was investigated *in vivo* utilising the immortalisation assay. The empty vector pcDNA 3.1 was also transfected as a negative control, while a GSE (genetic suppressing element) representing a p53 fragment (p53 GSE-56) was used as a positive control for immortalisation. p53 GSE-56 has been shown by Ossovskaya *et al.*, (Ossovskaya *et al.*, 1996) to be positive for immortalisation (Fig. 3.12).

When primary REFs were transfected with full length TUC-2 the number of colonies per dish was higher (13 colonies) but still comparable with the empty vector control (5 colonies). The difference in colony number observed after TUC-2 over-expression was definitely not as significant as the one observed for the positive p53 GSE-56 control, which gave 44 colonies (Fig. 3.12).

Therefore the over-expression of TUC-2 alone in primary proliferating REFs was not sufficient to induce immortalisation.

Three other proteins, TUC-4, TUC-4 $\beta$  and gelsolin, were also cloned into pcDNA 3.1, transfected into proliferating REFs (P2), and tested in the immortalisation assay. Due to the fact that these proteins were previously found to be up-regulated upon replicative senescence during the proteomic study, they were expected to suppress the colony formation in REFs, and to have an inhibitory effect on the immortalisation process. Therefore the number of colonies produced, upon their ectopic expression into primary cells, was expected to decrease. However, surprisingly, (as shown in Fig. 3.12) no substantial difference in the number of colonies was observed when these three proteins were transfected in primary cells. Moreover TUC-4, TUC-4 $\beta$  and gelsolin gave a higher number of colonies when compared to the empty vector used as a negative control. However the small fluctuations observed in the colony number could be due to either variability in the concentration of the cDNA transfected, or the size of the vectors used.





**Fig. 3.12** Immortalisation assay.

TUC-2, TUC-4, TUC-4 $\beta$  and gelsolin were over-expressed in primary REFs and their ability to immortalise primary REFs tested. The empty vector (Vector) was used as a negative control and the clone p53 GSE-56 (Ossovskaia *et al.*, 1996) was used as a positive control for immortalisation.

### **3.20.1 Cell line extension**

Since it has been observed that it is possible that some cDNAs ectopically expressed into primary cells do not increase the number of colonies but yield colonies that can be readily expanded into cell lines, six clones were picked for each transfected cDNA (the colonies were picked from different dishes) and were expanded to test their ability to produce cell lines.

According to the procedure each clone was picked and transferred into a 24 well plate. The cells were allowed to adhere and grow and at 80% confluence (that took from a couple of days to a week, according to the growth rate that was different for each clone), all the cells were then transferred into 3 cm dishes. When 80% confluence was reached the cells were transferred to 6 cm dishes, and eventually to 10 cm dishes and subsequently passaged to new 10 cm dishes after 1:6 dilution.

The six colonies picked from the transfections with the empty vector become growth arrested by the passage into 3 cm dishes; they were kept for 20-30 days, and freshly fed every three days, but their number no longer increased.

As expected all the six colonies picked from the transfection with p53 GSE-56 were readily expanded into cell lines; they reached the 10 cm dish stage and were further passaged 5 times in other 10 cm dishes, after 1:6 dilution, without showing any loss of proliferative potential. They were therefore classified as cell lines. Some of the cells have also been frozen, and they showed no decrease in their proliferative potential upon thawing.

None of the six colonies ectopically expressing TUC-2 showed any immortalisation capacity; they behaved like the empty vector and were growth arrested by passage into the 3 cm dishes, where they were kept for 20-30 days, changing the media every three days, but their number no longer increased.

Therefore it was possible to conclude that after the ectopic expression of the selected candidate proteins (TUC-2, TUC-4, TUC-4 $\beta$  and gelsolin) into primary REFs, no major phenotypes could be observed: neither differences in

colony number nor in their ability to yield cell lines could be observed.

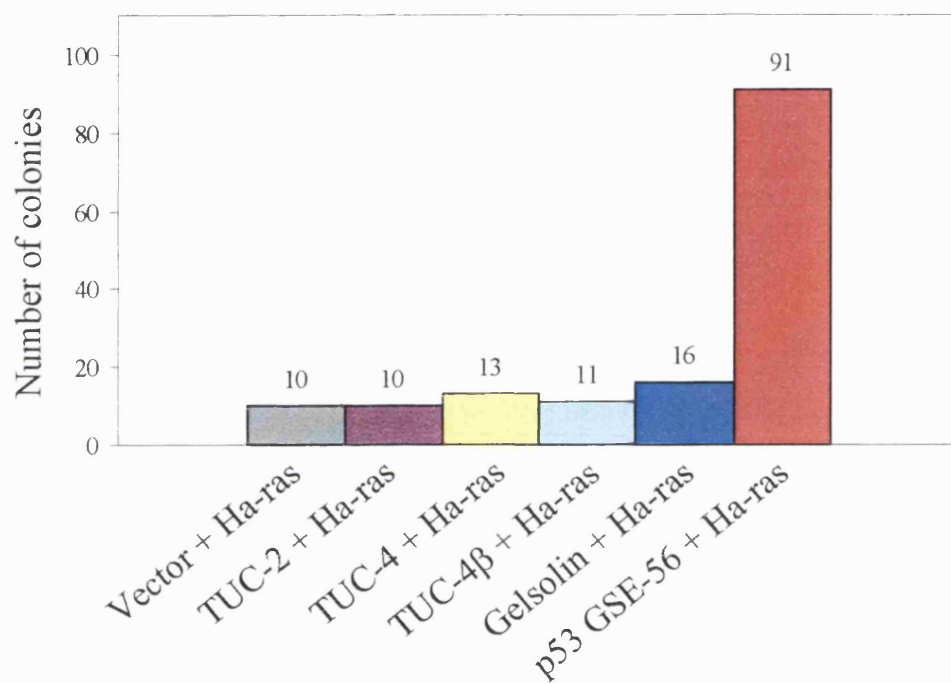
### **3.21 Ras co-operation assay**

In a parallel experiment the same candidate proteins were investigated in a ras co-operation assay.

The cDNAs corresponding to TUC-2, TUC-4, TUC-4 $\beta$  and gelsolin were tested for their ability to transform primary REFs in co-operation with activated ras (Ha-ras). The cDNAs of interest were co-transfected with Ha-ras, stable transfectants selected. The transfected dishes were then stained and the number of transformed colonies counted (Fig. 3.13).

None of the investigated cDNAs showed any substantial effect in co-operation with Ha-ras. The number of colonies was always comparable with that from the transfection of the empty vector together with ras (negative control). However the transfection of p53 GSE-56 together with ras gave substantially higher number of colonies (91 colonies).

In the case in which TUC-2, found to be down-regulated during replicative senescence, was an immortalising gene, it was expected to co-operate with ras in its ability to transform primary REFs, in a similar way to p53 GSE-56. However the number of colonies counted in the dishes co-transfected with TUC-2 and Ha-ras remained similar to the control dishes co-transfected with the empty vector and Ha-ras.



**Fig. 3.13** Ras co-operation assay.

TUC-2, TUC-4, TUC-4 $\beta$  and gelsolin were tested in a ras co-operation assay for their ability to transform primary REFs. The empty vector (Vector) was used as a negative control and the clone p53 GSE-56 (Ossovskaya et al., 1996) was used as a positive control.

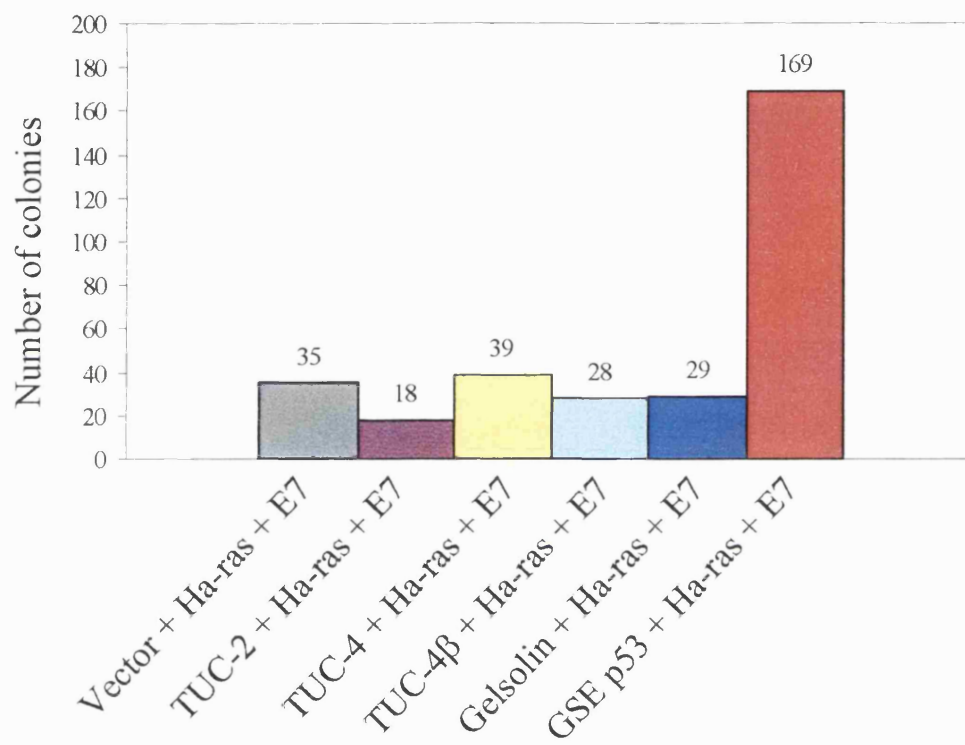
### 3.22 Ras and E7 co-operation assay

Since the proteins that were found to be up-regulated upon replicative senescence could be potential tumour suppressors, they were analysed in a ras and E7 co-operation assay, an assay previously used to show the tumour suppressor activity of p53 (Finlay *et al.*, 1989).

The ras and E7 co-operation assay was therefore performed to test whether the transformation activity of Ha-ras in combination with E7 was reduced by any of the cDNAs coding for the proteins found to be up-regulated during replicative senescence. In this assay the cDNAs of interest were co-transfected with Ha-ras and E7 into proliferating REFs (P2) and stable transfectants selected. The transfected dishes were then stained and colonies counted. As negative control the empty vector was co-transfected together with Ha-ras and E7. The activities of TUC-4, TUC-4 $\beta$  and gelsolin as possible tumour suppressors were therefore tested in the ras and E7 co-operation assay (Fig. 3.14). TUC-2 behaviour was also tested even though the TUC-2 protein was down-regulated upon replicative senescence.

No major difference in the colonies number could be detected when TUC-4, TUC4 $\beta$  and gelsolin (co-transfected with Ha-ras and E7) were compared with the empty vector (co-transfected with Ha-ras and E7). So it was possible to conclude that none of those selected proteins had a tumour suppressor activity. Surprisingly TUC-2, shown to be down-regulated upon replicative senescence, produced a 50% inhibitory effect on the overall colonies number (18 colonies against the 35 counted in the control). The potential role of TUC-2 as tumour suppressor remains to be investigated.

None of the proteins studied (TUC-2, TUC-4, TUC-4 $\beta$  and gelsolin) showed to have any effect in the immortalisation, the ras co-operation or the ras and E7 co-operation assays. It was therefore possible to conclude that none of these proteins were actively inducing cellular senescence.



**Fig. 3.14** Ras and E7 co-operation assay.

TUC-2, TUC-4, TUC-4 $\beta$  and gelsolin were tested in a ras and E7 co-operation assay to determine a potential tumour suppression role. The empty vector (Vector) was used as a negative control and the clone p53 GSE-56 (Ossovskaya *et al.*, 1996) was used as a positive control.

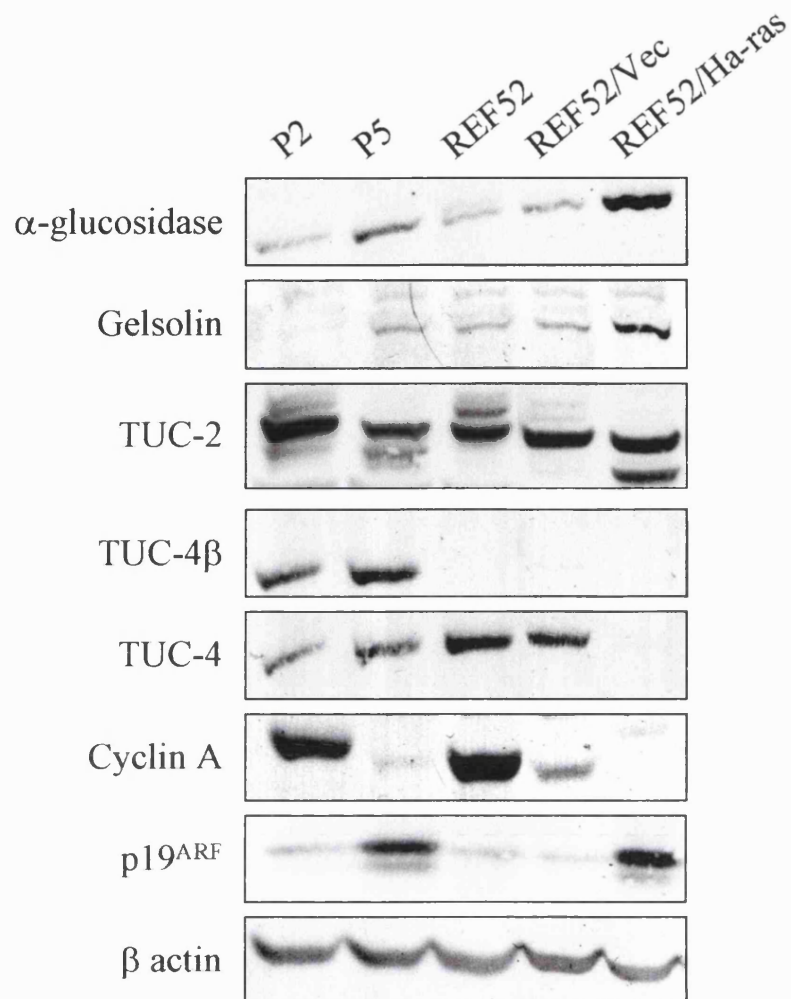
It can be possible that some of these proteins are involved in inducing or repressing replicative senescence but they cannot act alone. It is possible that some of these proteins have to be up-regulated, at the same time as others have to be down-regulated to produce any biological effect; in other words they may have to act in concert to produce any phenotype. This is very likely with the TUC family's members, of which some were found to be up and others down-regulated with senescence. It is also possible that a particular post-translationally modified version of these proteins is the biological player and when the proteins are over-expressed the equilibrium is pushed towards a differently modified, and therefore inactive, isoform or that modifications may or may not be occurring.

### **3.23 Premature senescence model**

The next step was to examine whether some of the changes observed in replicative senescence also occurred upon premature senescence, another commonly used system for studying senescence. In this model, REF52 cells, from an immortal rat cell line, are induced to senesce prematurely by ectopic expression of the activated Ha-ras oncogene (Serrano *et al.*, 1997).

First the REF52 model was tested for expression of cyclin A and p19<sup>ARF</sup> and compared with primary cells. The data shown in Fig. 3.15 confirmed that, as previously observed in primary REFs, p19<sup>ARF</sup> was up-regulated whereas cyclin A was down-regulated upon induction of premature senescence.

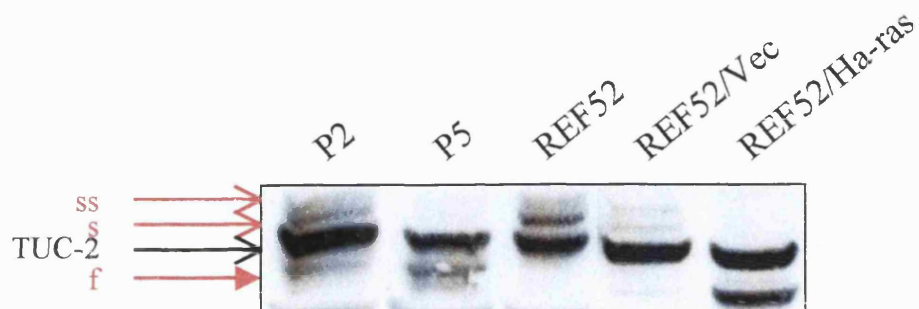
After having confirmed and compared the REF52 system with the primary REFs, it was decided to investigate the expression of some of the differentials in the REF52 model. In accordance with the data from the primary cells, both  $\alpha$ -glucosidase and gelsolin appeared to be up-regulated upon premature senescence (Fig. 3.15).



**Fig. 3.15** Premature senescence of REF52 cells.

Protein expression in REFs was compared with the premature senescence of REF52 (Serrano *et al.*, 1997) in which REF52 are induced to undergo senescence upon ectopic expression of activated Ha-ras. Cyclin A and p19<sup>ARF</sup>, known to be differentially expressed in both model systems, were used as a positive control;  $\beta$  actin was used to control for equal loading.





**Fig. 3.16** TUC-2 isoforms.

Four different bands corresponding to TUC-2 were detected in 1-D western blots. Other than the main TUC-2 band (black arrow) were also detected a super slow (ss) band (red open arrow) and a slow (s) (red open arrow). They both disappeared upon replicative senescence. A fast (f) band (red full arrow) appeared upon senescence.

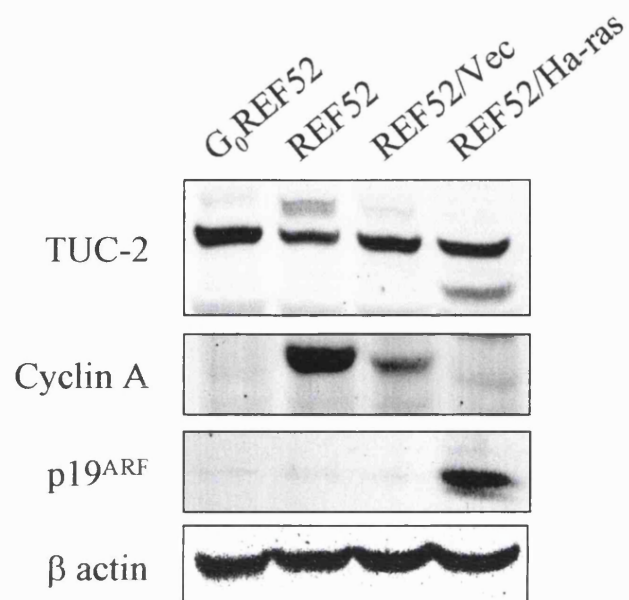
TUC-4 $\beta$  splice variant was not detected in REF52 cells and rather surprisingly TUC-4 was found to be down-regulated upon induction of premature senescence, contrary to the up-regulation previously observed upon replicative senescence in the primary fibroblast system (Fig. 3.15).

TUC-2 was also studied. In addition to the major TUC-2 band, previously found to be down-regulated in primary REFs upon senescence, two slower bands could be detected in proliferating REF52, as well as in P2 REFs. These two bands were called s, for slow, and ss, for super slow, and appeared on the X-ray films only after very long exposures (up to 2 hours), after which the decrease of the major TUC-2 band could no longer be detected, due to the fact that this major band resulted over-exposed (Fig. 3.16). The major TUC-2 band, previously detected and found to be down-regulated in the primary cells, and also the two slower bands (s and ss) were down-regulated when premature senescence was induced in REF52 in agreement with the down-regulation occurring upon replicative senescence of primary cells (Fig. 3.16).

A faster band, called f for fast (Fig. 3.16), also appeared when premature senescence was induced in REF52. The same band could be detected in primary fibroblasts at P5 while it was almost absent in P2 (a very faint band corresponding to the f band appeared in P2 only after very long exposures of the films, up to 2 hours).

### **3.24 Senescence versus quiescence**

As previously stated, all the cells used in the present study, were fed with fresh medium the day prior to extraction, so that all the lysates were prepared from cultures kept in fresh medium and sub-confluent. In this way it was ensured that cells were not in the quiescent state ( $G_0$  phase of the cell cycle). However it was interesting to check the expression of cyclin A and p19<sup>ARF</sup> in quiescent REF52 and compare the expression level of these proteins during quiescence with the senescent state.



**Fig. 3.17** Quiescence versus senescence.

Quiescent REF52 (starved in 0% serum for 5 days) were compared with growing and senescent REF52 by probing the gels with TUC-2, Cyclin A and p19<sup>ARF</sup>. β actin was used as control for equal loading. Senescence was demonstrated to be different and distinct from quiescence.

REF52 were induced to enter G<sub>0</sub> by starvation in 0% serum for 5 days. The cells were then lysed and the protein profiles compared by 1-D western blots with proliferating REF52 cells, REF52 cells transfected with the empty vector (and therefore still proliferating) and REF52 cells transfected with Ha-ras (and therefore prematurely senescent). As previously shown and discussed cyclin A was down-regulated upon senescence; cyclin A was also found to be down-regulated during quiescence (Fig. 3.17). As expected p19<sup>ARF</sup> was up-regulated with senescence, however it was not detectable in quiescent cells.

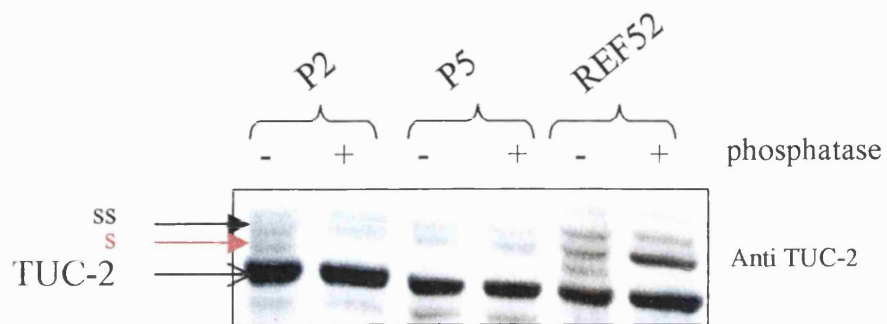
The different levels of p19<sup>ARF</sup> in senescence and quiescence demonstrated unquestionably that the senescent cells were not in G<sub>0</sub> (Fig. 3.17).

TUC-2 was also examined upon quiescence. It was found that the major band previously shown to be down-regulated upon replicative senescence in both primary cells and REF52 cell lines was slightly up-regulated in quiescence, while the two slower bands (ss and s) were clearly down-regulated upon quiescence; however the faster band (f), that appeared when premature senescence was induced, was not detectable with quiescence (Fig. 3.17)

### **3.25 TUC-2 loss of phosphorylation upon replicative senescence**

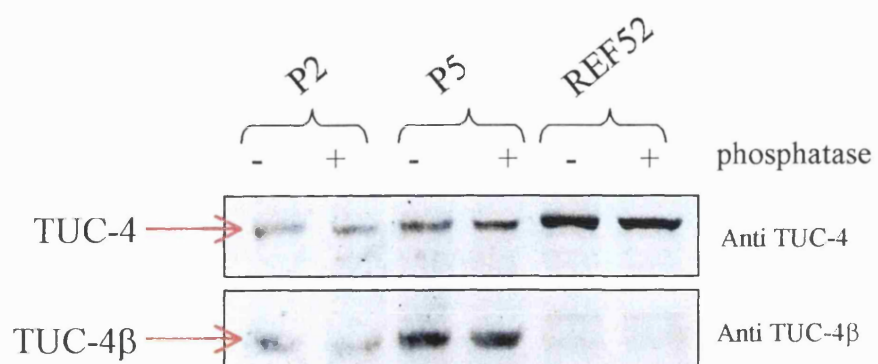
Because TUC-2 is known to be a phosphoprotein it was decided to investigate if any of the minor bands detected as differentially expressed upon senescence were possibly due to a change in the phosphorylation pattern of TUC-2. To address this question a phosphatase treatment of extracts was undertaken prior to western blotting.

The phosphatase treatment showed that the slow band s, detected in P2 REFs and growing REF52 cells, disappeared, indicating that at least one of the slower migrating bands observed in the proliferating REFs as well as in the proliferating REF52 cells, but not in the growth arrested cells, was due to phosphorylation (Fig. 3.18).



**Fig. 3.18** Phosphatase treatment of total extracts prior to blotting with anti TUC-2 antibody.

20 $\mu$ g of total cell lysates per sample were treated with Lambda Protein Phosphatase (BioLabs) prior to western blotting. TUC-2 was shown to be affected by phosphatase treatment. The s band (red arrow) was lost after phosphatase treatment.



**Fig. 3.19** Phosphatase treatment of total extracts prior to blotting with anti TUC-4 and TUC-4β antibodies.

20μg of total cell lysates per sample were treated with Lambda Protein Phosphatase (BioLabs) prior to western blotting. Neither TUC-4 nor TUC-4β were affected by phosphatase treatment.

An analogous phosphatase treatment was then undertaken prior to western blotting using the antisera directed against TUC-4 and TUC-4 $\beta$ . The bands corresponding to TUC-4 and TUC-4 $\beta$  were expected to disappear only if the antibodies were directed against the phosphorylation sites, alternatively if a massive amount of phosphate groups were lost upon phosphatase treatment, a shift in the migration of the protein was expected. In this experiment no bands failed to be detected after the phosphatase treatment and no differences in the molecular weights of the bands corresponding to TUC-4 or TUC-4 $\beta$  could be shown, strongly suggesting that no changes in the phosphorylation level of these proteins was occurring upon senescence (Fig. 3.19).

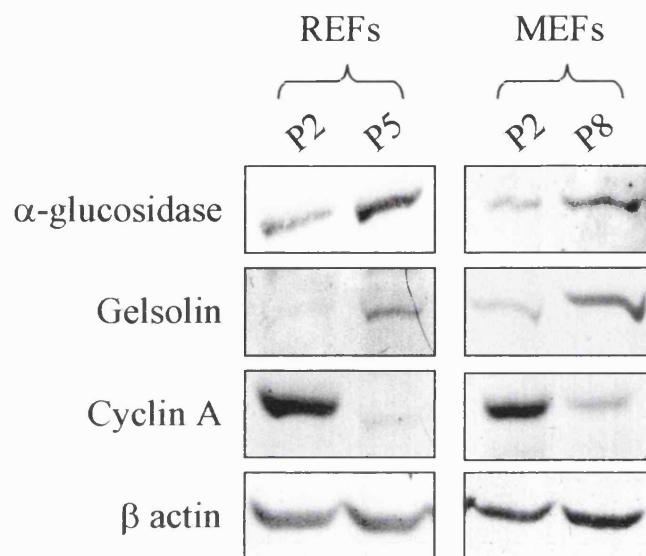
### 3.26 Mouse embryo fibroblasts

A third model system commonly used to study replicative senescence (other than REFs and REF52) was then used to check the behaviour of some of the proteins that were found to be differentially expressed in the 2-D gels upon replicative senescence of REFs, and then confirmed to be differential in the REFs, as well as in the REF52 model systems, by 1-D western blots.

Mouse embryo fibroblasts were prepared and serially passaged according to the 3T3 protocol of Todaro and Green (Todaro, 1963) (see Paragraph 2.1.3.4), and extracts were prepared from proliferating (passage 2 or P2) and senescent (passage 8 or P8) cells. It was necessary to passage the MEFs until passage 8 because they undergo senescence only by P8.

In accordance with the REF model system, both gelsolin and  $\alpha$ -glucosidase were found to be up-regulated when MEFs were serially passaged *in vitro* ceased dividing and underwent replicative senescence (Fig. 3.20).

Down-regulation of cyclin A upon senescence was used as a positive control whereas  $\beta$  actin was used as control for equal loading (Fig. 3.20).



**Fig. 3.20** Replicative senescence in mouse embryo fibroblasts.

Protein expression in P2 and P5 REFs was compared with P2 and P8 MEFs. Cyclin A expression was analysed as a positive control;  $\beta$  actin was used as control for equal loading.



### 3.26 Summary and discussion

A global 2-D differential proteome analysis of replicative senescence in serially passaged REFs was undertaken. Triplicate independent 2-D gels containing over 1200 spots each were run, curated and analysed.

This differential proteomic analysis revealed 49 spots (~4% of the total spots detected on an average 2-D gel), whose expression was altered more than two-fold upon replicative senescence. The differentially expressed proteins were then subjected to mass spectrometry analysis which yielded positive protein identification for 39 of these spots. The protein species identified can be assorted to several categories, that is, cytoskeletal, heat shock response and metabolism proteins as well as proteins involved in trafficking, differentiation and protein synthesis, turnover and modification.

Since proteomic studies do occasionally identify false positives, the proteomic data were validated by 1-D western blotting, where antisera were available. Furthermore some of the candidates were also shown to be differentially expressed in two other models commonly used to study senescence: Ha-ras induced premature senescence of REF52 cells and replicative senescence of MEFs.

Even though the proteins identified in this study have not, so far, been directly associated with replicative senescence, some of them, such as gelsolin,  $\alpha$ -glucosidase, heat shock proteins and the TUC family of proteins, have been linked to cell proliferation.

Gelsolin has been proposed to be a tumour suppressor for breast cancer (Asch *et al.*, 1996); while the 3'-untranslated region of a  $\alpha$ -glucosidase-related mRNA has been shown to be able to promote colony formation and immortalisation in REFs and cooperate with an immortalisation-defective mutant of SV40 LT-ag to immortalise REFs (Powell *et al.*, 1999).

The finding that heat shock proteins were differentially expressed may be particularly relevant because it has been proposed that replicative senescence

in rodent cells may be caused by the stress of *in vitro* tissue culture conditions (Serrano and Blasco, 2001; Wright and Shay, 2000). It has been observed that increased stress such as freeze/thawing and growth at lower temperatures can shorten the finite proliferative cellular life span (Ikram *et al.*, 1994). Furthermore Piotrowicz *et al.* (Piotrowicz *et al.*, 1995) showed that over-expression of HSP27 in bovine pulmonary endothelial cells can stimulate their growth rate and accelerate the rate at which the cultures reach senescence.

Four members of the TUC family of proteins (Fig. 3.10) were found to be differentially expressed upon replicative senescence, some of which were up and others down-regulated. TUC is a family of phosphoproteins critical for neuronal differentiation, and because it has been proposed that replicative senescence reflects a state of terminal differentiation (Bayreuther *et al.*, 1988) and that senescent fibroblasts are the equivalent of terminally differentiated end-stage cells, it was not surprising to find this family of proteins involved in the senescence process. However, the mechanisms by which these proteins modulate cell growth and differentiation remain unknown. In addition to the down-regulation of TUC-2, it was also shown that there was a loss of TUC-2 phosphorylation upon both replicative senescence of REFs and premature senescence of REF52 cells.

However it was shown that the ectopic expression of TUC-2 alone into primary cells was not sufficient for immortalisation, and therefore it was suggested that the loss of expression TUC-2 did not cause of replicative senescence. However the results presented in this thesis suggest that TUC-2 may be a very good marker for senescence, and therefore it will be very informative to determine whether the down-regulation at the RNA level is due to decreased transcription or changes in RNA stability and also identify the factors that are the cause of these changes.

In addition to the down-regulation of TUC-2, it was also shown that there was a loss of TUC-2 phosphorylation upon both replicative senescence of REFs and premature senescence of REF52 cells. Interestingly, Ihara and colleagues have previously shown that in Alzheimer's disease (AD) a

phosphorylated form of TUC-2 was present within neurofibrillary tangles and some plaque neurites and was significantly increased within the soluble fraction of AD brain extracts (Gu *et al.*, 2000). They further showed that the phosphorylation occurred at serine and threonine residues within the carboxy-terminal basic region and proposed that it may play a role in regulating its activity. It will therefore be very interesting to determine in growing cells, the sites of TUC-2 phosphorylation, identify the kinase responsible and finally determine whether the loss of phosphorylation has a role in cellular senescence.

It was previously shown that treatment of neuroblastoma cells with retinoic acid (RA) results in differential expression of the same TUC genes that have been observed to be differentially expressed in the present study (Gaetano *et al.*, 1997). RA treatment also affects expression of the p73 family of proteins, one of the two, together with p63, recently discovered family of proteins related to p53 (Irwin and Kaelin, 2001), and p73 knock out mice have a neuronal differentiation defect. It has already been shown that p63 proteins are differentially expressed upon replicative senescence and it was proposed that the p63 proteins might have a role in modulating p53 activity during replicative senescence (Djelloul *et al.*, 2002). It would therefore be very interesting to check whether the TUC and p73 proteins are constituents of the same cellular pathway, and whether the transcription of the TUC proteins is under p73 control or the TUC proteins are able to modulate p73 expression. Moreover since the TUC-2 gene has been shown to be down-regulated also at the level of mRNA, it would be very interesting to determine whether this occurs at the level of transcription or stability and to determine the cause.

However it was not possible to show that any of the differentially expressed proteins have any effect in regulating the entry into the senescence state when singly over-expressed in primary cells. It is possible to argue that it is not sufficient to singly over-express these proteins to see any biological effect and it can be suggested that these proteins may have to act in concert; in other words it is possible that some of these proteins have to be up-regulated and at the same time some others have to be down-regulated to produce any

biological effect. This hypothesis seems particularly true for the TUC family of proteins, some of which have been found to be up and some down-regulated upon senescence. As already discussed it is also possible that a particular post-translational modified version of these proteins is the biological player and when the proteins are over-expressed the equilibrium is pushed towards a different modified, and therefore inactive, isoform or that modifications may or may not be occurring.

In conclusion, even though none of the analysed proteins seemed to play an active role in inducing or preventing senescence they are novel markers for this complex biological process as it has been demonstrated that their expression levels was changing considerably and consistently upon senescence, and they may also be new markers for cancer diagnosis and therapeutics. The differentially expressed proteins also represent important starting points for determining the activities critical for the observed changes in expression whether they occur at the mRNA, or protein levels or are regulated by post-translational modification and also whether they are involved in replicative senescence of human cells.

## 4 Differential proteomic analysis of tsa cell lines

### 4.1 Objectives

To identify and study the changes in gene expression that were linked with the entry into the post-mitotic state, a differential proteomic study has been conducted on the conditionally immortalised cell lines (tsa), a system in which senescence can be induced synchronously by altering the growth temperature (see Paragraph 1.1.9.2).

The tsa cell lines were derived from primary REFs by retroviral infection with a thermolabile SV40 LT-ag, tsA58T. Therefore these cell lines grow continuously at the permissive temperature, 33°C, where LT-ag is active, but rapidly undergo a synchronous growth arrest upon shift up to the non-permissive temperature, 39.5°C, due to inactivation and probable degradation of tsA58T LT-ag (Gonos *et al.*, 1996).

Initially the proteomes of the conditionally immortalised cell lines kept at the permissive temperature, and therefore in a proliferating state, was compared against the proteome of the same cell lines kept at the non-permissive temperature, and therefore induced to enter into a senescent state, to identify proteins that were differentially expressed.

Afterwards the differentials identified during the analysis of the conditionally immortalised cell lines undergoing senescence were compared with the differentials identified in the differential proteome study of replicative senescence conducted in serially passaged primary REFs, and described in Chapter 3.

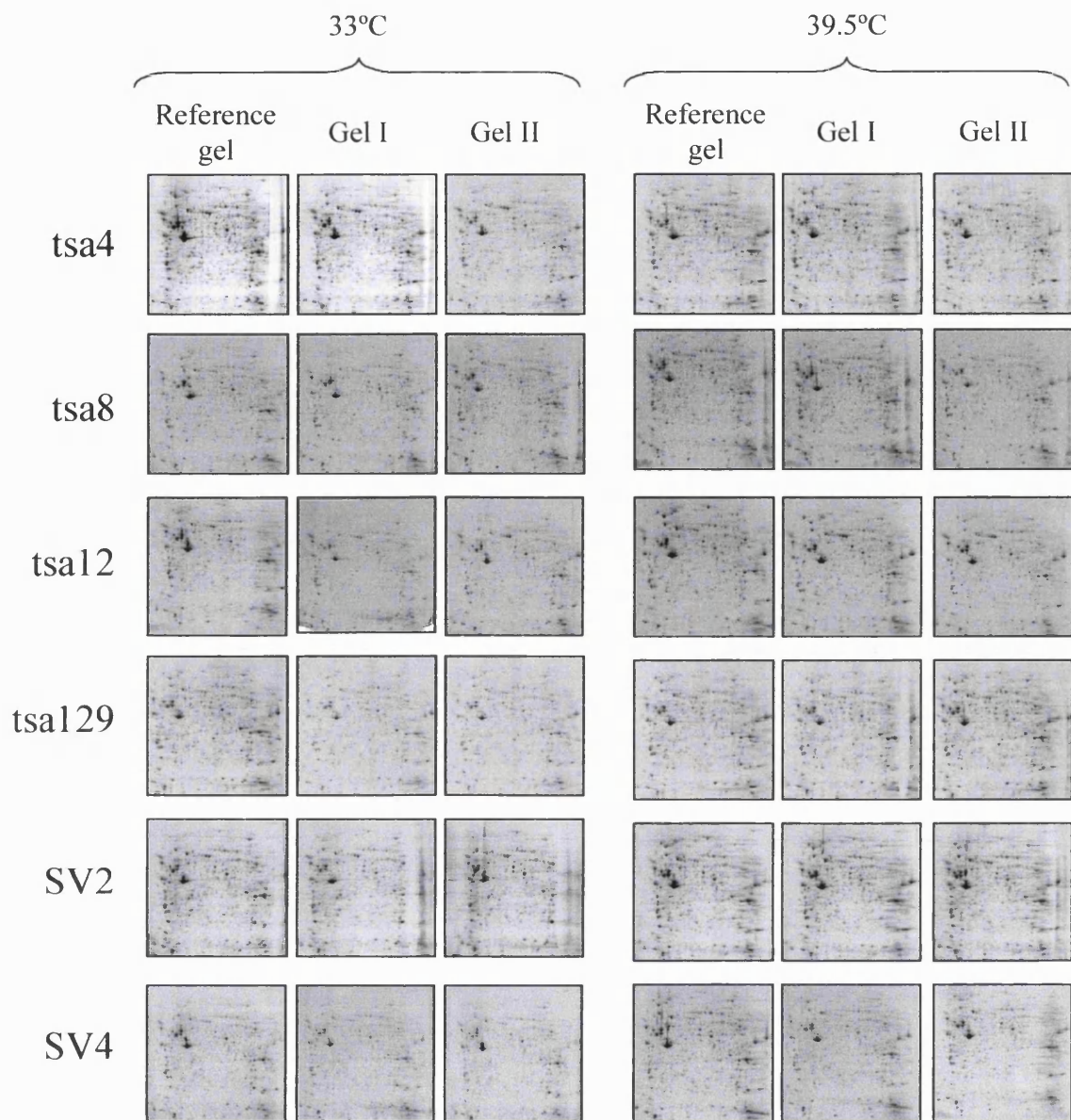
## 4.2 Experimental procedure

The loss of proliferative potential in the tsa cell lines was investigated in three different clonal cell lines, tsa4, tsa8 and tsa12. Only those changes in protein expression that were commonly observed in all the lines were further analysed.

Moreover, since this model system requires that cells are shifted to a higher growth temperature, and for this reason some genes could be differentially regulated by the increase in temperature, this study has been complemented by analysing two control clonal cell lines called SV2 and SV4 (Jat and Sharp, 1989). SV2 and SV4 were derived at the same time, from the same batch of Fischer REFs, and using the same protocol as for the tsa cell lines, except that the SV cell lines were isolated after infection with a recombinant retrovirus that transduced the wild type SV40 LT-ag. Since SV2 and SV4 cell lines were derived by immortalisation of REFs with wild-type SV40 LT-ag, they grow continuously at both temperatures, permissive (33°C) and non-permissive (and 39.5°C) and permit elimination of changes that are not related to loss of proliferative potential but are the consequence of the temperature shift.

Furthermore to ensure that the changes in expression were not specific to a particular genetic background, another clonal cell line, tsa129, derived using the same thermolabile tsA58 SV40 LT-ag used to generate tsa4, tsa8 and tsa14, but from Sprague-Dawley REFs (Mazars and Jat, 1997) instead from Fischer REFs was analysed.

Changes in protein expression profiles were monitored by high-resolution 2-D polyacrylamide gel electrophoresis (2-D PAGE). Features that were commonly differentially expressed between all the tsa cell lines, and not in the control cell lines were excised and identified by MALDI-MS and ESI-MS/MS.



**Fig. 4.1** Triplicate gels.

For each cellular condition three independent extracts were prepared and separated on three independent gels. t<sub>sa</sub>: conditionally immortalised cell line; SV: control cell line.

### 4.3 Sample preparation

Triplicate independent total protein extracts were prepared from the four different clonal conditionally immortalised cell lines (tsa4, tsa8, tsa12 and tsa129) grown at 33°C and, after shift up to the non-permissive temperature, at 39.5°C for 72 hours. An image of all the 2-D gels utilised in the study is represented in Fig. 4.1. Moreover, to ensure that only changes in expression due to senescence were being examined, cells were fed with fresh medium the day before extraction. In this way all lysates were prepared from cultures that were kept in fresh medium and sub-confluent and it was ensured that cells were not in the quiescent state ( $G_0$  phase of the cell cycle).

Moreover, as previously discussed (Paragraph 4.2), to eliminate any changes in protein expression due to the shift up to 39.5°C for 72 hours, extracts prepared from the two SV (SV2 and SV4) cell lines were analysed as a control.

When this study began, it represented the first investigation of tsa cell lines at the protein level; therefore it was decided to use total cellular extracts, to have a global view despite loosing sensitivity. Therefore the total protein extracts were fractionated on 2-D gels, to visualize changes in their proteome.

To be able to take into consideration during the experiment all the possible sources of variability (experimental and operator related variability, as well as the variability caused by the micro-environment) three independent cellular extracts were prepared for each cellular condition. The extracts were kept separate all through the process, and they were separated on different 2-D gels. This procedure yielded three independent 2-D gels for each clonal cell line at the two temperatures studied (33°C and 39.5°C).



#### **4.4 2-D gels: separation, detection and curation**

Total protein extracts prepared from the four tsa cell lines and the two controls at 33°C and 39.5°C were then fractionated on 2-D gels, to visualise changes in their proteome upon replicative senescence. The protein separation was performed using broad pH range gels in the first dimension, and gradient SDS-PAGE in the second dimension. Representative 2-D gels for tsa8 at 33°C is shown in Fig. 4.2.

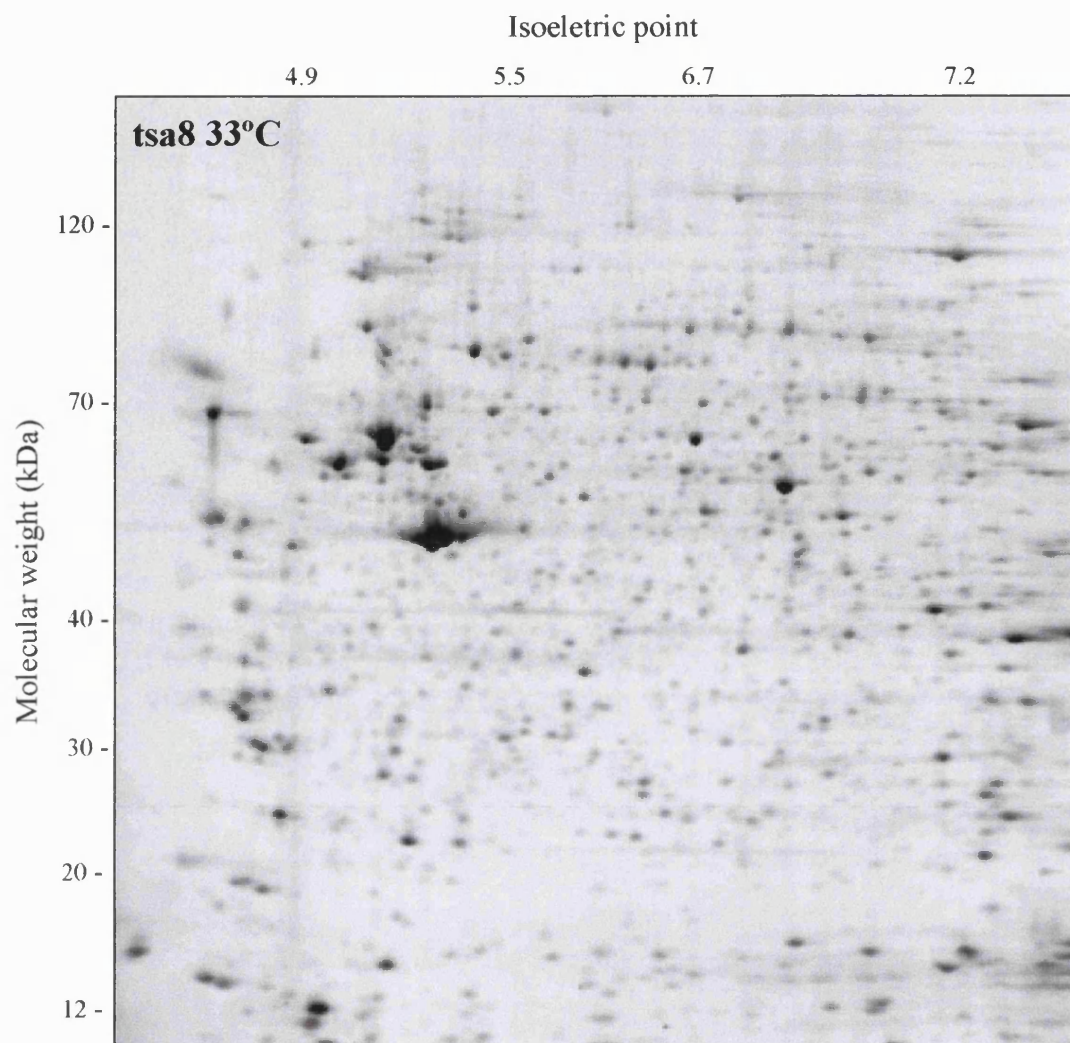
The fractionated proteins were then detected by staining the 2-D gels with the fluorescent dye OGT 1238 (proprietary of Oxford GlycoSciences), followed by scanning at a detection level of less than 1ng of protein.

The primary images were processed with a customised version of MELANIE II (GeneBio, Geneva, Switzerland) (see Paragraph 2.6.1) and a triplicate gel curation performed for each triplicate, has described in Paragraph 3.5.1. After curation, each gel was found to comprise over 1200 spots.

##### **4.4.1 Gel similarity**

After curation, the similarity among the gels inside each triplicate was then calculated as percentage of homology (%H) of the gels within each triplicate (as previously discussed in Paragraph 3.5.2). The triplicate gels representing the four tsa cell lines and the two controls showed an overall %H that was very high and therefore the gels were considered to be very reproducible within each triplicate (shown in Table 4.1).

The percentage of homology among the gels inside each triplicate was always above 80% except for tsa8 and tsa12, from whom it appeared to be slightly lower. In particular the percentage of homology for tsa8 gel II dropped to 66% when compared with the Reference gel.



**Fig. 4.2** Representative tsa 2-D gel.

A representative 2-D gel image of conditionally immortalised tsa8 cell line at 33°C, obtained upon fractionation of 150µg of total cellular extract.

**Table 4.1** Percent homology (%H) between the gels within each triplicate.

A)

33°C	Reference gel	Gel I	Gel II
<b>tsa8</b>	100	86.9	87.5
<b>tsa12</b>	100	84.9	76.8
<b>tsa4</b>	100	91.1	88.3
<b>tsa129</b>	100	82.3	94.3
<b>SV4</b>	100	84.8	80.3
<b>SV2</b>	100	89.4	87.0

B)

39.5°C	Reference gel	Gel I	Gel II
<b>tsa8</b>	100	66.0	87.1
<b>tsa12</b>	100	79.0	73.4
<b>tsa4</b>	100	97.8	94.0
<b>tsa129</b>	100	96.0	96.5
<b>SV4</b>	100	92.3	87.4
<b>SV2</b>	100	97.0	93.1

Triplicate 2D-gels were run for each cellular condition. The images were then curated and the percentage of homology (%H) among the gels within each triplicate calculated.

## 4.5 Analysis criteria

The curated gels were then submitted to CHIMAP (Harris *et al.*, 2002), to identify differentially expressed proteins (as described in Paragraph 3.6).

After the comparison of the proteome of each cell line at 33°C versus 39.5°C, the proteomes of the four clonal cell lines studied (tsa4, 8, 12 and 129) were compared, to check whether the different conditionally immortalised cell lines shared any changes in protein expression when each proliferative status was compared with the correspondent senescent status. It was then checked if the common changes were occurring in the same direction (i.e. up- or down-regulation) and if they had a similar magnitude.

During the analysis only those changes that were observed in all four tsa cell lines but not in the controls were considered relevant. Those changes that occurred both in the tsa cell lines as well as in the SV controls have not been considered as they were most likely to be due to the temperature shift, and not to cellular senescence *per se*.

The changes that occurred in the SV controls but not in the tsa cell lines have also been considered, hypothesizing that an opposite change was occurring in the tsa cell lines to balance the change observed in the controls due to the temperature shift.

Only protein spot changes that were greater than 1.5-fold in magnitude and observed in all three gels within each triplicate were considered (Table 4.2). It should be noted that for changes that were designated as n.c., it does not necessarily imply that there was no change, but that it was less than 1.5-fold in magnitude.

Representative 2-D gels of tsa8 at 33°C and 39.5°C, with highlighted some of the differential spots, are represented in Fig. 4.3, and representative 2-D gels of SV4 at 33°C and 39.5°C, with highlighted some of the differential spots, are represented in Fig. 4.4.

## 4.6 Differentially expressed proteins

The analysis of the conditionally immortalised cell lines identified a total of 43 spots that represented proteins differentially expressed upon shift up to 39.5°C and thus, in accordance with the model system utilised, represented features that were differentially expressed upon entry into replicative senescence. These differentials were grouped into 5 different sub-groups, according to how well they fit with the rationalisation presented above (see Paragraph 4.5).

Among the total of 43 spots identified as differentially expressed, 17 followed exactly the criteria that has been imposed for the analysis (Table 4.2 A). Spot 309 and 639 increased in all the tsa cell lines upon shift up from 33°C to 39.5°C, and did not change in the two control SV cell lines. Spot 408 behaved in an analogous way except that it decreased in one of the controls (SV2) upon shift up from 33°C to 39.5°C. Spot 295 (presented in Fig. 4.5) and 340 decreased in all the tsa cell lines upon shift up from 33°C to 39.5°C, and did not change in the two control SV cell lines. Spot 854 was found to remain flat in the tsa cell lines (all except tsa12 where it increased) upon transfer from 33°C to 39.5°C, and to decrease from 33°C to 39.5°C in the two SV control cell lines. All other 12 spots remain unchanged in all the tsa cell lines at both temperatures but increased in the control SV cell lines upon shift up from 33°C to 39.5°C, spot 112 (shown in Fig. 4.6) represents this category of changes. The 17 changes that followed exactly the analysis criteria, and described above, have been designated the “perfect matches”.

However during the analysis relevant those changes that did not fit exactly the above rationalisation and occurred in some of the cell lines, but not in all of them were also considered.

This was done because it was possible that the lack of a perfect match in some of the conditional cell lines was a reflection of the degree of conditionality of the tsa cell lines. It has been previously shown that the degree

of conditionality, shown as reduction in the proliferative potential upon shift up to the non-permissive temperature, varies between the cell lines.

In this way spots that were differential in all except one cell line were identified. This identified 13 differential spots (Table 4.2 B). Four changes that were mismatched in two of the cell lines considered in the study (Table 4.2 C) were also found. Similarly were also identified six spots that were mismatched in three cell lines (Table 4.2 D).

In accordance with the above hypothesis it was particularly interesting to note that the tsa cell line that had the lowest number mismatches with the rationale used for the analysis was tsa8 (no mismatches detected), while tsa12 had a single mismatch, and tsa129 and tsa4 had 3 and 5 mismatches respectively. This correlates exactly with the degree of conditionality of the cell lines; tsa8 is the most conditional cell line, followed by tsa12 and tsa4 and 129 are the least conditional in this group of tsa cell lines (Jat and Sharp, 1989).

The analysis identified a further 3 spots that showed a shift in their migration on the 2-D (Table 4.2 E). Spot 883 and 889 (Fig. 4.7) showed a shift in their migration in all the four tsa cell lines, while they migrated at the within the same gel coordinates in the two control SV cell lines. Spot 853 also showed a shift in its migration in the tsa cell lines but not in the control SV cell lines.

#### **4.7 Protein identification by Mass spectrometry**

The differentially regulated spots were then excised (using a customised OGS cutter, Apollo) and analysed by mass spectrometry to obtain their identity. To ensure that there was enough protein for the MS analysis, the features were excised from the gel in which they were most abundant. They were first subjected to MALDI-MS peptide mass mapping. The results are presented in

**Table 4.2** Fold change of the differentials.**A) All cell lines**

<b>ID</b>	<b>MW</b>	<b>pI</b>	<b>tsa4</b>	<b>tsa8</b>	<b>tsa12</b>	<b>tsa129</b>	<b>SV2</b>	<b>SV4</b>
61	113722	5.80	n.c.	n.c.	n.c.	n.c.	+ 2.5	+ 4.1
92	94474	6.50	n.c.	n.c.	n.c.	n.c.	+ 2.1	+ 1.5
112	92040	5.67	n.c.	n.c.	n.c.	n.c.	+ 1.8	+ 2.2
161	78807	6.44	n.c.	n.c.	n.c.	n.c.	+ 1.5	+ 1.8
209	71346	5.96	n.c.	n.c.	n.c.	- 4.9	+ 1.5	+ 1.6
236	60178	5.97	n.c.	n.c.	n.c.	n.c.	+ 2.1	+ 3.5
239	68141	5.90	n.c.	n.c.	n.c.	n.c.	+ 1.6	+ 2.3
295	63742	6.07	- 1.5	- 1.7	- 1.5	- 1.6	n.c.	n.c.
309	61908	6.90	+ 1.5	+ 1.5	+ 1.8	+ 1.8	n.c.	n.c.
340	61124	5.85	- 1.5	- 1.5	- 1.5	- 1.5	n.c.	n.c.
352	60276	5.54	n.c.	-1.5	n.c.	n.c.	+ 2.3	+ 1.5
408	54970	7.28	+ 2.1	+ 1.5	+ 1.9	+ 1.6	- 1.5	n.c.
448	53548	6.49	n.c.	n.c.	n.c.	n.c.	+ 2.0	+ 2.0
547	44443	5.34	n.c.	- 2.0	n.c.	- 1.5	+ 1.9	+ 2.6
639	42919	7.01	+ 1.7	+ 1.5	+ 1.6	+ 1.5	n.c.	n.c.
854	34256	7.19	n.c.	n.c.	+ 1.8	n.c.	- 1.5	-1.5
1009	21696	5.03	n.c.	- 1.8	n.c.	n.c.	+ 2.0	+ 1.3

**B) 5 cell lines**

<b>ID</b>	<b>MW</b>	<b>pI</b>	<b>tsa4</b>	<b>tsa8</b>	<b>tsa12</b>	<b>tsa129</b>	<b>SV2</b>	<b>SV4</b>
50	126542	5.49	n.c.	n.c.	- 4.3	+ 1.7	+ 1.7	+ 2.8
70	104809	4.92	n.c.	n.c.	n.c.	+ 1.5	+ 2.1	+ 1.7
75	109260	5.31	n.c.	- 2.0	+ 2.0	n.c.	+ 4.5	+ 4.3
88	99679	5.20	n.c.	n.c.	+ 1.6	n.c.	+ 8.3	+ 5.5
125	86088	5.20	+ 1.5	n.c.	n.c.	n.c.	+ 1.9	+ 1.5
130	84708	6.00	n.c.	n.c.	n.c.	n.c.	+1.5	n.c.
232	68929	7.01	+ 2.1	+ 1.7	+ 2.1	n.c.	n.c.	n.c.
282	63172	6.37	n.c.	- 1.8	n.c.	n.c.	n.c.	+ 2.9
454	46916	4.89	n.c.	n.c.	n.c.	- 2.2	+ 1.9	n.c.
503	50222	5.59	+ 2.1	-2.0	n.c.	n.c.	+ 4.0	+ 2.5
559	46471	5.62	+ 1.6	n.c.	n.c.	n.c.	+ 2.2	+ 1.8
667	39256	5.69	+ 1.9	n.c.	n.c.	n.c.	+ 1.6	+ 1.7
1060	24095	5.83	+ 1.5	- 1.5	- 1.5	- 1.5	n.c.	n.c.

### C) 4 cell lines

ID	MW	pI	tsa4	tsa8	tsa12	tsa129	SV2	SV4
68	104809	5.26	- 1.6	- 1.9	+ 2.1	+ 2.2	+ 7.5	+ 1.6
81	99504	7.15	n.c.	n.c.	+ 2.1	n.c.	n.c.	+ 1.9
693	38467	5.18	+ 2.5	- 3.3	+ 1.5	n.c.	n.c.	n.c.
740	38566	6.02	n.c.	-1.7	- 1.7	n.c.	n.c.	n.c.

### D) 3 cell lines

ID	MW	pI	tsa4	tsa8	tsa12	tsa129	SV2	SV4
75	102501	6.39	n.c.	+ 1.8	n.c.	n.c.	n.c.	n.c.
120	88201	7.29	n.c.	+ 1.8	+ 3.3	n.c.	- 1.6	n.c.
149	81062	6.17	n.c.	n.c.	+ 1.5	- 1.7	n.c.	+ 1.8
187	68832	7.38	+ 1.5	+ 2.0	n.c.	n.c.	- 1.8	n.c.
251	66472	5.45	n.c.	n.c.	- 2.3	- 1.5	+ 2.8	n.c.
380	58656	6.99	n.c.	n.c.	+ 1.9	n.c.	n.c.	n.c.

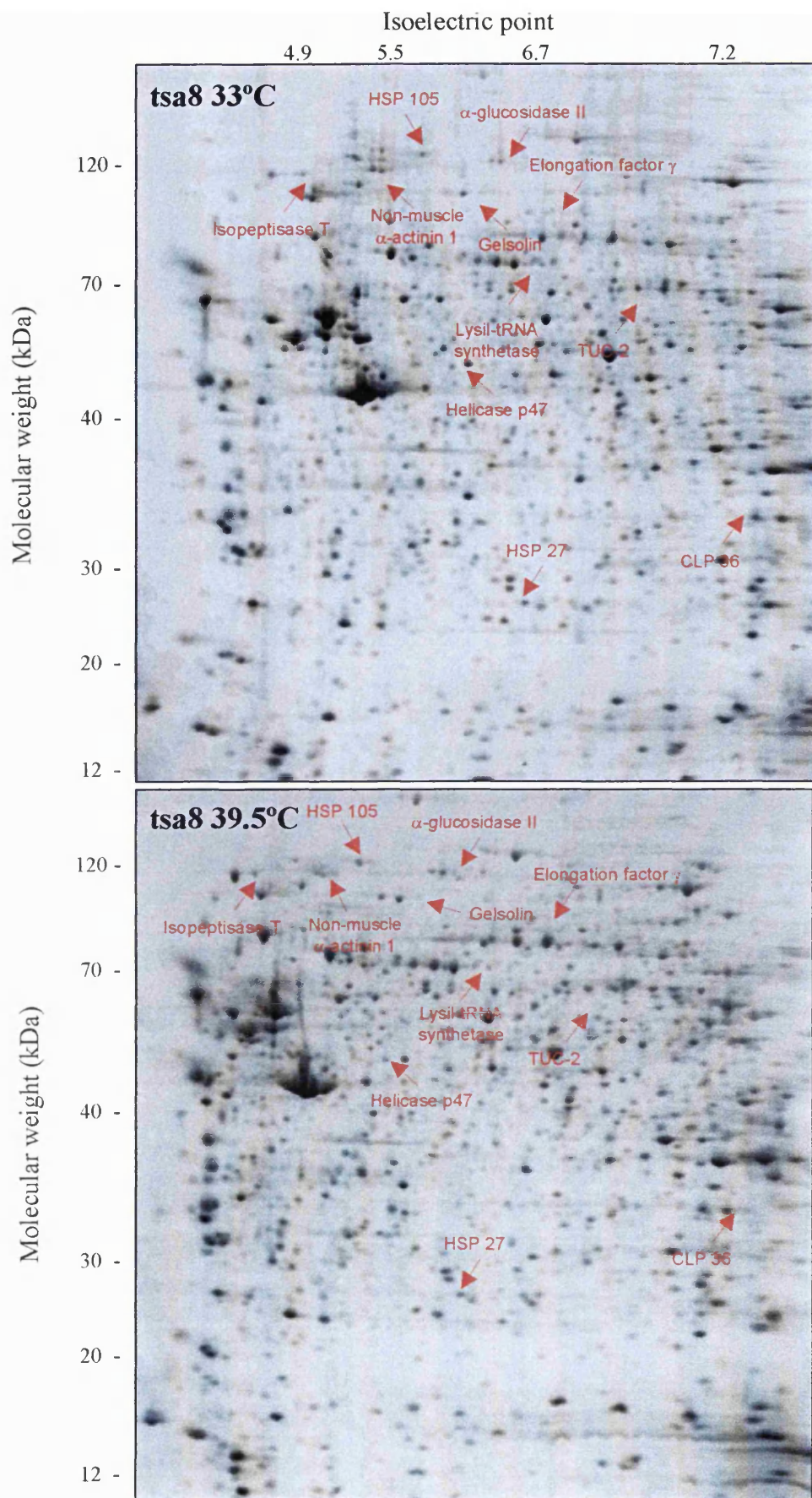
### E) Shift in the migration

ID	MW	pI	tsa4	tsa8	tsa12	tsa129	SV2	SV4
853	34374	6.17	SHIFT	SHIFT	SHIFT	n.c.	n.c.	SHIFT
883	31214	5.72	SHIFT	SHIFT	SHIFT	SHIFT	n.c.	n.c.
889	30933	5.75	SHIFT	SHIFT	SHIFT	SHIFT	n.c.	n.c.

ID: identity number  
n.c.: no changes

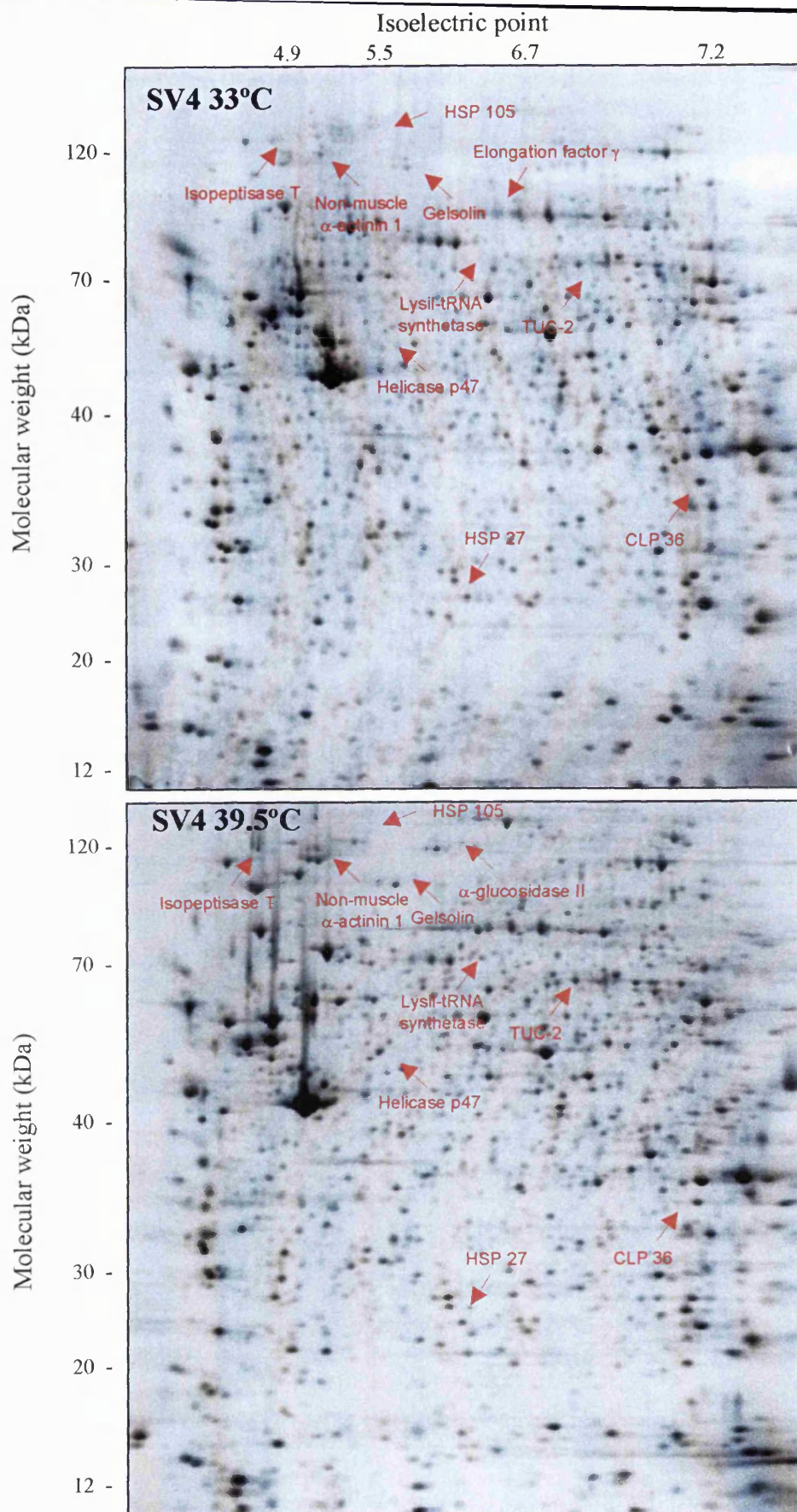
A) Representation of the magnitude of the changes for the features found to be differentially expressed in all the cell lines (A); in five cell lines (B); in four cell lines (C); in three cell lines (D) and with a shift in their migration (E).





**Fig. 4.3** Representative *tsa* 2-D gels with highlighted some differentially expressed features.

Representative 2-D gels from proliferating (33°C) and senescent (39.5°C) *tsa8* (150 $\mu$ g of total cell extracts) with highlighted the identities of some of the differential spots.



**Fig. 4.4** Representative SV 2-D gels with highlighted some differentially expressed features.

Representative 2-D gels from SV4 at 33 °C and 39.5°C (150 $\mu$ g of total cell extracts) with highlighted the identities of some of the differential spots.



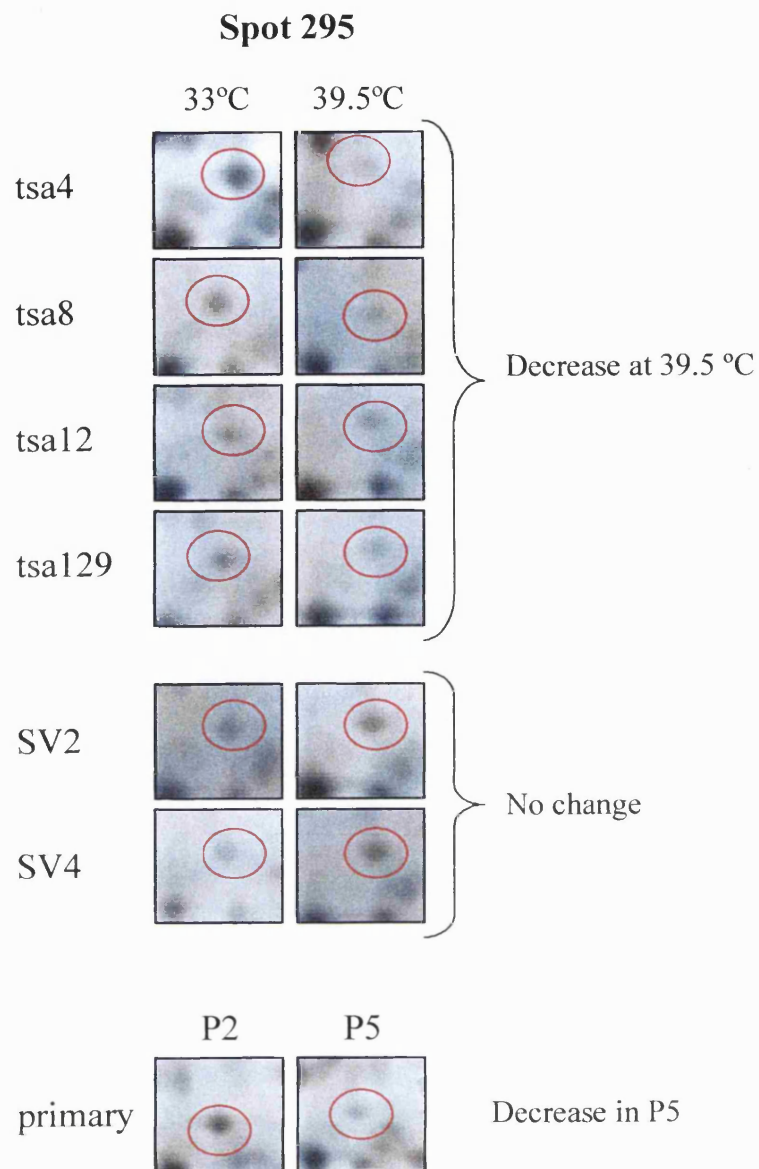
Table 4.3 and in greater detail in Supplementary information II, Table 4. For some of the spots where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous, ESI-MS/MS was performed. In addition, a few samples with sufficient MALDI-MS data for protein identification were also analysed by ESI-MS/MS to verify the MALDI-MS peptide mass mapping. These results are also summarised in Table 4.3 and in greater detail in Supplementary information II, Tables 5 and 6. Positive protein identification by MALDI-MS was confirmed in all cases where ESI-MS/MS was employed and all putatively identified proteins were confirmed.

#### **4.7.1 Peptide mass mapping by MALDI-MS**

Analysis of the differentially regulated spots by MALDI-MS yielded 29 single positive identifications, 5 double positive identifications and 4 single putative identifications (Tables 4.2 and in greater detail as Supplementary Information II, Table 4). In 2 cases more than 20 peptide ion signals were obtained, but database searches with these peptide mass lists did not identify any proteins. For the remaining 3 out of the 43 differential spots, the mass spectra recorded exhibited not more than 10 peptides; these data were classified as insufficient for peptide mass mapping. From all identified proteins only 22 were identified as rat proteins (*R. norvegicus*), 12 were mouse (*M. musculus*), 5 were human (*H. sapiens*) and 2 were from *Oryzolagus cuniculus*, 1 from *B. Taurus* and 1 from *Cricetulus longicaudatus*.

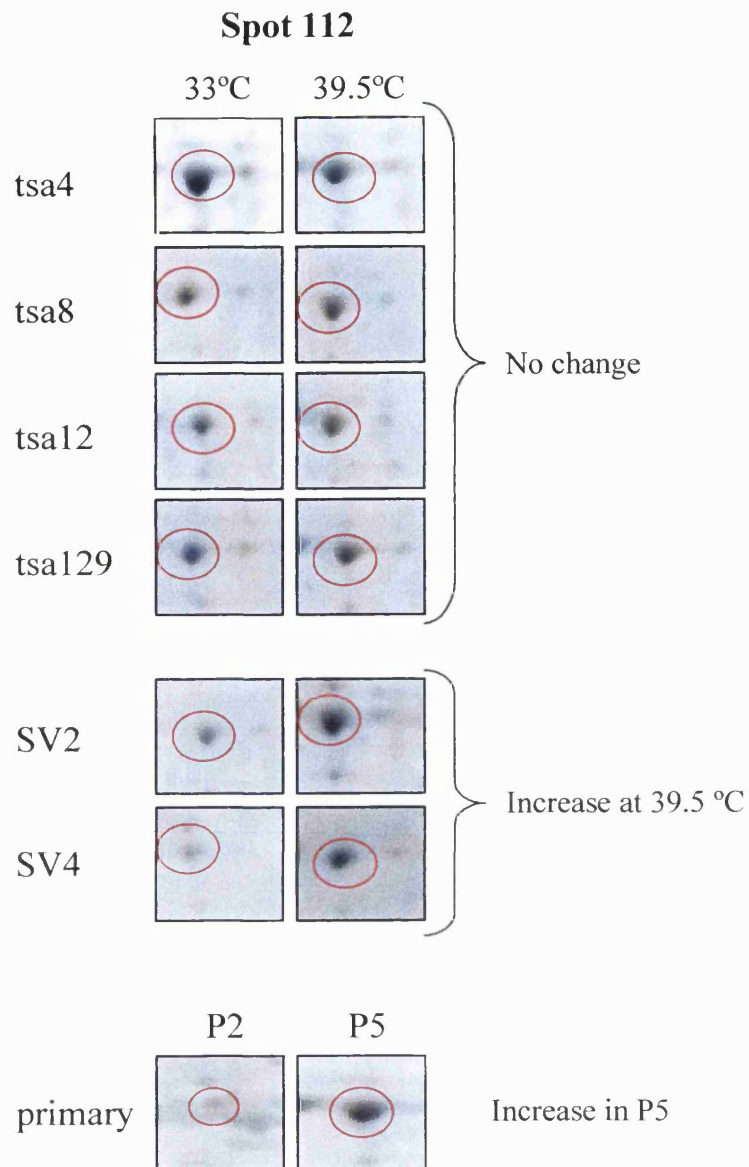
#### **4.7.2 Protein identification by ESI-MS/MS**

After MALDI-MS was performed for all the excised spots, nano-HPLC ESI-MS/MS was used for almost two thirds of the excised spots (in all cases



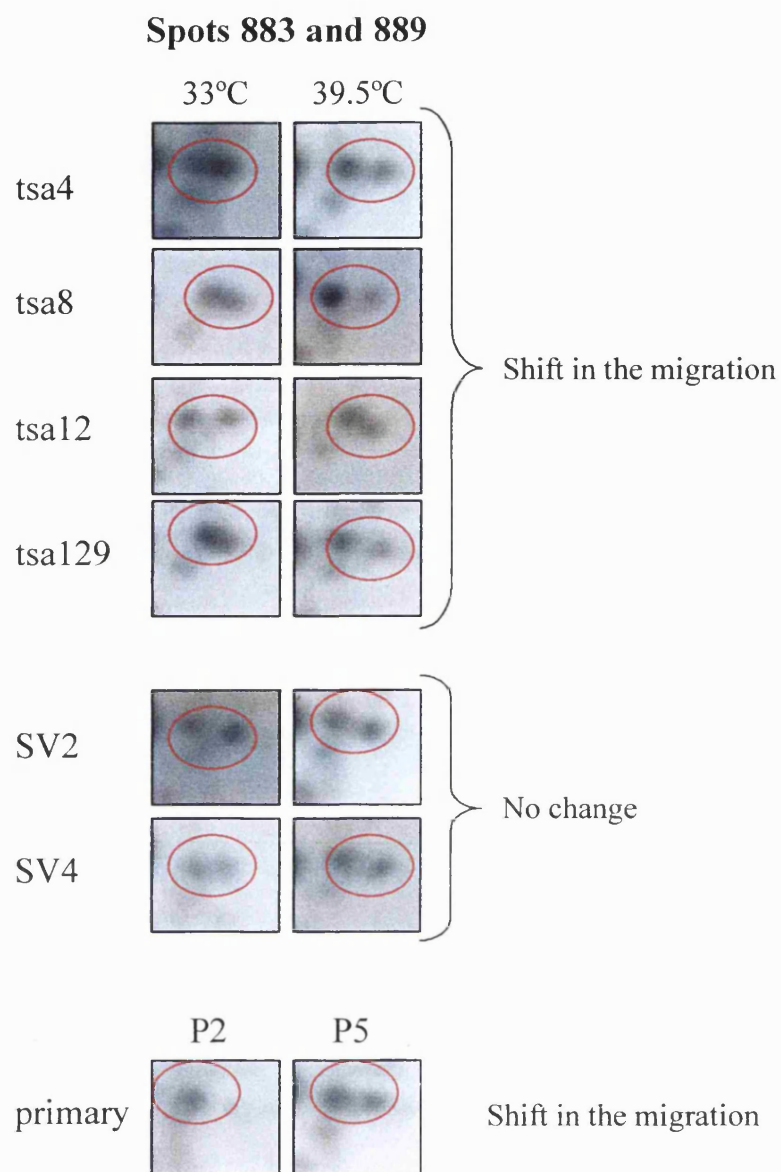
**Fig. 4.5** Representative down-regulated feature in the tsa cell lines.

2-D gel images showing a selected feature down-regulated in all the tsa cell lines and flat in the two controls. The same feature was down-regulated upon replicative senescence in the primary cells.



**Fig. 4.6** Representative up-regulated feature in the control cell lines.

2-D gel images showing a selected feature up-regulated in the SV control cell lines, and flat in all the tsa cell lines. The same feature was up-regulated upon replicative senescence in the primary cells.



**Fig. 4.7** Representative features that shift in their migration.

2-D gel images showing selected features with an altered migration. The same features showed a shift in their migration also in the primary cells upon replicative senescence.

where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous and for a few other spots as positive controls). The results for the protein identification by ESI-MS/MS (false positives excluded) are also summarised in Tables 4.2 and presented in greater detail as Supplementary Information II, Tables 5 and 6. From all the proteins identified by searching the NCBI protein database only 4 proteins were identified as rat proteins (*R. norvegicus*), whereas 9 were from mouse (*M. musculus*), 1 from *O. cuniculus*, 1 from *C. longicaudatus*, 1 from *X. laevis* and 6 were human (*H. sapiens*). Positive protein identifications obtained by MALDI-MS peptide mass mapping were confirmed in all cases where ESI-MS/MS was employed. All the putatively identified proteins by MALDI-MS peptide mass mapping were also confirmed by ESI-MS/MS whenever sufficient data was obtained. In addition to these confirmations the proteins of three more spots were identified (serine proteinase inhibitor mBM2A together with serine protease inhibitor 14 and 16 for spot 667, LIM protein for spot 854 and an hypothetical protein with accession number 8923710, for spot 883). As previously demonstrated for the REFs data (see Paragraph 3.8.2), the comparison of the results from the MALDI-MS and ESI-MS/MS data demonstrated that both techniques show comparable sensitivity in protein identification. Furthermore, this comparison showed that the criteria applied for protein identification was reliable and gave virtually no false identifications, with a minimal loss in analytical sensitivity.

#### **4.8 Differentially expressed proteins in common with primary cells**

The proteins found to be differentially expressed upon loss of proliferative potential in the conditionally immortalised tsa cell lines were then compared with the proteins found to be differentially expressed upon replicative senescence in the primary REFs.

This comparison identified 9 differentially expressed features that were in common between the two model systems.

The two studies of replicative senescence, one conducted on primary REFs and the other on the tsa cell lines, were conducted entirely separately. REFs and tsa gels were curated and analysed independently. The spots identified as differential were excised twice, once from the primary gels and once from the conditionally immortalised cell lines gels, and separated mass spectrometry analyses were conducted on each excised spot. Only in the end the lists of differentially regulated spots from the two studies, together with the identities of the spots obtained by MS, were compared.

It was possible to match and subsequently compare the tsa gels with the REF gels because the isoelectric point (pI) and molecular weight (MW) of each spot was calculated by bilinear interpolation between landmark spots on each image that had previously been calibrated with respect to the same *E. coli* proteins.

To begin with only the lists of the differentially expressed features in the two systems were compared; as follow up however it was necessary to manually check the common features on both the tsa and REFs Reference gels, to make sure that the features were truly differential and that no mismatch error occurred during the matching process.

The finding that the proteomic analysis of replicative senescence conducted on the primary REFs and the proteomic analysis conducted on the tsa cell lines identified nine common spots was very exciting. It was not only a support of the reliability of the proteome approach, but it was also a further justification of the necessity to use both model systems, primary cells and conditionally immortalised cell lines, to get a more comprehensive picture of replicative senescence.

From the 9 features that were in common between the tsa cell lines and the primary REFs, 6 pairs of spots identified the same protein from the two systems; tsa spot 50 and REFs spot 102 was alanyl-tRNA synthetase; tsa spot 61 and REFs spot 127 was  $\alpha$ -glucosidase II,  $\alpha$  subunit; tsa spot 112 and REFs spot 190 was gelsolin; tsa spot 295 and REFs spot 304 was TUC-2; tsa spot 503



**Table 4.3** Identification of the differentially expressed features by mass spectrometry.

ID	MW	pI	MALDI-MS SPOT IDENTITY	ESI SPOT IDENTITY
50	126542	5.49	Alanyl-tRNA synthetase (h)	Alanyl-tRNA synthetase (h) + Heat shock protein 105 kDa $\alpha/\beta$ (m)
61	113722	5.8	$\alpha$ -glucosidase II, $\alpha$ subunit (m)	
68	104809	5.26	Non-muscle $\alpha$ -actinin 1 (r)	
70	104809	4.92	Isopeptidase T (m)	
75	102501	6.39	Alix (m)	
75	109260	5.31	$\alpha$ -Actinin (r) + Non-muscle $\alpha$ -actinin 1 (r)	
81	99504	7.15	Elongation factor 2 (r)	
88	99679	5.2	Transitional endoplasmic reticulum ATPase (r)	
92	94474	6.5	Lysyl hydroxylase isoform 2 (m)	
112	92040	5.67	Gelsolin (m)	Gelsolin (h and m)
120	88201	7.29	DEAD box protein RB (h)	
125	86088	5.2	Eukaryotic polypeptide chain release factor 3 (oc)	Eukaryotic polypeptide chain release factor 3 (oc)
130	84708	6.0	Elongation factor $\gamma$ (r)	
149	81062	6.17	CDC47 (m)	CDC47p (xl)
161	78807	6.44	Radixin (m)	Radixin (m)
187	68832	7.38		&
209	71346	5.96	Glycl-tRNA synthetase (h)	
232	68929	7.01	Lamin A (r)	
236	60178	5.97	Leukotriene A4 hydrolase (r)	&
239	68141	5.9	Lysyl-tRNA synthetase (cl)	Lysyl-tRNA synthetase (cl)
251	66472	5.45	Annexin VI (r)	
282	63172	6.37	Lamin A (r) + Neural specific protein CRMP-2 (b)	
295	63742	6.07	TUC-2 (r)	
309	61908	6.9	Lamin A (m)	
340	61124	5.85	$\gamma$ -Butyrobetaine, 2-oxoglutarate dioxygenase (r)	

ID	MW	pI	MALDI-MS SPOT IDENTITY	ESI SPOT IDENTITY
352	60276	5.54	Copine I (h)	Copine I (h) + Chaperonin subunit (θ) (m) + T-complex protein (h) + κ-B motif-binding phosphoprotein (m)
380	58656	6.99	Tyrosyl-tRNA synthetase (h)	
408	54970	7.28	IMP dehydrogenase (m)	
448	53548	6.49	Aspartyl-tRNA synthetase (r)	
454	46916	4.89	ATP synthase β subunit (r) + Class I β-tubulin (r)	ATP synthetase β subunit (r) + Tubulin, β (m) + Tubulin, β (h)+ Vimentin (r)
503	50222	5.59	Eukaryotic polypeptide chain release factor 1 (oc) + Probable ATP-dependent RNA helicase p47 (r)	
547	44443	5.34	Translation initiation factor eIF-4A (m) + Actin β (r)	
559	46471	5.62		*
639	42919	7.01	Cytosolic NADP-dependent isocitrate dehydrogenase (r)	
667	39256	5.69		Serine proteinase inhibitor mBM2A (m) + Serine protease inhibitor (m) + Serine protease inhibitor (m)
693	38467	5.18	Guanine nucleotide-binding protein α subunit (r)	
740	38566	6.02		
853	34374	6.17	Cytosolic malate dehydrogenase (r)	
854	34256	7.19		LIM protein (r)
883	31214	5.72		Hypothetical protein (h)
889	30933	5.75	PA28 γ subunit (m)	
1009	21696	5.03	Mitotic checkpoint component Mad2 (m)	
1060	24095	5.83	Heat shock protein (r)	HSP27 (r)

ID: identity number; \*: no significant hits

(h): *Homo sapiens*; (m): *Mus musculus*; (r): *Rattus norvegicus*; (b): *Bos taurus*; (oc): *Oryctogalus cuniculus*;  
(cl): *Cricetulus longicaudatus*; (xl): *Xenopus laevis*

and REFs spot 677 identified eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47; tsa spot 1060 and REFs spot 1302 was HSP27. Moreover tsa spot 854 was identified as a LIM domain-containing protein whereas REFs spot 1076 was identified more specifically as CLP36, which contains a LIM domain.

One spot (tsa spot 309 which matched with REFs spot 504) yielded a different identity when analysed by MS in the two different systems. It was identified as lamin A from the tsa spot whereas from the REFs spot it was identified as MPAST. The last spot did not yield any protein identity in the REF sample (tsa spot 239 which matched with REFs spot 369) while it was identified as lysyl-tRNA synthetase from the tsa spot.

One possibility for the lack of correlation between the identities for spot 309/504 could be the presence of multiple proteins and the relative abundance of each component of that spot being slightly different in the two different cell models, and only the most abundant protein being identified by mass spectrometry.

Since it was hypothesised that the tsa cell lines were a model for studying the transition to the post-mitotic state of senescence, the features found to be differential in both model systems (primary cells and conditionally immortalised cell lines) were likely to correspond to proteins involved in the entry into senescence.

#### **4.9 Differentially expressed features in the tsa but not in the primary cells**

While the primary cells were a model for studying both the counting mechanism hypothesised to be involved in senescence and the entry into the post-mitotic state, the tsa cell lines were a model for studying only the transition to the post-mitotic state of senescence. According with this rationale it is possible to speculate that the features found to be differential in both model

systems (primary cells and conditionally immortalised cell lines) are likely to correspond to proteins that are involved in the entry into the post-mitotic state.

On the other hand, the differentials only occurring in the primary model, and not in the conditionally immortalised cell lines, were likely to represent those proteins involved in the counting mechanism.

It was more difficult to explain those changes that were occurring in the tsa but not in the primary cells. It could be possible that the changes detected only in the conditionally immortalised cell lines were in reality occurring also in the primary cells, but they were not detected because of the slightly different analysis criteria adopted. For the analysis of the primary cells it was decided to consider as relevant those changes that were at least 2 fold in magnitude, while during the tsa cell lines study it was decided to drop the fold change to 1.5. This was done because during the analysis of tsa cell lines more conditions were compared at the same time.

Alternatively it was possible that the changes detected in the tsa cell lines and not in the primary cells were actually occurring also in the primary cells but the magnitude of these changes was lower than the cut-off set for the REF analysis.

The changes occurring only in the tsa cell lines and not in the primary cells might also be due to ectopic expression of LT-ag in the cell lines. They might be genes down-stream of LT-ag, switched on when LT-ag was expressed in the cells at 33°C and remaining in the cells after the LT-ag inactivation for 72 hours at the not permissive temperature (39.5°C), after which the conditionally immortalised cell lines can not be reverted into mortality with the shift back to the permissive temperature.

#### **4.10 Candidates for the entry into the post-mitotic state**

The proteins that were found to be differentially expressed in the tsa cell lines when they ceased proliferating upon inactivation of tsA58 LT-ag comprised a variety of cytoskeletal, heat shock and metabolic proteins as well as proteins involved in differentiation and protein synthesis, turnover and modification.

As previously stated, 9 features were in common between the primary REFs and the tsa cell lines. Three of these features fit perfectly the analysis criteria adopted in each model system used; therefore they were perfect potential candidates for the entry into the post-mitotic state. They will be described in greater detail in the following section.

#### **4.11 TUC-2**

tsa spot 295 (matched with REFs spot 304) was identified as TUC-2 (Tables 3.3 B and 4.3). It was identified as a down-regulated spot in all four tsa cell lines while it remained unchanged in the two control SV cell lines upon shift up of the temperature (Fig. 4.5). TUC-2 was also down-regulated in the primary REFs upon serial passaging (Fig. 3.7 B, Fig. 3.8 and Fig. 4.5).

The down-regulation of TUC-2 was also shown to occur when premature senescence was induced in REF52 cells upon ectopic expression of activated Ha-ras (Fig. 3.15 and Fig. 3.16).

Moreover the down-regulation of TUC-2 in senescent REFs was demonstrated to occur at the RNA level (Fig. 3.11).

Since it was shown that ectopic expression of TUC-2 alone was not sufficient for immortalisation of REFs (previously discussed in Paragraph 3.20 and shown in Fig. 3.12) it was suggested that the loss of expression TUC-2 was not a cause of replicative senescence, however TUC-2 down-regulation remains an excellent marker for senescence. It was previously shown (Paragraph 3.25

and Fig. 3.18) a loss of phosphorylation of TUC-2 upon replicative senescence of REFs as well as premature senescence of REF52. It remains to be investigated whether the same loss of phosphorylation occurs also in the tsa cell lines.

#### **4.11.1 *Lamin A***

Another feature that was differential according with the criteria adopted in the analysis was tsa spot 309 (corresponding with REFs spot 504). It was up-regulated in all the tsa cell lines and remained unchanged in the control SV cell lines upon shift up in temperature. The same spot has previously found to be up-regulated in senescent REFs. This feature was identified in the tsa gels as lamin A whereas in the REF gels it was identified as MPAST together with lamin A or C.

Lamin A is a member of a protein family that includes three known mouse A-type lamins (A, C and C2), encoded within a single genomic locus (Nakajima and Abe, 1995). They are the major components of the nuclear lamina, a two-dimensional filamentous network at the periphery of the nucleus in higher eukaryotes, directly underlying the inner nuclear membrane (Gotzmann and Foisner, 1999). Lamins as well as lamin-binding proteins appear to be important for various steps of post-mitotic nuclear reassembly, including cross-linking of chromatids, nuclear membrane targeting, nuclear lamina assembly, and also the formation of a replication-competent nucleus, however their specific role remains unknown (Gotzmann and Foisner, 1999).

Further work is thus necessary to determine which of these candidate proteins is truly differentially expressed, whether the up-regulation is due to up-regulation at the RNA level and what is the cause of this up-regulation.

**Table 4.4** Differentially expressed features that were in common between the primary cells and the tsa cell lines.

REFs ID	MW	pI	REFs IDENTITY	tsa ID	MW	pI	tsa IDENTITY
102	125386	5.48	Alanyl-tRNA syntetase	50	126542	5.49	Alanyl-tRNA synthetase + Heat shock protein 105 kDa $\alpha/\beta$
127	113345	5.82	$\alpha$ -glucosidase II, $\alpha$ subunit	61	113722	5.8	$\alpha$ -glucosidase II, $\alpha$ subunit
190	91608	5.66	Gelsolin + myosin heavy chain	112	92040	5.67	Gelsolin
304	63329	6.09	TUC-2	295	63742	6.07	TUC-2
369	67881	5.90		239	68141	5.9	Lysyl-tRNA syntetase
504	60158	6.94	MPAST	309	61908	6.9	Lamin A
677	47888	5.57	Eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47	503	50222	5.59	Eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47
1076	33986	7.22	CLP36 (rat LIM domain containing protein)	854	34256	7.19	LIM protein
1302	24177	5.58	HSP27	1060	24095	5.83	HSP27

#### 4.11.2 CLP36

Another feature that showed a perfect fit with the rationale used for the analysis was tsa spot 854 (matched with REFs spot 1076).

This feature remained unchanged in three of the tsa cell lines (tas4, 8 and 129) and was up-regulated in tsa12 cells while it was down-regulated in the two control SV cell lines. This suggested that it had to have been up-regulated upon senescence to compensate for the down-regulation due to temperature shift. The up-regulation described in the conditionally immortalised cell lines was in accordance with the finding that the REFs matched spot (spot 1076) was up-regulated upon senescence.

REFs spot 1076 was identified by mass spectrometry as CLP36, a LIM domain containing protein, while the matching tsa spot (spot 854) was described as a generic LIM protein, when analysed by MS.

CLP36 was originally identified as a gene down-regulated by chemical hypoxia in a subtractive screen of normoxic versus hypoxic rat hepatocytes. It encodes a 327 amino acid protein that contains a conserved LIM domain: a cysteine-rich domain initially identified in homeodomain proteins, Lin-11 (Freyd *et al.*, 1990) Isl-1 (Karlsson *et al.*, 1990) and Mec-3 (Wang *et al.*, 1995; Way and Chalfie, 1988). The LIM domain is a zinc finger motif present in several types of proteins, including homeodomain transcription factors and kinases. Proteins containing LIM domains have been discovered to play important roles in a variety of fundamental biological processes including cytoskeleton organisation, cell lineage specification and organ development, and may also be involved in oncogenesis. The LIM domain is a protein-protein interaction motif critical for these processes (Bach, 2000). CLP36 has been shown to bind, via  $\alpha$ -actinin-1, to actin filaments and stress fibres in activated human platelets and endothelial cells (Bauer *et al.*, 2000). In the rat, CLP36 mRNA is mainly expressed in heart, lung and liver, less in spleen and skeletal



muscle, and at extremely low levels in testis and brain tissues (Bauer *et al.*, 2000).

Further experiments are now necessary to determine the cause of the up-regulation of CLP36 and whether the up-regulation contributes towards replicative senescence.

#### **4.11.3 Gelsolin**

There were three other features that were commonly differentially expressed but did not fully fit the analysis criteria. tsa spot 112 (matched with REFs spot 190) identified as gelsolin was one of these (Fig. 4.6).

Gelsolin has been previously shown to be up-regulated upon replicative senescence in both rat and mouse embryo fibroblasts (described in Chapter 3). However the data obtained from the analysis of the conditionally immortalised cell lines showed that gelsolin remained unchanged in the tsa cell lines upon shift up to the non-permissive temperature, while it was up-regulated in the two control SV cell lines. This suggested that either the tsa cells did not respond to the temperature shift or there was a compensatory down-regulation. Since it has been previously shown that tsa cell lines respond to heat shock, the most likely cause of this result was that a compensatory down-regulation of the gelsolin protein was occurring upon shift up to 39.5°C.

However the regulation described in the conditionally immortalised system was not in accordance with the previous finding that gelsolin levels increased upon replicative senescence in primary cells (Fig. 3.7 A and Fig. 3.8) and this increase was due to protein stability (Fig. 3.11). It remains therefore intriguing to determine how and why gelsolin protein becomes stabilised in senescent primary REFs and why the stabilisation process does not occur when the tsa cells cease dividing.

#### **4.11.4 $\alpha$ -glucosidase II**

Another differential spot in common between the conditionally immortalised cell lines and the primary cells was tsa spot 61 (corresponding with REFs spot 127). These features were both identified to be  $\alpha$ -glucosidase II,  $\alpha$  subunit. However this spot did not fully fit the rationale used for the analysis.

$\alpha$ -glucosidase II,  $\alpha$  subunit was up-regulated upon replicative senescence (discussed in Chapter 3) in both primary rat (Fig. 3.7 A and Fig. 3.8) and mouse embryo fibroblasts (Fig. 3.20) and also in premature senescence of REF52 cells upon ectopic expression of activated Ha-ras (Fig. 3.15).

In the conditionally immortalised cell lines  $\alpha$ -glucosidase II was shown to be differentially expressed in the tsa cell lines upon inactivation of LT-ag and entry into senescence. As for gelsolin,  $\alpha$ -glucosidase II was not up-regulated in the tsa cells upon shift up, rather it was up-regulated in the control SV cell lines, suggesting that either the tsa cells did not respond to the temperature shift or there was a compensatory down-regulation, as for the gelsolin protein.

#### **4.11.5 Others**

Another common differential feature between tsa and REFs was tsa spot 239 (matched with REFs spot 369), which was identified as lysyl-tRNA synthetase in the conditionally immortalised cell lines. Unfortunately it was not possible to obtain any identity by mass spectrometry analysis from the spot excised from the REFs gel, because it did not contain sufficient protein and therefore produce a poor ion signal. Further work is necessary to determine the identity of the protein that was differential within this spot.

The analysis identified three other features that were in common between the two systems, tsa spot 50 (REFs spot 102) (alanyl-tRNA synthetase + heat shock protein 105kDa  $\alpha/\beta$ ), tsa spot 503 (REFs spot 677) (eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47) and tsa spot 1060 (REFs spot 1302) (heat shock protein 27). Unfortunately these three spots were not differential in all the tsa cell lines and were thus not considered as prime candidates. Interestingly two of them, tsa spots 50 and 503 behaved as spots whose expression was up-regulated only in the control SV cell lines.

The data for spot 1060 was rather confusing because even though it was a heat shock protein, it remained unchanged in the control SV cells upon temperature shift, and it appeared down-regulated in tsa8, 12 and 129 and up-regulated in tsa4 cells upon shift up.

#### **4.12 Summary and discussion**

A high-resolution differential proteomic analysis to study the entry into the post-mitotic state of the conditionally immortalised cell lines was undertaken. This research was aimed at identifying proteins that were differentially expressed when REF tsa cell lines immortalised with the thermolabile tsA58 SV40 LT-ag underwent replicative senescence upon its inactivation.

The study identified 43 proteins as differentially expressed, which were subsequently identified by mass spectrometry. The proteins that were found to be differentially expressed in the tsa cell lines when they ceased proliferating upon inactivation of tsA58 LT-ag comprised a variety of cytoskeletal, heat shock and metabolic proteins as well as proteins involved in differentiation and protein synthesis, turnover and modification.

Comparison of the differentially expressed features identified during the proteomic tsa analysis with those identified during the differential proteomic

study of replicative senescence upon serial passaging of primary REFs, identified 9 features that were in common between the two systems. It was previously shown that the conditionally immortalised cell lines are committed to undergo senescence but are prevented from undergoing this process by the presence of the tsA58 LT-ag and therefore are a model for studying the entry into cellular senescence upon its inactivation. For this reason the features differentially expressed in the two model systems are likely to correspond to proteins that are either involved in or are markers for the entry into the post-mitotic state.

Three of these common features fit perfectly the criteria as potential candidates for entry into replicative senescence. These three features comprised tsa spot 295 that was identified as TUC-2 (Fig. 4.5). It was down-regulated in all four tsa cell lines and remained unchanged in the control SV cell lines upon shift up; it was also down-regulated in REFs upon serial passaging. Moreover the down-regulation of the TUC-2 protein in senescent REFs was associated with down-regulation at the RNA level.

However it was shown that the ectopic expression of TUC-2 alone into primary cells was not sufficient for immortalisation, and therefore it is suggested that the loss of expression TUC-2 did not cause of replicative senescence. However the results presented in this thesis (see also Chapter 3) suggest that TUC-2 may be a very good marker for senescence.

Another feature that fit perfectly the criteria adopted in the analysis was tsa spot 309. This feature was found to be up-regulated in all the tsa cell lines while it remained unchanged in the control SV cell lines upon shift up and was up-regulated in senescent REFs. This feature was identified in the tsa gels as Lamin A whereas in the REF gels it was identified as MPAST together with Lamin A or C. Further work is thus necessary to determine which of these candidate proteins is truly differentially expressed, whether the up-regulation is due to up-regulation at the RNA level and what is the cause of this up-regulation.

Another feature that showed a perfect fit with the analysis criteria was tsa spot 854. It remained unchanged in three of the tsa cell lines and was up-regulated in tsa12 cells. Interestingly it was down-regulated in the control SV cells indicating that it had to be up-regulated upon senescence to compensate for the down-regulation due to temperature shift. This up-regulation was in accordance with the finding that the same spot was up-regulated in senescent REFs. This feature was identified to be CLP36, a LIM domain containing protein. Further experiments are now necessary to determine the cause of the up-regulation of CLP36 and whether the up-regulation contributes towards replicative senescence.

Three other features were identified as differentially expressed in the primary cells and the tsa cell lines, however they did not fully fit the analysis criteria adopted. tsa spot 112 identified as gelsolin was one of these. Gelsolin was found to be up-regulated upon replicative senescence in both REFs and MEFs. However gelsolin remained unchanged in the tsa cells upon shift up but it was up-regulated in the control SV cells suggesting that either the tsa cells did not respond to the temperature shift or a compensatory down-regulation was occurring. Since it was previously shown that tsa cells respond to heat shock, the most likely cause of these results is that there was a compensatory down-regulation of the gelsolin protein upon shift up. However this was not in accordance with the data obtained from the REFs proteomic study which showed that gelsolin was up-regulated in senescent cells and that the increase was due to protein stability. It will therefore be intriguing to determine how and why this protein becomes stabilised in senescent cells and why this does not occur when the tsa cells cease dividing.

Another common differential spot that did not fully fit the analysis criteria was tsa spot 61 identified to be  $\alpha$ -glucosidase II.  $\alpha$ -glucosidase was found to be up-regulated upon replicative senescence in both REFs and MEFs and also in premature senescence of REF52 cells upon ectopic expression of activated Ha-ras. It was also shown to be differentially expressed in the tsa cell lines upon inactivation of SV40 LT-ag and entry into senescence. As for

gelsolin, it was not up-regulated in the tsa cells upon shift up, rather it was up-regulated in the control SV cell lines. Interestingly it was also shown by RT-PCR analysis of RNA extracted from serially passaged REFs that  $\alpha$ -glucosidase levels did not vary upon senescence suggesting that the increase in protein level is most likely to be due to protein stability.

The third feature that comprises this group of common differentials was tsa spot 239, which was identified as lysyl-tRNA synthetase. Unfortunately there was insufficient protein within the REF spot for identification by mass spectrometry. Therefore further work is necessary to determine the identity of the protein that was differential within this spot.

The differential proteomic analysis presented here has allowed for the identification of some candidate proteins that have previously not been linked with replicative senescence. Even though it remains to be unequivocally demonstrated that any of these differentially expressed proteins either singly or in combination are the cause of replicative senescence, they provide novel markers for the senescent phenotype.

## 5 Microarray analysis

### 5.1 Objectives

cDNA microarrays allow the monitoring of expression levels for thousands of genes at the same time. In a classic experiment, two mRNA samples are isolated from a pair of cell cultures to be compared, reverse transcribed into cDNA and labelled using two different fluorescent dyes (usually Cy3 and Cy5) and then hybridised simultaneously to a cDNA array (chip). The chip is then washed and scanned for fluorescent intensities. Comparison of the intensity of the two dyes provides relative expression values for thousands of genes in one single experiment (Taniguchi *et al.*, 2001; Yang *et al.*, 2002).

Having studied replicative senescence at the protein level, it was decided to investigate the same biological process at the mRNA level using cDNA microarrays. This was done in an attempt to increase sensitivity and to study the same biological process at a different level. It was also interesting, but not the ultimate aim of this study, to compare whether any of the differentially expressed proteins identified during the proteome analysis were also differentially regulated at the mRNA level.

### 5.2 Specification of the arrays used

Having studied the changes in the proteome profiles of proliferating versus senescent REFs it would have been best to study the changes in the expression profile upon replicative senescence using the same model system, therefore employing microarrays prepared from rat cDNAs. Unfortunately when this study was done, rat cDNA chips were not available. For reasons of

resources other than costs it was possible to work with only mouse or human microarrays. As mice are more closely related to rats than humans, the changes in the expression profile upon replicative senescence were investigated in the mouse embryo fibroblasts (MEFs) model system. Moreover, even though changes in the proteome profile of MEFs were not directly analysed, it was shown (see Paragraph 3.26) that some of the changes in protein expression detected in REFs also occurred in MEFs (Fig. 3.20).

However it has to be taken into consideration that MEFs and REFs model systems have unique characteristics each. MEFs diverge from REFs in that MEFs do not senesce until passage 8 (P8), and also because the loss of proliferative potential in mouse cells is not as clear-cut as in rat cells. Mouse cells can undergo spontaneous immortalisation at a measurably frequency (1 in  $10^4$  -  $10^6$  cells) while rat cells immortalise much less frequently.

The mouse chips were also preferred to the human chips for other reasons. First a major problem while analysing human senescence directly would have been the extent of epigenetic variation between cells obtained from different individuals. Clearly this was not an issue using a rodent model system in which the cells were obtained from inbred lines. The use of primary rodent cells meant also that there were almost no limitations to the sources of embryos and therefore it was possible to prepare fresh extracts whenever necessary. Rodent cells were also a more suitable system for reasons of time: while human fibroblasts are capable of undergoing 50-60 divisions before undergoing replicative senescence, rodent embryo fibroblasts senesce in culture after 20-30 divisions.

Rodent and human cells differ also in the biological counting mechanism. The progressive shortening of telomeres throughout the cellular life span has been proposed to be the mitotic clock in human cells (Harley *et al.*, 1990). However telomerase is not required for the immortalisation and transformation of rodent cells where growth inhibition, that occurs in mouse cells after 10-15 doubling in culture, is most likely to be regulated by a telomere-independent clock (Wright and Shay, 2000). As previously discussed,



telomerase negative mice were shown to be viable for 5 generations of homozygosity and their fibroblasts to undergo normal senescence. Moreover *M. musculus* with a shorter life span than humans, have 5-20 times longer telomeres (Blasco *et al.*, 1997; Prowse and Greider, 1995). Therefore it is possible to conclude that telomere based replicative senescence does not exist in mouse but evolved in larger and longer lived organisms as an additional restraint against tumour formation (Wright and Shay, 2000).

A future aspect of this project would be to extend the study to both human and rat cells and check if the differential candidates identified in the mouse system are also differential in both human and rat replicative senescence.

In the present study high-density cDNA microarrays, manufactured at the Wellcome Trust Sanger Institute, Hinxton UK (<http://www.sanger.ac.uk>) were used. The Sanger Centre made and provided the microarrays as part of a jointly supported Wellcome Trust, Cancer Research UK and the Ludwig Institute for Cancer Research microarray consortium.

The microarrays were prepared using PCR products as targets. The PCR products were “spotted” onto a glass matrix (slide) using a spotting technology that utilises covalent attachment of cDNA molecules to a glass support via an amino-link located at the 5’ end of the coding strand of gene segment. The arrayers used to spot the cDNA onto the chips were BioRobotics MicroGrid II ([http://www.affymetrix.com/products/417\\_arrayer.html](http://www.affymetrix.com/products/417_arrayer.html) and <http://www.biorobotics.com/>).

The mouse microarrays employed during this study had the dimension of 54 mm x 18 mm. The spot sizes varied from sub-array to sub-array, generally from 150  $\mu$ m to 230  $\mu$ m, depending on the pin that had been used for spotting.

### 5.3 Gene library

The cDNA microarrays used were known as mouse arrays 1.1.1 or Mver 1.1.1. They were made by spotting cDNA elements from the NIA Mouse 15K cDNA clone collection [<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>] and (Kargul *et al.*, 2001)].

On each microarray were represented 11500 “unique” clones from among 52374 3’ expressed sequence tags (ESTs) that were derived from early embryonic cDNA libraries from pre- and peri-implantation embryos, embryonic day (E) 12.5 female gonad/mesonephros, and newborn ovary (Tanaka *et al.*, 2000). The average insert size of each cDNA clone spotted onto the glass slide was 1.5kb.

Up to 50% of the clones were fragments derived from new genes, and therefore represented genes with no known function. The overall redundancy of the set was 1.3, which meant that some genes were represented on the array by more than one spot (although the same gene might be represented in different spots by a different fragment of its coding sequence). To avoid any possible confusion, the spots on the microarrays will here be referred to as clones.

The fact that some genes were represented on the microarray by different clones was used to control for the homogeneity of the hybridisation across the same slide.

### 5.4 Sample preparation

As discussed in the introduction (see Chapter 1) there are mainly two sources of variability that may occur while performing a microarray experiment, the experimental variability and the biological variability.

To take into account the experimental variability, two separate samples of RNA (called RNA A and RNA B) were extracted from the same embryo

preparation but from independently passaged cultures. To confirm that the changes in expression were not related to that particular donor animal, and therefore to consider the biological variability, a second set of RNA (RNA C) was extracted from cells obtained from a different embryo preparation conducted by a different operator at a different time.

In this way both, the experimental variability (for example the possible operator-related variations that could occur during the RNA extraction even though the same protocol was followed) as well as the biological variability between samples (that ultimately reflects the real differences between individual samples) for each cellular condition (each passage) were taken into account.

#### **5.4.1 Cellular conditions**

To be able to perform the microarray experiment MEFs were serially passaged using the 3T3 passaging regime (Todaro, 1963), already utilised for culturing REFs and previously described (Paragraph 2.1.3.4). Total RNA was extracted from cells from passages P2, P7 and P8. The RNA concentration was then measured using a spectrophotometer and the RNA quality was checked on an agarose/formaldehyde gel (Fig. 5.1).

P2 (passage 2) represented the cellular condition in which the cells were actively proliferating, and therefore at this stage the population of cells was considered as dividing. It must be noted however, as for REFs, that any population of cells is asynchronous; therefore amongst the dividing cells there may have been some cells that were already senescent. P8 represented the cellular condition in which the majority of the cells were no longer dividing and the population of cells was designated as senescent, even though there might have been present some cells that were still proliferating. P3, P4, P5 and P6 represented the intermediate passages from dividing to senescent cells, while

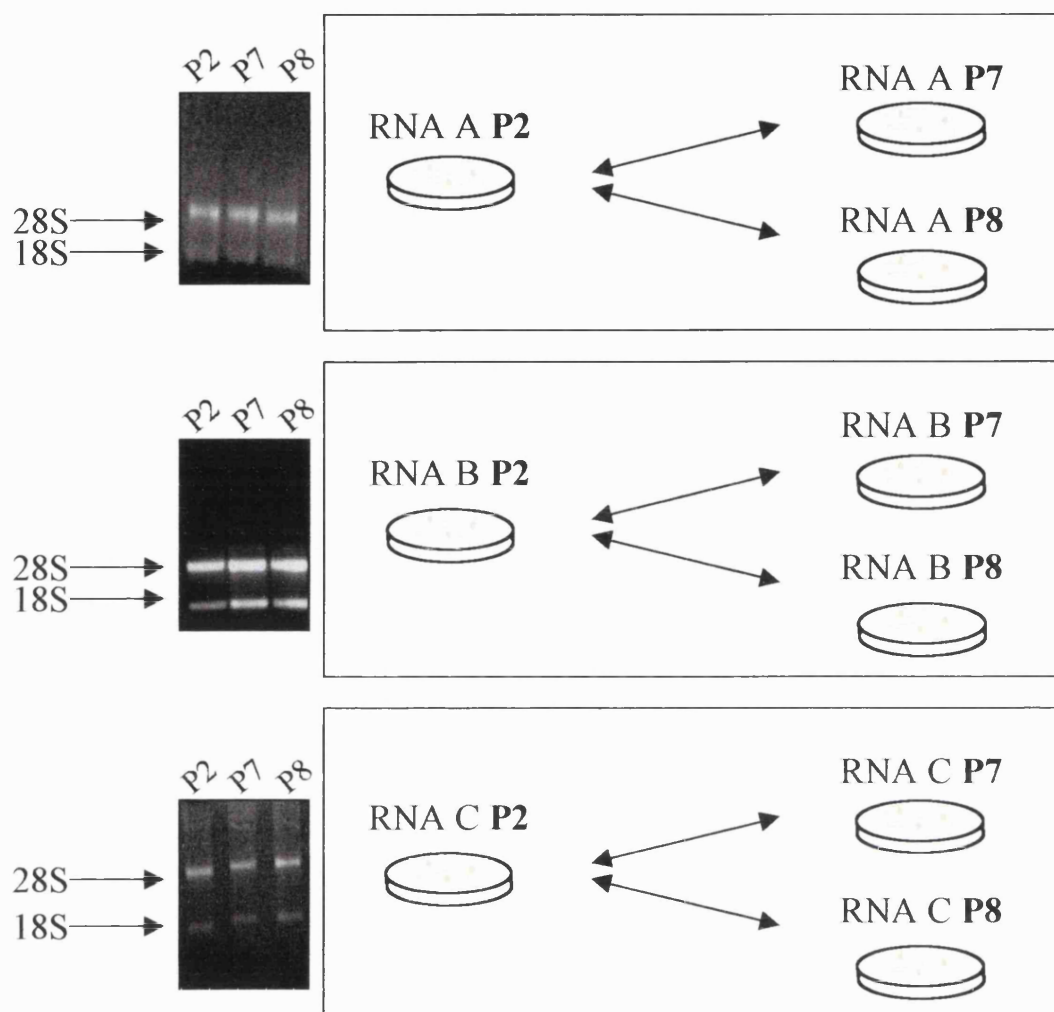
P7 represented the condition where cells were close to entering the post-mitotic or senescent state.

Not only passage P2 (proliferating) versus P8 (senescent), were analysed but P7 was also included in the study. As previously mentioned P7 corresponded to a nearly senescent state, in which not all the cells plated onto the dishes were fully stopped, but they had already undergone a major reduction in their proliferative potential, and they were therefore considered as entering the senescent phase. It was interesting to investigate how the genes found to be differentially expressed in P8 were behaving in P7, and therefore to study the trend of the changes upon the progressive loss of proliferative potential occurring while serially culturing MEFs.

## **5.5 Reciprocal labelling**

It was decided to perform the experiments using direct comparisons, where the RNAs from two different cellular conditions were directly compared on the chips, rather than using indirect comparisons, where each condition was compared to the same control RNA sample.

To take into consideration the variations that were due to the unequal rate of dye incorporation (Yang *et al.*, 2002), it was necessary to repeat the same experiment by inverting the labelling (see Paragraph 1.3.7). For example in a P2 versus P8 comparison, if P2 was labelled with Cy3 and P8 with Cy5 in the first hybridisation, in the second hybridisation P2 was labelled with Cy5 and P8 with Cy3. This procedure is commonly known as “reciprocal labelling” or “flip fluor” labelling. In Fig. 5.2 a successful reciprocal labelling (P2 versus P8) is represented. In experiment (A) the RNA extracted from P2 was labelled with Cy3 (coloured in red in the image) and the RNA from P8 with Cy5



**Fig. 5.1** Outline of the microarray analysis.

Three sets of RNA (RNA A, B and C) were prepared from MEFs passages P2, P7 and P8. The hybridisation performed are indicated by the arrows, the double headed arrows indicate that all the experiments have been repeated also with the reciprocal labelling. On the left side of the slide is represented the quality control of the RNA samples on agarose/formaldehyde gel (0.5µg each lane).

(coloured in green in the image). The dot detected as red in the composite image implied that the corresponding RNA was down-regulated in P8. In experiment (B) the same RNA samples were reverse labelled (P2 with Cy5 and P8 with Cy3). Consequently the same spot appeared to be green in the composite image in the reciprocal labelling experiment. In Fig. 5.3 it is highlighted as an up-regulated clone in a reciprocal labelling experiment. The clone indicated with the arrow was clearly up-regulated in P8, in fact it appeared to be bright green when P2 was labelled with Cy3 and bright red when P2 was labelled with Cy5.

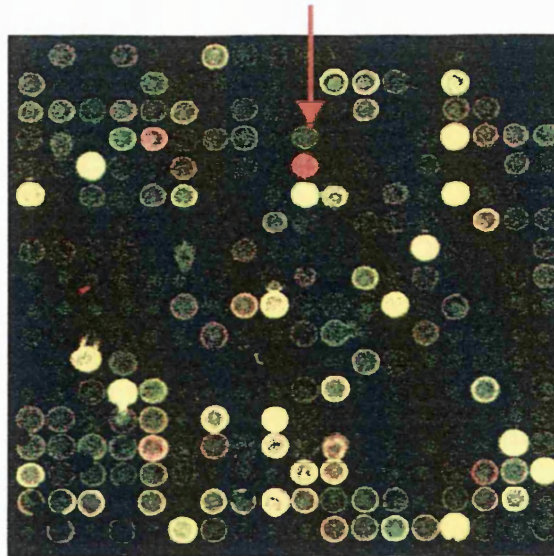
## 5.6 Normalisation and threshold

As previously explained different sources of systematic variation may affect the measured gene expression levels during a cDNA microarray experiment. The term normalisation refers to the process of removing such variations. A constant adjustment is used to force the distribution of the intensity log ratios to have a median of zero for each slide.

In order to accurately measure gene expression changes it is important to take into account the random (experimental) and systematic variations that occur in any microarray experiment.

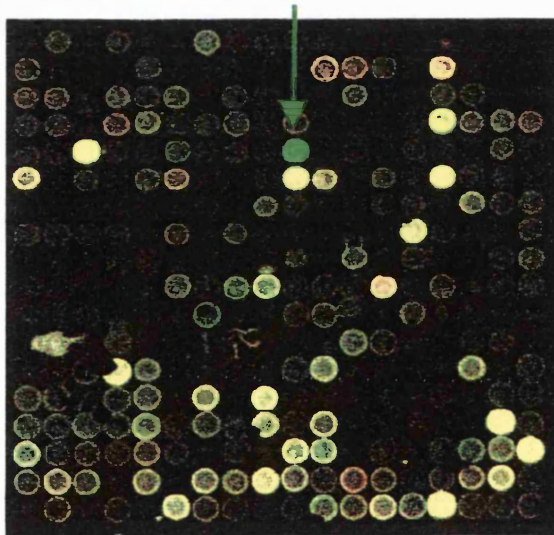
A source of systematic variation arises from biases associated with the different fluorescent dyes. Even though these biases are usually relatively small they may be confounding when searching for subtle biological differences (Yang *et al.*, 2002). Dye biases arise from different factors: physical properties of the dyes (such as heat and light sensitivity together with half life), efficiency of dye incorporation, experimental variability in hybridisation processing procedures and scanner settings at the data collection step (Yang *et al.*, 2002).

Experiment A)



P2 labelled with Cy3 and P8 with Cy5

Experiment B)

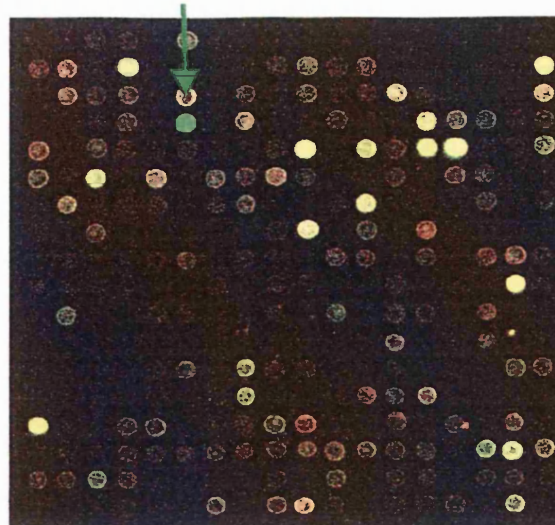


P2 labelled with Cy5 and P8 with Cy3

**Fig. 5.2** Reciprocal labelling of a down-regulated spot.

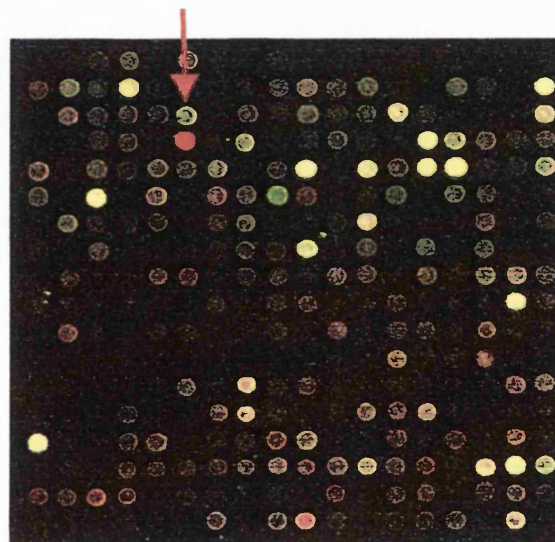
In experiment A sample P2 was labelled with Cy3 (red) and sample P8 with Cy5 (green), the indicated red dot (arrow) detected on the composite image implied that the mRNA represented was down-regulated in P8. The same samples were then reverse labelled (P2 labelled with Cy5 and P8 labelled with Cy3), consistently that same spot appeared green in the reciprocal composite image.

Experiment A)



P2 labelled with Cy3 and P8 with Cy5

Experiment B)



P2 labelled with Cy5 and P8 with Cy3

**Fig. 5.3** Reciprocal labelling of an up-regulated spot.

In experiment A sample P2 was labelled with Cy3 (red) and sample P8 with Cy5 (green), the indicated green dot (arrow) on the composite image implies that the mRNA represented was up-regulated in P8. The same samples were then reverse labelled (P2 labelled with Cy5 and P8 labelled with Cy3), that same spot appeared red in the reciprocal composite image.



The aim of normalisation is to minimise the systematic variations in the gene expression levels of two co-hybridised samples, so that biological differences can be detected more easily.

After the hybridisation the images from the two different dyes were acquired and quantified to obtain data. The power and gain of each laser was adjusted to avoid saturation. The data sets from the two different dyes were then normalised to each other. Once the channels were normalised, a ratio was obtained for each spot where the signal from P8 or P7 was divided by the signal from P2.

Because with the samples studied, it could be assumed that there were no major changes in the total number of expressed genes or the average level of the majority of the genes, the data from the two channels were normalised to each other by equalising the median value of all the spots present on the microarray slides. This method is known as “global normalisation” and does not rely only on those genes expected to remain unchanged in the samples compared (house keeping genes).

Therefore in the present study a global normalisation procedure, which considered all the spots present on the array, was used. This method can be adopted only when a relatively small proportion of the genes will vary significantly in expression between the two hybridised mRNA samples. In conclusion it can be applied only when the samples studied are biologically very similar and therefore very few genes are expected to be differentially expressed.

The bottom tenth percentile was used as a test for correct background subtraction. This was never less than the negative of the synthetic positive controls.

To control for artefacts caused by non-linear dye incorporation rates and inconsistent relative fluorescent intensities of the dyes, Lowess normalisation (also called “intensity dependent” or “non-linear”) method was used in combination with global normalisation (Schwarze *et al.*, 2002) (Fig. 5.4 and 5.5). Before the normalisation process a clear curvature in the scatter plot

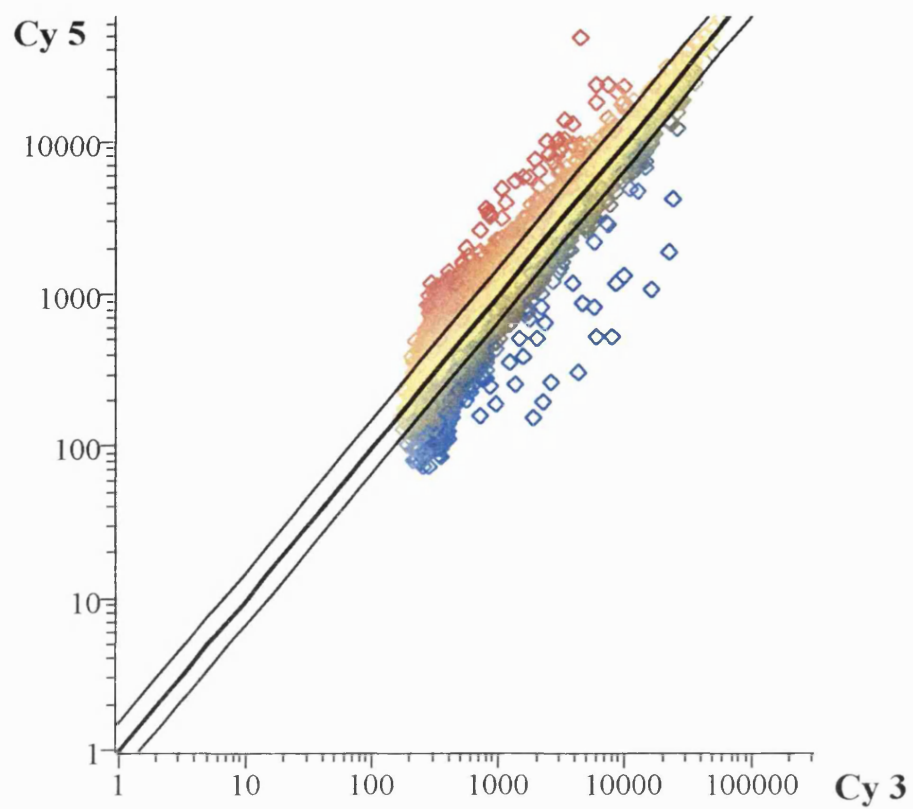
(referred to as the “banana effect”) was present and suggested the existence of an intensity-dependent dye bias (Fig. 5.4). The “banana effect” was first described by Dudoit *et al.* in a self-self comparison where the same mRNA sample was labelled with Cy3 and Cy5 and then hybridised to the same slide (Dudoit, 2002). The authors showed that the curvature detected in the scatter plot of a self-self comparison experiment could be removed when Lowess normalisation was applied to the raw data (Fig. 5.5).

After the normalisation process, before studying the data, it was necessary to set a cut-off for the analysis, which allowed the discrimination between the genes that were truly differentially expressed and those that were not.

Within any microarray there is always a level of background and noise. Therefore while it is relatively easy to study those genes that are expressed at high and medium copy numbers, it is much more difficult to study those genes expressed at low levels, that are ultimately the majority of the genes. Therefore small differences in setting the cut-off can severely influence the spectrum of differentially expressed genes, which can be detected.

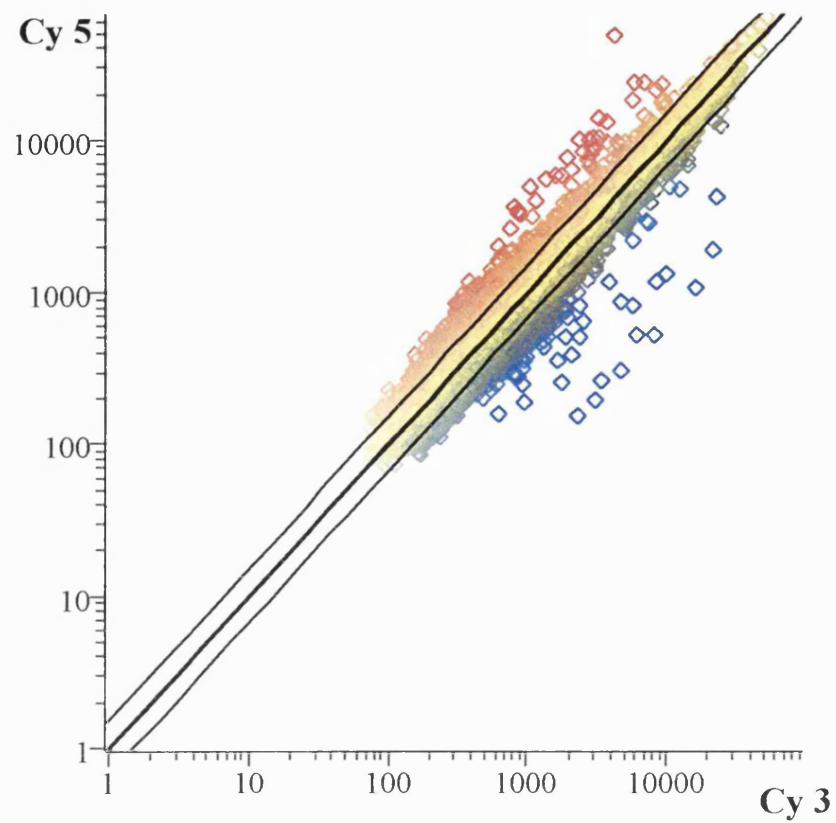
In the present study it was decided to use 1.5-fold change as cut-off threshold to achieve a compromise so that the cut-off was set high enough that few genes would reach the arbitrary threshold by chance alone, but that it would not be too stringent to identify genes whose regulation was relevant to the study (Schwarze *et al.*, 2002).

In determining differences in gene expression using the mRNAs obtained from the first serially passaged mouse embryo fibroblasts (RNA A and B) were considered up-regulated those clones with a ratio above 1.5 in all four chips, and down-regulated those spots with a ratio below 0.66666 in all four chips. However the addition of the data from the second set of serially passaged mouse embryo fibroblasts (RNA C) allowed this restriction to be decreased so that genes with ratios of above 1.5 in 3 out of 4 chips from RNA A and B were included as long as the ratios from RNA C were both above 1.5.



**Fig. 5.4** Scatter plot of a microarray experiment comparing P2 versus P8 prior to Lowess normalisation.

Before the normalisation it was possible to see a curvature in the scatter plot (referred to as the “banana effect”) that suggested the presence of an intensity-dependent dye bias.



**Fig. 5.5** Scatter plot of a microarray experiment comparing P2 versus P8 after Lowess normalisation.

The Lowess normalisation eliminates the “banana effect”

Ultimately the restriction used was that the ratio had to be above 1.5 fold, for the up-regulated clones (or below 0.66666 fold for the down-regulated clones) in 5 out of 6 chips; this is still considerably stringent and is unlikely to be obtained by chance alone.

## **5.7 Outline of the analysis**

To investigate replicative cellular senescence in primary mouse fibroblasts by microarrays, total RNA was extracted from proliferating (P2) and senescent cells (P8). The RNA samples were reverse transcribed and at the same time labelled with fluorescent dyes, and then compared on the same chip. The analysis was then extended by comparing RNA extracted from cells at P7 with RNA from cells at P2. This was done in an attempt to check whether the trend of up- or down-regulation detected in P8 was already appearing in P7.

Three sets of RNA were prepared, RNA A and B were made from different plates obtained during the same embryo preparation, and RNA C was obtained from a different embryo preparation, made at a different time and by a different operator (I would like to thank G. Chu for kindly providing RNA C). Total RNA was therefore extracted from P2 (proliferating cells), P8 (senescent state) and P7 (pre-senescent state) MEFs (Fig. 5.1).

All the samples utilised in the experiment are listed below:

- RNA A, P7
- RNA A, P8
- RNA B, P7
- RNA B, P8
- RNA C, P7
- RNA C, P8

Subsequently hybridisations were performed as indicated below:

- P2 versus P8 using the RNA A (reciprocal labelling)
- P2 versus P7 using the RNA A (reciprocal labelling)

- P2 versus P8 using the RNA B (reciprocal labelling)
- P2 versus P7 using the RNA B (reciprocal labelling)
- P2 versus P8 using the RNA C (reciprocal labelling)
- P2 versus P7 using the RNA C (reciprocal labelling)

The results obtained from the different hybridisations were then analysed. Ultimately the clones differentially expressed upon replicative senescence in MEFs were the ones up- or down-regulated in P8 and common in all the three RNA used (Tables 5.1 and 5.2) (see Paragraph 5.6 for the analysis criteria).

#### ***5.7.1 Clones up-regulated P2 versus P8***

Fifty-five clones were found to be up-regulated from P2 to P8 (more than 1.5 fold, in 3 out of 4 chips for RNA A and B and 2 out of 2 chips in RNA C) (Table 5.1).

In Table 5.1 the differentially expressed clones are ranked by fold change. This table indicates the clone name (clone ID), the fold change (F.C.) and t-test P-value for P2 versus P8, together with the clone description where known and the suggested function of the encoded protein. In the table are also shown the F.C. and t-test P-value of the same clones found when P2 was compared with P7.

The differentially expressed clones could be grouped into two different categories: validating clones and novel candidates. The category of the validating clones is comprised of those clones, which were known to play some role in senescence or tumour formation from the literature. The novel candidates group included a selection of clones previously not known to play any role in replicative senescence but that were likely to be actively involved in this process, or alternatively that were possibly new markers of the process.

#### 5.7.1.1 Validating clones

Fibronectin (FN) (clone H3116A10\_1) found up-regulated by 2.80 fold (when P8 was analysed against P2) and gelsolin (clone H 3120B07\_1) up-regulated by 2.09 fold were classified as validating clones (when P8 was analysed against P2).

##### *Fibronectin (FN)*

The extracellular matrix is composed of collagens, glycosaminoglycans, such as heparan sulphate, and other macromolecules; among these one of the major components is fibronectin (FN). FN plays an important role in cell adhesion, spreading and cellular movements.

Several changes in the functional characteristic of FN have been noted as cells become senescent in culture, with the greatest changes in level expression occurring near the end of a culture's proliferative potential.

It was also shown by Kumazaki that fibroblasts from donors of higher age have a larger cell area and express a higher level of FN (Kumazaki, 1992), thus it was proposed a clear correlation between the increased mRNA content and *in vivo* aging. It has also been reported that FN produced by old cells is 5 to 10kDa bigger than that of young cells (Sorrentino and Millis, 1984) and that FN secreted from old cells is less capable than that from young, for mediating adhesion and spreading.

A clear correlation between increased FN mRNA content both *in vitro* cellular senescence and *in vivo* aging has been demonstrated (Fodil-Bourahla *et al.*, 1999; Gonos *et al.*, 1998; Kumazaki *et al.*, 1993; Pagani *et al.*, 1993).

##### *Gelsolin*

Gelsolin was represented on the arrays by clone H3120B07\_1. In the microarray analysis gelsolin mRNA was found to be up-regulated upon

**Table 5.1** Clones up-regulated.

CLONE ID	F.C. P7	t-test P-value	F.C. P8	t-test P-value	DESCRIPTION	FUNCTION
H3153C09_1	2.97	0.001	3.87	0.000	tissue inhibitor of metalloproteinase 2 (Timp2)	Matrix/Structural Pt
H3014G02_1	3.08	0.002	3.32	0.007	tissue factor (MTF)	Matrix/Structural Pt
H3116A10_1	1.45	0.294	2.80	0.002	fibronectin (FN)	Matrix/Structural Pt
H3156A10_1	2.09	0.036	2.68	0.001	unknown	Unknown
H3009F05_1	2.12	0.000	2.61	0.000	phosphatidylserine decarboxylase	Matrix/Structural Pt
H3008H06_1	2.35	0.002	2.61	0.001	cathepsin D	Signal Transduction
H3010H10_1	1.83	0.032	2.51	0.005	receptor (calcitonin) activity modifying protein 2 (Ramp2)	Signal Transduction
H3138H10_1	2.68	0.001	2.48	0.000	cathepsin D	Signal Transduction
H3138H09_1	2.67	0.000	2.43	0.000	cathepsin D	Signal Transduction
H3127D03_1	1.24	0.467	2.33	0.003	PGI (biglycan)	Unknown
H3124D03_1	1.75	0.002	2.26	0.005	unknown	Matrix/Structural Pt
H3132G02_1	1.70	0.022	2.23	0.016	hippocampal amyloid precursor protein	Energy/Metabolism
H3091E10_1	1.76	0.018	2.22	0.001	p8 protein (p8)	Heat Shock/Stress
H3036D07_1	1.29	0.288	2.21	0.000	catenin src (Catns)	Signal Transduction
H3151H12_1	1.89	0.007	2.20	0.015	LAG protein (Lag)	Matrix/Structural Pt
H3106H03_1	1.98	0.001	2.19	0.007	p8 protein	Unknown
H3128D02_1	1.81	0.020	2.16	0.020	thioredoxin interacting factor (Vdup1)	Matrix/Structural Pt
H3013D11_1	2.35	0.000	2.14	0.000	metallothionein II (MT II)	Signal Transduction
H3131D09_1	2.02	0.001	2.12	0.001	steroid sensitive gene-1 protein (SSG-1)	Signal Transduction
H3120B07_1	1.87	0.005	2.09	0.001	gelsolin	Matrix/Structural Pt
H3132H02_1	1.69	0.004	2.08	0.007	unknown	Energy/Metabolism
H3022G01_1	1.56	0.188	2.08	0.074	catenin src (Catns)	Unknown
H3135D01_1	1.71	0.012	2.05	0.010	tumor differentially expressed 1 (Tde1)	Signal Transduction
H3114C10_1	1.90	0.001	2.01	0.000	protective protein for $\beta$ -galactosidase (Ppgb)	Unknown
H3159B03_1	1.63	0.015	2.01	0.030	fasciculation and elongation protein zeta 1 (zygin I) (FEZ1)	Signal Transduction
H3031H04_1	1.56	0.005	2.01	0.006	integrin $\beta$ -5	Energy/Metabolism
H3110F10_1	1.84	0.000	1.97	0.001	epoxide hydrolase 1, microsomal (Ephx1)	Transcription



CLONE ID	F.C. P7	t-test P-value	F.C. P8	t-test P-value	DESCRIPTION	FUNCTION
H3134H04_1	1.51	0.084	1.96	0.004	FLJ22290 fis	Signal Transduction
H3028F03_1	2.05	0.001	1.94	0.025	cathepsin L (Ctsl)	Matrix/Structural Pt
H3010E09_1	2.11	0.007	1.94	0.002	unknown	Cell Cycle
H3102F08_1	2.01	0.000	1.92	0.015	cathepsin L (Ctsl)	Unknown
H3153D08_1	1.55	0.188	1.92	0.002	N-acylsphingosine amidohydrolase 1 (Asah1)	Matrix/Structural Pt
H3129C10_1	1.12	0.621	1.89	0.008	C2-H2 zinc finger protein	Matrix/Structural Pt
H3158D11_1	1.96	0.003	1.84	0.003	steroid sensitive gene-1 protein (SSG-1)	Unknown
H3133F12_1	1.49	0.002	1.81	0.002	matrix metalloproteinase 2 (Mmp2)	Cell Cycle
H3048G02_1	1.30	0.234	1.80	0.001	cyclin D2 (CCND2)	Cell Cycle
H3025E04_1	1.16	0.383	1.80	0.078	unknown	Energy/Metabolism
H3134H10_1	1.53	0.002	1.79	0.009	nidogen 1 (Nid1)	Cell Cycle
H3077D05_1	1.44	0.101	1.78	0.002	integrin $\beta$ subunit	Signal Transduction
H3151C09_1	1.64	0.006	1.77	0.014	epididymal secretory protein	Matrix/Structural Pt
H3034F01_1	1.70	0.011	1.76	0.001	Kcnq1, Ltrpc5, Mash2, Tapa-1, Tssc4 and Tssc6	Matrix/Structural Pt
H3077H03_1	1.25	0.366	1.73	0.240	unknown	Unknown
H3136G08_1	1.22	0.254	1.72	0.022	cyclin G	Unknown
H3133B06_1	1.21	0.225	1.71	0.004	caldesmon	Matrix/Structural Pt
H3059F01_1	1.51	0.026	1.70	0.012	cyclin D2 (CCND2)	Matrix/Structural Pt
H3047G12_1	1.58	0.041	1.69	0.118	UCH-L1	Matrix/Structural Pt
H3034H06_1	1.36	0.044	1.67	0.009	C7-1 protein (C7-1)	Unknown
H3052B11_1	1.42	0.149	1.67	0.050	cyclin G	Matrix/Structural Pt
H3119F01_1	1.77	0.004	1.66	0.006	PCTAIRE-motif protein kinase 3 (Pctk3)	Signal Transduction
H3159C08_1	1.43	0.114	1.66	0.105	transposon-like element	Signal Transduction
H3113C03_1	1.27	0.205	1.62	0.001	type IV collagen $\alpha$ 5 chain (Col4a5 )	Signal Transduction
H3085G07_1	1.08	0.591	1.60	0.061	imprinted and ancient (Impact)	Signal Transduction
H3127E08_1	1.13	0.589	1.60	0.001	trans-golgi network protein 2 (Ttgn2)	Unknown
H3152D07_1	1.42	0.073	1.59	0.007	unknown	Matrix/Structural Pt

replicative senescence, both in P7 and P8, respectively by 1.87 and 2.09 times (normalised average values in all the six chips analysed) with a very significant t-test P-value, always below 0.001.

In the differential proteomic analysis of replicative senescence in REFs described in Chapter 3, gelsolin, represented on the 2-D gels by spot 102, was found to be up-regulated 4.8 fold when P2 (proliferating REFs) was compared with P5 (senescent REFs) (Fig. 3.7 A). Moreover gelsolin was confirmed to be up-regulated with senescence in primary REFs by 1-D western blots (Fig. 3.8), and was also shown to be up-regulated in the REF52 system when premature senescence was induced by ectopic expression of Ha-ras (Fig. 3.15). It was also shown by semi-quantitative RT-PCR that the gelsolin up-regulation detected at the protein level during the proteomic study was a transcriptional regulation; gelsolin mRNA was shown to be slightly up-regulated upon cellular senescence (Fig. 3.11).

#### 5.7.1.2 Novel candidates

Five of the most interesting clones found to be up-regulated upon replicative senescence during the microarray study were: metallothionein II (MT II), tissue inhibitor of metalloproteinase 2 (Timp2), metal-responsive element-binding transcription factor-1 (MTF-1) and epoxide hydrolase. These up-regulated clones were classified as novel candidates.

##### *Metallothionein (MTs I + II)*

Metallothionein II (MT II), represented by clone H3013D11\_1, was found to be up-regulated 2.14 fold upon senescence. Metallothionein (MTs I + II) are cysteine-rich, low molecular weight proteins, which bind zinc, copper and cadmium. They play a pivotal role in metal-related cell homeostasis. The main function of MTs is to sequester and/or dispense zinc, participating in zinc homeostasis in cells. Consistent with this role, MT gene expression is

transcriptionally induced by a variety of substances known to cause oxidative stress, and the major role of MTs is to protect cells from reactive oxygen species (ROS). Substances causing oxidative stress, such as ethanol and iron, glucocorticoids, as well as changes in the dietary zinc supply, acute administration of various metals, severe food restriction, infections, stress and endotoxin treatment induce the synthesis of metallothioneins (Cousins, 1983). MTs exercise their oxidative stress response by inducing secretion of pro-inflammatory cytokines by immune and brain cells for a prompt response. These cytokines are then involved in new synthesis of MTs in the liver and brain. This protective mechanism occurs in early PD stages, when stresses are transient. However in old age the stress-like condition is constant, and therefore MTs are constantly induced with the result that cells are deprived of their intracellular zinc and consequently the bio availability of zinc ions becomes very low. Because free zinc ions are required for optimal efficiency of the immune-endocrine-nervous network, zinc-bound MTs (I+II) may play a novel role during ageing, switching from a protective to a deleterious function (Mocchegiani *et al.*, 2001; Mocchegiani *et al.*, 2000). It has been also shown that animals deficient in metallothionein isoforms exhibit greater susceptibility to oxidative stress (Ebadi *et al.*, 1996).

In the present analysis MT II was found to be up-regulated by 2.14 fold in P8. If replicative senescence is considered to be the result of a stress condition, it is not surprising that MT II was found to be up-regulated.

#### *Tissue inhibitor of metalloproteinase 2 (Timp2)*

Tissue inhibitor of metalloproteinase 2 (Timp2), represented on the arrays by clone H3153C09\_1, was found to be up-regulated by 3.87 fold.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, whose enzymatic activity is directed against components of the extracellular matrix (ECM) (Kleiner and Stetler-Stevenson, 1999). MMPs play a key role in the normal physiology of connective tissue during development, morphogenesis and wound healing, and their unregulated activity has been

implicated in numerous diseases including arthritis, cancer, metastasis and arteriosclerosis. An important mechanism for the regulation of the activity of MMPs is via binding to a family of homologous proteins referred to as the tissue inhibitors of metalloproteinases (Timp1, Timp2, Timp3 and Timp4) (Brew *et al.*, 2000). Timp2, together with Timp1, is capable of inhibiting the activities of MMPs and it can also inhibit tumour growth, invasion and metastasis together with angiogenesis and programmed cell death. The other members of the family are Timp3 (which is tightly bound to ECM and whose main role is the inhibition of TNF- $\alpha$  (tumour necrosis factor  $\alpha$ ) converting enzyme and the induction of programmed cell death through the stabilisation of TNF- $\alpha$  receptors on the cell surface) and Timp4 (whose main role is to maintain ECM homeostasis) (Giannelli and Antonaci, 2002; Mannello and Gazzanelli, 2001).

Degradation of the extracellular matrix around tumour cells is an essential step in the process of tumour invasion and metastasis and ultimately the major players in this process are MMPs and Timps (Kugler, 1999).

In the present study Timp2 was found to be up-regulated upon senescence, which could be a reflection of the changes in the ECM composition that occur upon aging.

#### *Metal-responsive element-binding transcription factor-1 (MTF-1)*

Metal-responsive element-binding transcription factor-1 (MTF-1), represented by clone H3014G02\_1, was up-regulated by 3.32 fold upon senescence. As previously stated the expression of MT I and II is induced at the transcription level by heavy metals. The promoter region of metallothionein genes contains metal responsive elements (MRE), which are responsible for induction by heavy metals (Lichtlen and Schaffner, 2001). The transcription factor binding the MRE promoter sequence has been described as MTF-1 (metal-responsive element-binding transcription factor-1). When cells are treated with heavy metal, MTF-1 is activated, binds to MRE promoter

sequences and induces transcription of target genes, all of which have a role in toxicity and cell stress response (Lichtlen *et al.*, 2001).

MTF-1 is a 70kDa transcription factor and contains six zinc finger domains and at least three separate transcription activation domains (Muller *et al.*, 1995; Radtke *et al.*, 1993). MTF-1 was demonstrated to be essential for embryonic development and homozygous knock-out mouse embryos for MTF-1 die *in utero* at day 14 of gestation, due to liver decay (Chen *et al.*, 2002).

Therefore it was not surprising that a clone representing MTF-1 (clone H3014G02\_1), together with the clone previously described representing MT-II (clone H3013D11\_1) were up-regulated with senescence.

### *Epoxide hydrolase*

Epoxides are organic three-membered oxygen compounds that arise from oxidative metabolism of endogenous, as well as xenobiotic compounds via chemical and enzymatic oxidation processes, including the cytochrome P450 mono-oxygenase system. The resultant epoxides are typically unstable in aqueous environments and chemically reactive. In the case of xenobiotics and certain endogenous substances, epoxide intermediates have been implicated as ultimate mutagenic and carcinogenic initiators (Sayer *et al.*, 1985). Therefore, it is of vital importance for biological organisms to regulate levels of these reactive species. The epoxide hydrolases are a class of proteins that catalyse the hydration of chemically reactive epoxides to their corresponding dihydrodiol products to transform the very reactive epoxide into the more stable and less reactive intermediates. In mammalian species, there are at least five different epoxide hydrolase: microsomal cholesterol 5,6-oxide hydrolase, hepxilin A (3) hydrolase, leukotriene A (4) hydrolase, which is soluble, and microsomal epoxide hydrolase (Fretland and Omiecinski, 2000). It was therefore not surprising to find that epoxide hydrolase 1 (clone H3134H04\_1) was up-regulated upon senescence by 1.97 fold.

### *p8 protein*

Two clones representing the p8 protein (clones H3091E10\_1 and H3106H03\_1) were identified as up-regulated, by 2.22 and 2.19 fold respectively.

p8 mRNA was initially cloned from a Rat pancreatic cDNA library based on its over-expression in pancreatic acinar cells during the acute phase of pancreatitis (Mallo *et al.*, 1997). The finding that p8 contained the canonical bipartite signal for nuclear targeting suggested its nuclear location; furthermore the analysis of p8 primary structure classified p8 as a DNA-binding protein (Mallo *et al.*, 1997). p8 has been recently described as a co-transcription factor able to enhance the transcription activity of the Smad proteins in response to TGF- $\beta$  (Garcia-Montero *et al.*, 2001). It was previously shown not only that p8 expression was strongly induced in pancreatic acinar cells during the acute phase of pancreatitis (Mallo *et al.*, 1997) but p8 was also described as a protein with a strong growth-promoting activity (Su *et al.*, 2001).

The fact that during the present microarray analysis p8 expression level was found to increase upon replicative senescence was in net disagreement with Su *et al.* (Su *et al.*, 2001).

However the finding that p8 mRNA expression was strongly induced also in brain after transient ischaemic injury, suggested that p8 mRNA over-expression might be part of a ubiquitous defence programme against cellular injury or cellular stress (Vasseur *et al.*, 1999).

### **5.7.2 Clones down-regulated P2 versus P8**

The expression profile of replicative senescence of MEFs highlighted thirty-one clones, which were down-regulated from P2 to P8, (with fold change below 0.66666 in 3 out of 4 chips for RNA A and B and 2 out of 2 chips in RNA C) (Table 5.2).

In Table 5.2 the differentially expressed clones are ranked by fold change. Also included are the clone name (clone ID), the fold change (F.C.) and t-test P-value for P8, together with the clone description, where known, and the suggested function of the encoded protein. In the table are also shown the F.C. and t-test P-value for the same clones when P2 was compared with P7.

As for the up-regulated clones the down-regulated clones were grouped into two different categories: validating clones (already known to play some role in senescence and cancer) and novel candidates (not previously implicated but likely to be involved in senescence). However it was necessary to create a new category to represent those clones that were undergoing a regulation, which seemed to occur in the opposite direction of what expected from the literature. These last clones were named opposite regulated clones.

#### 5.7.2.1 Validation clones

The down-regulated clone histone acetyltransferase 1 (HAT 1) (clone H3126B05\_1), down-regulated by 0.15 fold in P8 and 0.18 in P7, was classified as validating clone.

##### *Histone acetyltransferase 1 (HAT 1)*

The DNA in eukaryotic chromosomes is packaged with proteins to form chromatin; the nucleosomes are the first level of chromatin organisation. They consist of 146 bp of DNA wrapped 1.75 times around the core histone octamer, which is comprised of two molecules of each histone proteins H2A, H2B, H3 and H4 (Parthun *et al.*, 1996). The octamer is held together through protein-protein and protein-DNA interactions (Brownell and Allis, 1996).

The state of chromatin has been recognised to have major effects on the levels of gene expression (Stern and Berger, 2000), and many different chromatin-altering strategies, including histone modifications, are employed by

**Table 5.2** Clones down-regulated.

CLONE ID	F.C. P7	t-test P-value	F.C. P8	t-test P-value	DESCRIPTION	FUNCTION
H3123G09_1	0.07	0.000	0.06	0.000	unknown	Synthesis/Translation
H3140G12_1	0.08	0.000	0.08	0.000	H19 and muscle-specific Nctc1	Synthesis/Translation
H3133G06_1	0.08	0.000	0.11	0.000	H19 and muscle-specific Nctc1	Synthesis/Translation
H3129B08_1	0.08	0.000	0.08	0.000	H19 and muscle-specific Nctc1	Transcription
H3144B07_1	0.09	0.000	0.11	0.000	H19 and muscle-specific Nctc1	Synthesis/Translation
H3005A04_1	0.09	0.000	0.10	0.000	H19 and muscle-specific Nctc1	Synthesis/Translation
H3126A04_1	0.10	0.000	0.07	0.000	IGF-II	Signal Transduction
H3144B06_1	0.12	0.001	0.14	0.001	H19 and muscle-specific Nctc1	Synthesis/Translation
H3024B07_1	0.12	0.000	0.08	0.000	IGF-II	Signal Transduction
H3130H06_1	0.14	0.003	0.16	0.003	H19 and muscle-specific Nctc1	Synthesis/Translation
H3130E06_1	0.17	0.007	0.20	0.008	H19 and muscle-specific Nctc1	Synthesis/Translation
H3126B05_1	0.18	0.000	0.15	0.000	histone acetyltransferase 1 (HAT 1)	
H3123G05_1	0.18	0.001	0.35	0.068	similar to downstream neighbor of SON	Unknown
H3126C05_1	0.20	0.000	0.29	0.008	unknown	Unknown
H3003G06_1	0.20	0.000	0.24	0.000	stromal cell derived protein-1	
H3126F05_1	0.26	0.010	0.32	0.006	insulin-like growth factor binding protein 5 (IGFBP5)	Signal Transduction
H3114F06_1	0.33	0.005	0.28	0.001	retinoic acid-responsive protein (MK-2)	
H3097D03_1	0.40	0.010	0.38	0.005	p57 <sup>KIP2</sup> protein	Cell Cycle
H3026D12_1	0.42	0.001	0.44	0.005	1700012A18Rik	Unknown
H3018D03_1	0.42	0.000	0.46	0.003	1700012A18Rik	Unknown
H3027E02_1	0.57	0.006	0.52	0.023	H2A histone family, member Z (H2afz)	Transcription
H3009F03_1	0.57	0.006	0.53	0.023	H2A histone family, member Z (H2afz)	Transcription
H3009D10_1	0.59	0.014	0.58	0.008	H2A histone family, member Z (H2afz)	Transcription
H3027E01_1	0.59	0.025	0.55	0.068	H2A histone family, member Z (H2afz)	Transcription
H3103B03_1	0.60	0.005	0.59	0.056	somatic histone binding protein NASP	Transcription
H3001D04_1	0.65	0.019	0.61	0.050	replication-dependent histone H2A.1	
H3106B10_1	0.65	0.003	0.53	0.001	centromere autoantigen A (Cenpa)	Cell Cycle
H3048G06_1	0.67	0.021	0.67	0.026	spermidine synthase	
H3106C10_1	0.72	0.111	0.57	0.001	unknown	Unknown
H3027D07_1	0.74	0.133	0.63	0.004	high mobility group protein 1 (Hmg1)	Transcription
H3101G01_1	0.76	0.357	0.57	0.330	unknown	Unknown



cells to control the transcription level. The histone acetylation status is regulated by the equilibrium of histone acetyl-transferase activity (HAT) and the histone deacetylase activity (HDAC). Histone acetyltransferases (HATs), members of a large multi-protein complex, are the enzymes that catalyse the acetylation of histones (Brown *et al.*, 2000; Sterner and Berger, 2000).

HATs function by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the  $\epsilon$ -amino group of certain lysine side chains within the basic N-terminal tail region of histones (Sterner and Berger, 2000). Within the histone octamer these basic regions are involved in binding the DNA, where the positively charged histone tails binds with the negatively charged DNA. The lysine acetylation neutralises part of the positive charge present on the tail and therefore weakens the histone-DNA interaction, and thus affects directly the higher-order chromatin structure, giving access to other nuclear factors and ultimately leading to chromatin de-repression and transcription activation (Brownell and Allis, 1996; Sterner and Berger, 2000). Thus a high degree of acetylation corresponds to a high degree of transcription (Kleff *et al.*, 1995).

HATs play a major role in cells in regulating transcription by establishing active chromatin, directing cytoplasmic synthesis and nuclear deposition of histones and also histone replacement during germ cell maturation (Christensen *et al.*, 1984).

Histone acetyltransferases (HATs) can be classified with respect of their intracellular location and substrate specificity as either nuclear A-type (HAT A) or cytoplasmic B-type (HAT B). Hat1 was the first HAT enzyme identified in yeast. It was originally classified as a B-type HAT involved in the cytoplasmic acetylation of histones, before their deposition on DNA after translocation in the nucleus. Hat1 has been shown to acetylate lysine 12 of free histone H4 N-terminal tail region, but not of H4 packaged in chromatin. It has been shown that *S. cerevisiae* Hat1 null mutants have no obvious growth defects or phenotypes other than the enzyme defect itself, suggesting that Hat1 function may be redundant with other HATs (Kleff *et al.*, 1995; Parthun *et al.*, 1996; Sterner and Berger, 2000).

Alterations in histone acetylation status appear to play a central role in the regulation of neoplasia, tumour suppression, cell cycle control, hormone responsiveness and senescence (Dressel *et al.*, 2000).

It has been previously shown by Matuoka *et al.* (Matuoka *et al.*, 2001) that histone acetyltransferase activity in aged cells, together with histone H4 acetylation, were significantly lower, and this is consistent with the gene down-regulation of expression shown in this study.

#### 5.7.2.2 Novel candidates

H19 together with IGF-II were the two most interesting clones found to be down-regulated upon replicative senescence during the microarray study. H19 was represented on the microarrays by 8 clones, H3130E06\_1, H3130H06\_1, H3144B06\_1, H3133G06\_1, H3144B07\_1, H3005A04\_1, H31229B08\_1 and H3140G12\_1, all down-regulated more than 0.06 fold. IGF-II was represented on the microarrays by 2 clones, H3024B07\_1 and H3126A04\_1) down-regulated in P8 by 0.08 and 0.07 fold respectively.

#### *H19 and IGF-II*

The mouse H19 gene encodes one of the most abundant RNAs in the developing mouse embryo. After birth this gene is expressed in all tissues except skeletal muscle. A cellular fractionation experiment performed by Brannan *et al.* (Brannan *et al.*, 1990) showed that H19 mRNA is cytoplasmic but not associated with the translational machinery and thus it has been suggested that H19 RNA is not a classical mRNA, and the functional product of this unusual gene may be an RNA molecule (Brannan *et al.*, 1990).

The mouse H19 gene maps to the distal segment of chromosome 7 together with IGF-II, Mash2 and Ins2 (Viville and Surani, 1995). It has been demonstrated that the H19 gene and IGF-II (Sotomaru *et al.*, 2002) are parentally imprinted (Bartolomei *et al.*, 1991; Taniguchi *et al.*, 1995), a process

by which genes are differentially expressed on the maternal and paternal chromosomes. Moreover they are reciprocally imprinted (Sasaki *et al.*, 2000).

The expression of the linked but reciprocally imprinted IGF-II and H19 genes has been shown to be activated in the course of liver tumour development. Moreover both IGF-II and H19 RNAs have been shown to be expressed in the majority of the neoplastic nodules and hepatocellular carcinomas, while H19 has been shown to be highly activated in smaller and less distinct hyperplastic regions (Vernucci *et al.*, 2000). Moulton *et al.* (Moulton *et al.*, 1994) proposed a role for H19 as a tumour suppressor gene in Wilms' tumours (WTs).

In the present study both H19 and IGF-II appeared to be down-regulated upon replicative senescence. The fact that both genes appeared to be regulated in the same direction was quite intriguing, because despite H19 and IGF-II undergoing reciprocal imprinting it appears now that they might be commonly regulated.

#### 5.7.2.3 Oppositely regulated clones

p57<sup>KIP2</sup> (represented on the microarrays by clone H3097D03\_1) was found to be down-regulated by 0.38 fold in P8. From the previous literature the regulation of p57<sup>KIP2</sup>, upon replicative senescence, would have been expected to occur in the opposite direction, therefore it was classified as opposite regulated clone.

#### *p57<sup>KIP2</sup>*

p57<sup>KIP2</sup> is a member of the Cip/Kip sub-family of cyclin-dependent kinase inhibitors together with p21<sup>WAF1/Sdi1/CIP1</sup> and p27<sup>KIP1</sup> (Lee and Yang, 2001). p57<sup>KIP2</sup> inhibits the kinase activities of cyclin D-cdk4/6, cyclinE-cdk2/3 and cyclin A-cdk2. It was shown that ectopic expression of p57<sup>KIP2</sup> in lung cells or in Saos-2 osteoclastoma cells leads to G<sub>1</sub> arrest (Lee *et al.*, 1995;

Matsuoka *et al.*, 1995). It therefore appeared rather surprising to find p57<sup>KIP2</sup> down-regulated upon replicative senescence.

## 5.8 Further confirmation of the differentials by analysis of P7 RNAs

The microarray analysis of proliferating (P2) versus senescent (P8) cells was then extended to investigate how the clones that met the analysis criteria and were therefore considered up-regulated upon replicative senescence were behaving in P7, which represented the condition in which cells were close to entering the post-mitotic or senescent state, in which not all the cells plated onto the dishes were fully stopped, but they had already undergone a major reduction in their proliferative potential. It was interesting to include P7 in this study to investigate how the genes found to be differentially expressed in P8 were behaving in P7, and therefore to study the trend upon the progressive loss of proliferative potential occurring while culturing MEFs. The results are presented in Table 5.1.

The study of the behaviour of the up-regulated clones at passage 7 showed that all the clones up-regulated in P8 were already up-regulated in P7. Interestingly some clones, such as cathepsin D (clones H3138H10\_1 and H3138H09\_1), metallothionein II (clone H3131D09\_1), cathepsin L (clones H3028F03\_1 and H3028F04\_1), steroid sensitive gene-1 protein (clone H3129C10\_1) and PCTAIRE-motif protein kinase (clone H3052B11\_1), were found to have a higher fold change when P2 was compared with P7 than with P8.

A similar analysis was then applied for the down-regulated clones. The clones identified during the comparison of P2 versus P8 were therefore investigated in P7 (Table 5.2). This analysis showed that all the clones down-regulated in P8 were also down-regulated in P7.

It would be worth completing the present study with the other intermediate passages (P3, P4, P5 and P6) to investigate the trend of the differential regulations.

## **5.9 Summary and discussion**

After having studied replicative senescence at the protein level it was decided to investigate the same biological process at the mRNA level using cDNA microarrays. This was done in attempt to gain sensitivity and to study the same biological process at a different level.

It was also interesting, but not the ultimate aim of this study, to compare whether any of the differentially expressed proteins identified during the proteome analysis were also differentially regulated at the mRNA level. The analysis of the microarray data obtained from the MEFs model system has therefore been extended by comparing the genes found to be differentially regulated at the mRNA level with the proteins identified to be differentially expressed during the previous proteomic study of REFs.

The comparison between the proteomic and the microarray data is summarised in Table 5.3, in which for each gene are represented the proteomic spot number (2-D ID) and the fold change of the regulation found when P2 was compared with P5 in the proteomic study; also included are the microarray clone number and the fold changes found when P2 was compared with P7 and P8, together with the respective t-test P-values, in the microarray study.

A preliminary comparison of the differentials identified during the proteomic study with the differentials identified during the microarray analysis revealed only gelsolin (2-D spot 102 and microarray clone H3120B07\_1) as a common differential. The gelsolin protein was found to be up-regulated 4.8 fold from P2 to P5 in REFs while gelsolin mRNA appeared in MEFs to be up-regulated both in P7 and P8, respectively by 1.87 and 2.09 (normalised average values in all the six chips).

**Table 5.3** Proteomics versus microarrays.

2-D ID	F.C.	CLONE	IDENTITY	P7		P8	
				F.C.	t-test P-value	F.C.	t-test P-value
102	+ 3.2	H3059D07_1	Alanyl-tRNA synthetase	0.86	0.369	0.96	0.866
108	> 10	H3029G09_1	O-GLCNAC Transferase p110 subunit	1.25	0.154	1.21	0.182
108	> 10	H3119G12_1	O-GLCNAC Transferase p110 subunit	1.18	0.415	1.40	0.036
110	> 10	H3020E01_1	105-kDa heat shock protein	0.83	0.516	0.85	0.711
110	> 10	H3049B07_1	105-kDa heat shock protein	0.84	0.354	0.77	0.296
122	> 10	H3138G09_1	$\alpha$ -glucosidase II, $\alpha$ subunit	1.01	0.971	1.11	0.546
190	+ 4.8	H3155G10_1	Myosin heavy chain	1.06	0.672	1.02	0.937
190	+ 4.8	H3001A08_1	Myosin heavy chain	1.05	0.810	0.98	0.913
190	+ 4.8	H3046C03_1	Myosin heavy chain	0.99	0.956	1.12	0.727
190	+ 4.8	H3120B07_1	gelsolin	1.87	0.000	2.09	0.000
290	+ 2.0	H3109B05_1	Transferrin	0.88	0.598	1.29	0.315
460	- 2.0	H3117H04_1	IMP dehydrogenase	0.75	0.131	0.89	0.522
493	+ 2.0	H3043F10_1	$\gamma$ -butyrobetaine, 2-oxoglut. Dioxyg.	1.02	0.889	0.85	0.438
495	+ 3.3	H3024B05_1	Chaperonin containing TCP-1, $\gamma$ -sub.	0.90	0.424	0.89	0.514
497	+ 2.0	H3013C11_1	T-complex polypeptide 1	0.82	0.300	0.99	0.959
497	+ 2.0	H3023G04_1	T-complex polypeptide 1	0.90	0.466	0.92	0.724
504	+ 2.2	H3017F05_1	LaminA or C2	0.97	0.840	0.97	0.873
504	+ 2.2	H3002B06_1	MPAST1	1.31	0.105	1.46	0.021
677	+ 3.0	H3028A12_1	ATP-dependent RNA helicase p47	0.53	0.024	0.70	0.202
713	+ 3.0	H3016E05_1	Similar to cdc37	1.12	0.374	1.08	0.636
736	- 2.6	H3146D03_1	30kDa protein	1.17	0.218	1.27	0.309
773	+ 3.1	H3114H03_1	Elongation factor-1- $\gamma$	0.84	0.242	0.93	0.754
773	+ 3.1	H3126H04_1	Elongation factor-1- $\gamma$	0.84	0.272	1.01	0.938
773	+ 3.1	H3114H05_1	Elongation factor-1- $\gamma$	0.87	0.294	0.99	0.935
773	+ 3.1	H3114H04_1	Elongation factor-1- $\gamma$	0.82	0.208	0.96	0.794
773	+ 3.1	H3014C02_1	Elongation factor-1- $\gamma$	0.83	0.316	0.94	0.814
863	- 4.7	H3080D03_1	Tubulin $\beta$ chain 15	0.96	0.748	1.03	0.899
863	- 4.7	H3043H01_1	Tubulin $\beta$ chain 15	1.19	0.407	0.65	0.531
902	+ 2.0	H3076F09_1	Arp2	1.01	0.928	0.96	0.851
907	+ 2.1	H3025C08_1	26S proteasome subunit p40.5	0.92	0.572	0.96	0.821
1054	- 2.0	H3131H01_1	$\rho$ GDP dissociation inhibitor	0.87	0.588	1.17	0.570
1054	- 2.0	H3014C09_1	$\rho$ GDP dissociation inhibitor	0.99	0.941	1.17	0.370
1075	+ 2.0	H3046C10_1	Isopentenyl diphosphate isomerase	0.90	0.430	0.89	0.580
1076	+ 2.0	H3015E11_1	CLP36	1.03	0.821	1.36	0.026
1295	- 2.0	H3145H10_1	PRx IV	0.70	0.105	0.84	0.388

The lack of any other common differential genes could be purely due to the fact that the genes found to be differentially expressed during the proteomic analysis were not represented on the microarray slides employed in this work.

It was therefore necessary to manually check whether the genes found to be differentially expressed at the protein level were represented on the array and eventually how they were behaving upon replicative senescence at the mRNA level.

Twenty-three genes, other than gelsolin, were found to be represented on the chips used in the present study (12% of the total differentials identified from the proteomic analysis), some of which were represented by more than 1 spot on the array (Table 5.3).

The proteins represented by 1 clone on the chip were: alanyl tRNA synthetase (clone H3059D07\_1),  $\alpha$ -glucosidase (clone H3138G09\_1), chaperonin containing TPC 1- $\gamma$  subunit (clone H3024B05\_1), lamin A (clone H3017F05\_1), PRx IV (clone H3145H10\_1), CLP38 (clone H3015E11\_1), IMP dehydrogenase (clone H3117H04),  $\gamma$ -butyrobetaine, 2-oxoglutarate dioxygenase (clone H3043F10\_1), isopentenyl diphosphate: dimethylallyl diphosphate isomerase (clone H3046C10\_1), Arp2 (clone H3076F09\_1), MPAST1 (clone H3002B06\_1), probable ATP-dependent RNA helicase p47 (clone H3028A12\_1), similar to cdc37 (clone H3016E05\_1) and 30kDa protein (clone H3146D03\_1). Five proteins were represented by two clones on the chips; they were: O-GLNAC transferase p110 subunit (clones H3029G09\_1 and H3119G12\_1), 105 kDa heat shock protein (clones H302E01\_1 and H3049B07\_1), T-complex polypeptide 1 (clones H3013C11\_1 and H3023G04\_1), tubulin  $\beta$  chain 15 (clones H3080D03\_1 and H3043H01\_1) and GDP dissociator inhibitor for the  $\rho$  protein (clones H3131H01\_1 and H3014C09\_1). Myosin heavy chain was represented by three clones (clones H3155G10\_1, H3001A08\_1 and H3046C03), and elongation factor-1- $\gamma$  from five (clones H3114H03\_1, H3126H04\_1, H3114H05\_1, H3114H04\_1, H3014C02\_1).

Among these clones O-GLCNAC transferase, whose protein was previously shown to be up-regulated by 2.3 fold upon replicative senescence during the proteomic study of REFs, was found to be up-regulated 1.4 fold in one of the two clones on the chips, clone H3119G12\_1. The up-regulation however was not found to occur in the other clone representing the same protein, clone H 3029G09\_1.

MPAST1, a protein that was up-regulated by 2.2 fold in REFs upon senescence, was found to be slightly, but not significantly up-regulated upon senescence at the mRNA level in MEFs; it was up-regulated 1.46 fold in P8. A minor up-regulation was found also for CLP36 represented on the chips by clone H3015E11\_1, and previously found up-regulated by 2.0 fold during the proteomics study.

Probable ATP-dependent RNA helicase p47 was identified in a multiple up-regulated spot (together with ERF1) during the proteomic study; this spot was up-regulated 3.0 fold upon replicative senescence at the protein level in REFs, while it appeared to undergo a very modest up-regulation at the mRNA level, and only in P7 (up-regulation of 0.53). Due to the fact that probable ATP-dependent RNA helicase p47 was identified in a multiple spot, it still remains to be demonstrated which of the proteins identified in the multiple spot are really truly differential and furthermore if there is any correlation between protein and mRNA regulation.

None of the other differentials identified during the proteomic study and represented on the cDNA array used, appeared to undergo any regulation at the mRNA level.

Therefore it is possible to conclude that the comparison of the data from the differential proteomic analysis of primary REFs with the data obtained from the microarray study of primary MEFs upon replicative senescence showed a substantial lack of correlation between the protein and the mRNA regulation. Although it is generally accepted that there is a connection between the function of a gene product and its expression pattern, and it has been proposed as a general rule by Brown and Bostein that each gene is expressed in a cell at a



certain time and under specific conditions to ensure that its product has a role (Brown and Botstein, 1999), the mRNA level does not always correlate with protein level. As previously shown by Anderson and Anderson (Anderson and Seilhamer, 1997) and Gygi *et al.* (Gygi *et al.*, 1999), mRNA levels correlate poorly with corresponding protein levels in *S. cerevisiae* and human liver.

During this microarray study of replicative senescence in MEFs a number of differentially regulated genes have been identified, some of which have been classified as validating clones and others as novel candidates. The validating clones represent genes that have been previously implicated to have a role in senescence while the novel candidates represent genes not known to have a role in cellular senescence, and therefore are novel candidates for the senescent phenotype.

When cells are explanted from the organism and separated from one another for cultivation, they face radical changes in their physical environment. In culture cells lose most of their cell-cell contacts and all the heterotypic interactions between different cell types, together with all the paracrine and endocrine stimulations. Cells in culture undergo a constant loss of metabolites, since the ratio of medium volume to cell volume is thousands of times higher in culture than the ratio of body fluids to tissues in the organism, and are also continuously subjected to hyperoxia: the oxygen concentration is higher in cells bathed in a thin layer of medium exposed directly to air than cells inside a tissue (the partial oxygen pressure reaches 40% in culture against the estimated 3% *in vivo*). While cultivated, cells are also continuously subjected to trypsin treatments, or other methods of dissociation.

It has been proposed that the limit to the replicative lifespan observed in culture is an artefact that reflects the failure of diploid cells to adapt to the trauma of dissociation and to the radically foreign environment of cell culture (Rubin, 1997). Therefore it has been proposed that the loss of proliferative potential in rodent and human normal diploid cells is a response to inappropriate culture conditions or “culture shock” (Sherr and DePinho, 2000). There is now strong evidence which suggests that stress is a key factor in

determining cellular lifespan (Rubin, 2002). The loss of proliferative capacity of rodent cells and a large fraction of human cells to replicate in culture results from the transfer of the cells from their natural environment in the body to the conditions of culture.

Therefore it was very interesting, but rather not surprising, to find that the majority of the up-regulated genes were genes involved in stress response, such as metallothionein II (MT II), tissue inhibitor of metalloproteinase 2 (Timp2), metal-responsive element-binding transcription factor-1 (MTF-1) and epoxide hydrolase.

As previously described (see Chapter 1) the “oxidative stress” theory of aging proposed that cellular senescence is the result of a random accumulation of cellular damage (Sherwood *et al.*, 1988). According to this hypothesis, respiration and other physiological processes within cells, generate a variety of products, which are toxic for cells themselves. Anti-oxidant cellular defences are not always sufficient to completely eliminate these toxic products which are therefore free to attack both DNA and protein molecules, causing an irreversible cellular damage that ultimately induces senescence.

It has been shown that normal human diploid fibroblasts (HDFs) exposed to various types of noncytotoxic oxidative stress display a senescent phenotype called “stress induced premature senescence” or SIPS (Toussaint *et al.*, 2000). Many biomarkers of cellular senescence appear in SIPS: enlarged and flat morphology (Toussaint *et al.*, 1992), irreversible growth arrest, lack of response to mitogenic stimuli (Chen and Ames, 1994), decrease of DNA synthesis and increase in cells positive for the senescent-associated  $\beta$ -galactosidase (Dumont *et al.*, 2000). An up-regulation of the CKI p21<sup>WAF1/Sdi1/CIP1</sup> (Dumont *et al.*, 2000), together with a hypophosphorylation of pRB was observed during SIPS, which explained the block of the cell cycle.

The “oxidative stress” theory of aging together with the concept of SIPS can somehow explain the fact that so many stress associated genes appeared to be up-regulated upon cellular senescence.

The same conclusion that aging resulted in a differential gene expression pattern indicative of a marked stress response was also reached by other groups who studied senescence by microarrays. Lee *et al.* (Lee *et al.*, 1999a) showed a marked stress response in mice skeletal muscle with age while Weindruch *et al.* reached the same conclusion studying mice brain (Weindruch *et al.*, 2001)

The results presented here from the microarray experiments are very preliminary and they need to be validated, with either northern blots or RT-PCR. It would also be very interesting to investigate the promoter regions of the differentially regulated genes to check whether they are under the control of the same regulatory elements. It would be also interesting to further investigate the differentially regulated genes at the protein level to look for correlation between mRNA and protein regulation. Future studies also will be required to explore whether any of those genes are active players in the senescence process by overexpressing/inhibiting them in primary cells to explore if any of those genes are able to prevent/induce cellular senescence.

## **6 Summary and final discussion**

Replicative cellular senescence was described for the first time more than 30 years ago by Hayflick and Moorhead (Hayflick, 1961). They observed that human diploid fibroblasts show a spontaneous decline in growth rate when kept in culture, which is not related to the elapsed time but to an increasing number of population doublings (PD), and they eventually stop dividing after 50-70 PD, and remain in a viable state which has been named replicative or cellular senescence (Wynford-Thomas, 1999).

Even though replicative senescence has been extensively studied the molecular basis underlying this complex biological phenomenon are still not fully understood.

Proteomics and microarrays are the two most widely used “post-genomic era techniques”; they offer a systematic approach to survey changes in gene expression at the protein, for proteomics, and the transcriptional level, for microarrays. They simultaneously investigate changes in the expression of hundreds or even thousands of genes at a time and produce a list of genes whose expression varies in the examined samples, and allow taking a global view on any biological phenomenon. These techniques are particularly suitable for studying a multigenetic and complex process such as replicative senescence, and therefore it was decided to use both proteomics and microarrays in the attempt to study replicative senescence “globally”. Two differential proteomic studies, one on primary REFs and the other on tsa cell lines, and a microarray analysis on MEFs were undertaken.

In the first part of this thesis (Chapter 3) a high-resolution differential proteome analysis of replicative senescence in serially passaged primary REFs has been presented.

This study revealed 49 spots (~4% of the total spots detected on the average 2-D gel), whose expression was altered more than two-fold according to

the number of passages in culture, and whose differential regulation was occurring in both directions (up or down-regulation). Mass spectrometric analysis was undertaken on the differentially expressed spots and the protein identification obtained for 39 of these spots. Since proteomic studies do occasionally identify false positives, this study was then validated by 1-D western blots performed not only within the REFs system but also in two other model systems commonly used to study replicative senescence: Ha-ras induced premature senescence of REF52 cells and replicative senescence of primary MEFs.

The second part of this thesis (Chapter 4) described a high-resolution differential proteomic analysis aimed at identifying proteins that were differentially expressed when tsa cell lines immortalised with the thermolabile SV40 tsA58 LT-ag underwent replicative senescence upon its inactivation. This study identified 43 differentially expressed features that were subsequently identified by mass spectrometry.

Comparison of the differentially expressed features identified during the differential proteomic study of replicative senescence upon serial passaging of primary REFs and the complimentary proteomic analysis on tsa cell lines, identified 9 features that were in common between the two systems.

It was previously shown that the conditionally immortalised cell lines are committed to undergo senescence but are prevented from undergoing this process by the presence of the tsA58 LT-ag and therefore are a model for studying the entry into cellular senescence upon its inactivation. Therefore these common features are likely to correspond to proteins that are either involved in or are markers for the entry into the post-mitotic state. On the contrary the differentials that only occur in the primary model, and not in the tsa cell lines, are likely to represent those proteins involved in the counting mechanism.

No proteins with a regulatory function such as transcription factors, protein kinases or regulatory elements of the cell cycle were identified. This was somehow expected as all these types of proteins are expressed at very low

levels in cells and therefore are extremely difficult to separate on 2-D gels prepared from total cellular extracts. All the proteins identified in this study are relatively abundant in cells and are likely to have long half-lives. As previously discussed (Chapter 1) the major limit of the current proteomic technique is sensitivity. Proteomics allow the simultaneous separation hundred or thousands of proteins, which represent the major proportion of the proteome of relatively small and simple organisms but it is a small fraction of the whole protein content for more complex organisms (Celis *et al.*, 1992). As previously discussed there are several methods, which can be used to increase the number of detectable low abundant proteins. However when this study was initiated, it represented one of the first analysis of replicative senescence by proteomics and therefore it was decided to use total cellular extracts to have the more global view as possible of this phenomenon despite losing sensitivity. A follow-up of this study would be to perform some sort of pre-fractionation prior to 2-D gels separation.

After having studied replicative senescence at the protein level it was decided to investigate the same biological process at the mRNA level using cDNA microarrays.

cDNA microarray is a fast and very sensitive technique compared with proteomics.

Quantitative analysis of global mRNA levels is often used to describe the state of cells and tissue. The technique is fast and very sensitive when compared with proteomics (quantitative analysis of protein expression levels), which is more time consuming and limits the analysis to the relatively highly expressed proteins. Proteomics however gives types of information that are of critical importance for the description of a biological system and that cannot be obtained by studying the mRNA expression level.

However mRNA expression patterns are necessary but are by themselves not sufficient for the quantitative description of biological systems. Several post-translational mechanisms control gene expression. Such mechanisms at the mRNA level include: control of the translational rate, when mRNA is

synthesised from the template DNA, and control of the half-lives of mRNA messengers. An analogous regulation occurs at translation, when messengers are used to synthesise proteins. As for mRNA the rate of protein synthesis together with the half-lives of the proteins are used to control protein abundance; also the intracellular localisation and molecular association of the protein products are used to modulate gene expression.

During the microarray analysis fifty-five clones were found to be up-regulated and thirty-one down-regulated upon replicative senescence. Some of these differentials have been previously implicated with cellular senescence, thus they were classified as validating clones, while some others represented novel candidates.

It was also interesting, but not the ultimate aim of this study, to compare whether any of the differentially expressed proteins identified during the proteome analysis resulted differentially regulated also at the mRNA level.

The differential proteomic and microarray analysis presented here has allowed the identification of some candidate proteins that have previously not been linked with replicative senescence. Even though it remains to be unequivocally demonstrated that any of these differentially expressed genes, either singly or in combination are the cause of replicative senescence, they can be considered novel markers for this complex biological process as it has been demonstrated that their expression levels was changing considerably and consistently within senescence. They represent as well important starting points and they may provide new markers for cancer diagnosis and therapeutics.

## 7 References

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## Supplementary Information I

### RT PCR analysis

#### Gelsolin:

forward primer: 5' AATGAGGTGGTGGTCCAGAG 3'; reverse primer: 5' GCTTGCCTTTCCAAACAAAG 3'; annealing temperature: 57; number of cycles: 32; MgCl<sub>2</sub>: 3mM

#### α-glucosidase:

forward primer: 5' GCCTGAGACTGAAGGTCCTG 3'; reverse primer: 5' TCAATGTCCAACCAAATGACA 3'; annealing temperature: 56; number of cycles: 33; MgCl<sub>2</sub>: 2.5mM

#### TUC-2:

forward primer: 5' GAACCAGTTTGTGGCTGTGA 3'; reverse primer: 5' CACCAGACAAGCTGAAACCA 3'; annealing temperature: 57; number of cycles: 29; MgCl<sub>2</sub>: 2.5 mM

#### TUC-4:

forward primer: 5' CACTGGGAAGATGGATGAGAA 3'; reverse primer: 5' CCATGCCTTCAAAGATGTTGT 3'; annealing temperature: 57°C; number of cycles: 31; MgCl<sub>2</sub>: 2.5 mM

#### p21:

forward primer:

5'GAGAGAAAGCTTATGTCCAATCCTGGTGATGTCCGA3';

reverse primer: 5'GAGAGAGGATCCTCAGGGTTTTCTCTTGCAGAAGAC 3'; annealing temperature: 55°C; number of cycles: 36; MgCl<sub>2</sub>: 1.5 mM

β-actin:

forward primer: 5' CGGTGGACGATGGAGGGGCCG 3'; reverse primer: 5' GCCGAGCGGGAAATCGTGCGTG 3'; annealing temperature: 55°C; number of cycles: 17; MgCl<sub>2</sub>: 1.5 mM

## Supplementary Information II

Supplementary Information II, Table 1: MALDI-MS protein identification by peptide mass mapping (REFs)

Gel Spot #	Gel Spot Mass <sup>a</sup>	Gel Spot pI <sup>a</sup>	Protein Name	Species	Protein Mass <sup>b</sup>	Protein pI <sup>b</sup>	NCBI Acc. #	1 <sup>st</sup> ID <sup>c</sup>	2 <sup>nd</sup> ID <sup>d</sup>
15	158986	6.38	Keratin contamination <sup>e</sup>						
102	125386	5.48	Alanyl-tRNA synthetase ?	Human*	106802	5.31	4501841	9;13%	
104	125386	6.37	??						
108	124240	6.53	Keratin contamination <sup>e</sup>						
110	123105	5.53	105kDa heat shock protein	Mouse*	96408	5.39	1001009	20;32%	
122	114390	5.78	$\alpha$ -glucosidase II, $\alpha$ subunit	Mouse*	109405	5.75	6679891	16;20%	
127	113345	5.82	$\alpha$ -glucosidase II, $\alpha$ -subunit	Mouse*	109405	5.75	6679891	21;25%	
153	79874	4.79	Sec23 protein ? / Ischemia responsive 94kDa protein ?	Human* / Rat	86147 / 94057	6.64 / 5.13	5454042 / 3360521	13;18%	10;14%
158	98305	5.01	???						
177	93348	6.52	Lysyl hydroxylase isoform 2	Mouse <sup>#</sup>	84527	6.34	6755108	23;32%	
185	91608	4.96	Heat shock protein 90- $\alpha$	Human <sup>#</sup>	84660	4.94	72219	15;23%	
190	91608	5.66	??						
223	83776	5.31	???						
248	81062	7.08	???						
278	64858	5.09	Preimmunoglobulin heavy chain binding protein	Rat	72347	5.07	121574	13;28%	
290	74833	6.64	Transferrin	Bovine <sup>#</sup>	77754	6.75	2501351	12;25%	
304	63329	6.09	TOAD-64 (TUC-2)	Rat	62278	5.95	1351260	22;42%	



331	71199	6.67	Moesin ?	Rat	67739	6.16	2218139	6;15%	
332	71199	6.56	Moesin / Guanosine 5'- monophosphate synthetase ?	Rat / Human*	67739 / 76716	6.16 / 6.42	2218139 / 4504035	32;52%	10;16%
369	67881	5.9	???						
426	64712	6.19	Vesicle transport-related protein (RA410)	Rat	72263	6.09	1377856	13;24%	
460	53263	7.39	IMP dehydrogenase	Mouse*	55785	6.84	124427	12;22%	
493	60767	5.76	$\gamma$ -butyrobetaine, 2-oxoglutarate dioxygenase	Rat	60925	5.70	1709530	18;35%	
495	60767	6.47	Chaperonin containing TCP-1, $\gamma$ - subunit / Ulip (TUC-4)	Mouse <sup>#</sup> / Mouse <sup>#</sup>	60630 / 61937	6.28 / 6.04	6753320 / 6681219	35;56%	14;37%
497	60462	6.06	Seryl-tRNA synthetase	Human*	58751	6.05	5730029	10;22%	
504	60158	6.94	MPAST1	Mouse*	60603	6.35	7106303	13;30%	
639	42919	7.01	Cytosolic NADP-dependent isocitrate dehydrogenase	Rat	46735	6.53	1170478	23;62%	
677	47888	5.57	ERF1 / Probable ATP-dependent RNA helicase p47	Human* / Rat	49031 / 48972	5.51 / 5.51	4759034 / 2500531	19;54%	15;42%
693	38467	5.18	Guanine nucleotide-binding protein $\alpha$ subunit	Rat	40499	5.28	121025	11;39%	
713	45110	5.14	Similar to cdc37	Rat	44377	5.38	1197180	8;29%	
736	39212	6.13	30kDa protein	Human*	29952	6.33	7688693	7;26%	
773	45386	6.54	Elongation factor-1- $\gamma$	Human*	50119	6.25	4503481	15;35%	
863	34304	5.41	Tubulin $\beta$ chain 15	Rat	49937	4.79	92930	6;21%	
882	34172	5.57	Transitional endoplasmic reticulum ATPase ?	Rat	89350	5.14	1174637	13;21%	
902	40216	6.58	Arp2	Human*	44761	6.29	5031571	10;29%	
907	40010	5.71	26S proteasome subunit p40.5	Mouse*	42810	5.46	6755210	18;39%	
1054	25301	5.01	GDP diss. inhibitor for the $\rho$ protein	Bovine*	23421.5	5.12	121107	13;54%	

1075	24213	5.79	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase	Rat	26402	5.57	6225530	11;50%
1076	33986	7.22	CLP36	Rat	35525	6.56	1705900	8;49%
1093	23343	5.84	Thiol-specific antioxidant protein	Rat	24819	5.64	9506385	20;74%
1095	33385	6.42	???					
1117	22014	6.19	???					
1124	32329	5.34	Keratin contamination <sup>c</sup>					
1239	14189	5.66	Proteasome subunit RC10-II ?	Rat	22965	6.15	730380	7;36%
1264	25789	6.05	Keratin contamination <sup>c</sup>					
1295	24425	5.87	PRx IV ?	Rat	31008	6.18	4336879	7;39%
1299	24259	5.78	??					
1302	24177	5.83	Heat shock 27 protein	Rat	22822	6.12	478327	9;53%
1319	23370	5.57	Keratin contamination <sup>c</sup>					
1348	22134	6.2	Proteasome subunit RC10-II	Rat	22965	6.15	730380	7;40%
1351	22134	6.1	??					

<sup>a</sup> calculated from protein markers; <sup>b</sup> calculated from protein data base sequence; <sup>c</sup> number of matched peptides and their combined sequence coverage for the identified protein of the 1<sup>st</sup> search; <sup>d</sup> number of matched peptides and their combined sequence coverage for the identified protein of the 2<sup>nd</sup> search; <sup>e</sup> positive IDs only for Keratins (predominantly Keratin 1, 2, 9, 10 and 16); / separates 1<sup>st</sup> ID from 2<sup>nd</sup> ID; ? marks putative ID; ?? denote good ion signal intensity, but no positive ID; ??? denote poor ion signal intensity; # indicates that a rat homologue was present in the NCBI protein database; \* indicates that no rat homologue was present in the NCBI protein database. Proteins identified as TOAD-64 and Ulip in the data base searches are referred to as TUC-2 and 4 in accordance with Quinn et al [Quinn, 1999 #49].



**Supplementary Information II, Table 2:** Nano-HPLC-ESI-MS/MS protein identification by Mascot database searching (REFs)

Gel Spot	Gel Spot Mass <sup>a</sup>	Gel Spot pI <sup>a</sup>	Protein Name (# of analysed peptides)	Species	Protein Mass <sup>b</sup>	Protein pI <sup>b</sup>	NCBI Acc. #	EST DB searches
15	158986	6.38	No MS/MS <sup>c</sup>					
102	125386	5.48	No MS/MS					
104	125386	6.37	2-Oxoglutarate dehydrogenase precursor (1)	Human*	113477	6.62	2160381	B
108	124240	6.53	O-GLCNAC Transferase p110 subunit (1)	Rat	115607	6.18	3914191	-
110	123105	5.53						
122	114390	5.78						
127	113345	5.82	$\alpha$ -glucosidase II, $\alpha$ subunit (1)	Mouse*	109405	5.75	6679891	C
153	79874	4.79	Sec23 protein (3) / Ischemia responsive 94kDa protein (1) / HSP70 (1)	Human* / Rat / human	86147 / 94057 / 78996	6.64 / 5.13 / 5.12	5454042 / 3360521	C / -
158	98305	5.01	No MS/MS					
177	93348	6.52						
185	91608	4.96						
190	91608	5.66	Gelsolin (5 <sup>♦</sup> ) / Myosin heavy chain (5 <sup>♦</sup> )	Human & Mouse* / Chicken <sup>#</sup>	85698 & 80878 / various	5.9 & 5.53 / various	4504165 & 6754078 / 238271, 86350, 86358	A / B
223	83776	5.31	No MS/MS					
248	81062	7.08	No MS/MS					
278	64858	5.09						



290	74833	6.64						
304	63329	6.09	TOAD-64 (5)	Rat	62278	5.95	1351260	-
331	71199	6.67	Moesin (6 <sup>♦</sup> )	Rat	67739	6.16	2218139	
332	71199	6.67	Moesin (2) / Guanosine 5'-monophosphate synthetase (2)	Rat / Human*	67739 / 76716	6.16 / 6.42	2218139 / 4504035	- / C
369	67881	5.9	No MS/MS					
426	64712	6.19						
460	53263	7.39						
493	60767	5.76						
495	60767	6.47						
497	60462	6.06	seryl-tRNA synthetase (1) / T-complex polypeptide 1 (1)	Human* / Rat	58751 / 60360	6.05 / 5.86	5730029 / 6981642	C / -
504	60158	6.94	MPAST1 (3) / LaminA or C2 (1)	Mouse* / Rat	60603 /	6.35 /	7106303 / 453180, 1556433	A / -
639	42919	7.01						
677	47888	5.57						
693	38467	5.18						
713	45110	5.14						
736	39212	6.13						
773	45386	6.54	Elongation factor-1- $\gamma$ (1)	Human*	50119	6.25	4503481	B
863	34304	5.41	$\beta$ -Tubulin (13 <sup>♦</sup> )	Rat & Chinese Hamster <sup>#</sup>	49963 & 49742	4.79 & 4.85	135451 & 109431	- & A
882	34172	5.57	No MS/MS					
902	40216	6.58	Arp2 (5 <sup>♦</sup> ) / hnRNP-E2 (1)	Human* / Human*	44761 / 38580	6.29 / 6.33	5031571 / 4826886	A / A
907	40010	5.71	26S proteasome subunit p40.5 (2)	Human*	42919	5.53	4506223	C



1054	25301	5.01	p GDP dissociation inhibitor (8)	Human & Bovine*	~23000	~5	36038 & 121107	A
1075	24213	5.79						
1076	33986	7.22						
1093	23343	5.84						
1095	33385	6.42	No MS/MS <sup>c</sup>					
1117	22014	6.19	No MS/MS <sup>c</sup>					
1124	32329	5.34	No MS/MS <sup>c</sup>					
1239	14189	5.66	Proteasome subunit RC10-II (2) / PRx III (2)	Rat / Rat	22965 / 28322	6.15 / 7.15	8394082 / 4336877	- / -
1264	25789	6.05	No MS/MS <sup>c</sup>					
1295	24425	5.87	PRx IV (4) / Heat shock 27 protein (1)	Rat / Rat	31008 / 22822	6.18 / 6.12	4336879 / 478327	- / -
1299	24259	5.78	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (2)	Rat	26402	5.57	6225530	-
1302	24177	5.83						
1319	23370	5.57	No MS/MS <sup>c</sup>					
1348	22134	6.2	Proteasome subunit RC10-II (3)	Rat	22965	6.15	730380	-
1351	22134	6.1	PRx III (3)	Rat	28322	7.15	4336877	-

<sup>a</sup> calculated from protein markers; <sup>b</sup> calculated from protein data base sequence; <sup>c</sup> positive IDs only for Keratins (predominantly Keratin 1, 2, 9, 10 and 16), Trypsin, and/or standards through crossover contamination; / separates 1<sup>st</sup> ID from 2<sup>nd</sup> ID; # indicates that a rat homologue was present in the NCBI protein database; \* indicates that no rat homologue was present in the NCBI protein database; ♦ at least one peptide has been matched by extracting amino acid sequence information and MS-Pattern searching; A at least one rat EST was found with MS-Tag searching the NCBI EST database; B at least one rat EST was found with MS-Pattern searching the

NCBI EST database with the first 8 amino acids obtained from the initial MS-Tag search allowing for one amino acid substitution; C no rat ESTs were found by either method A or B.

**Supplementary Information II, Table 3:** Amino acid sequences of peptides analysed by nano-HPLC-ESI-MS/MS and Mascot database searching (REFs)

Gel Spot #	Amino Acid Sequence (Peptide Mass)
15	
102	
104	IEQLSPFPFDLLKL
108	LVSIVAEQLEK
110	
122	
127	VLLVLELQGLQK
153	...NLTDLLGELQR (M <sub>w</sub> : 1645.8) <sup>#</sup> ...VPVAALFTPLK (M <sub>w</sub> : 1400.8) <sup>#</sup> TTYLEFIQQNEER EFSITDVVPYPISLR
158	
177	
185	
190	AGALNSNDAFVLK QAEKDALISQLSR QVEKDALISQLSR EVQGFESSTFQGYFK ...TPSAAYLWVG... (M <sub>w</sub> : 1824.6) <sup>#</sup> MADREMAAFGAGAPFLR ...VADENPF... (M <sub>w</sub> : 1692.4) <sup>#</sup> QTQVSVLPEGGETPLFK ASSDAEMAVFGAAPYLR ...MAAFGEAA... (M <sub>w</sub> : 1923.5) <sup>#</sup>
223	
248	
278	
290	
304	GSPLVVISQGK IVLEDGTLHVTEGSGR SITIANQTNCPYVTK KPFPDFVYK GLYDGPVCEVSVTPK
331	KAQEELEEQTR AQEELEEQTR ISQLEMAR FVIKPIDKK AQMVEDLEK APDFVFYAPR
332	TQEQLASEMAELTAR ELDLPEELVSR AQMVEDLEK EPPTDVTPTFLTGTGLSTLR



369	
426	
460	
493	
495	
497	TICAI LENYQTEK EVGDGTTSVVHIAAELLK
504	MQQQQLDEYQELLDIK LDISDEFSEVIKI LFEAEEQDLFK EPELFQTVAEGLR
639	
677	
693	
713	
736	
773	STFVLDEFKR
863	AILVDLEPGTMDSVR ...AILVDLEPGTMDSVR (M <sub>w</sub> : 1672.9) <sup>#</sup> IMNTFSVVPSPK IREEYPDR EIVHIQAGQCGN ISVYYNEATGGK ISVYYDEATGGK INVYYNEATGGK AVNMVPFPR SLGGGTGSGMGTLISK IMNTFSVVPSPK FPGQLNADLR LAVNMVPFPR ALTVPELTQQVFDAK VSDTVVEPYNATLSVH GHYTEGAELVDSVLDVVR
882	
902	KVVVCDNGTGFK ILLTEPPMNPTK DLMVGDEASELR SMLEVNYPMENGIVR HIVLSGGSTMYPGLPSR GYAFNHSADFETVR IITLAGPTNAIFK
907	QMTDPNVALTFLEK LYENFISEFEHR
1054	YIQHTYR IDKTDYMGVSYGPR TDYMGVSYGPR SIQEIQELDKDDESLR VAVSADPNVNPVIVTR

	AEEYEFLTPMEEAPK
	AEEYEFLTPMEENPK
1075	
1076	
1093	
1095	
1117	
1124	
1239	HLSVNDLPVGR FGPYYTEPVIAGLDPK DYGVLLESAGIALR LYIGLAGLATDVQTVAQR
1264	
1295	AQIGGPESEQSGAK LVQAFQYTDK DYGVYLEDSGHTLR QITLNDLPVGR
1299	AELGIPLEEVDLNEMNYLTR AFSVFLFNTENK
1302	
1319	
1348	LYIGLAGLATDVQTVAQR DAVSGMGVIVHIIK FGPYYTEPVIAGLDPK
1351	GLFIIDPNGVIK HLSVNDLPVGR DYGVLLESAGIALR

# only partial sequence could be obtained, modifications and substitutions unknown. Please note that many methionine residues have been identified in their oxidised form and N-terminal glutamine residues also as pyro-glutamic acid.



**Supplementary Information II, Table 4: MALDI-MS protein identification by peptide mass mapping (tsa)**

Gel Spot #	Gel Spot Mass <sup>a</sup>	Gel Spot pI <sup>a</sup>	Protein Name	Species	Protein Mass <sup>b</sup>	Protein pI <sup>b</sup>	NCBI Acc. #	1 <sup>st</sup> ID <sup>c</sup>	2 <sup>nd</sup> ID <sup>d</sup>
50	126542	5.49	Alanyl-tRNA synthetase ?	Human	106802	5.31	4501841	10;15%	
61	113722	5.8	$\alpha$ -Glucosidase II	Mouse	109405	5.75	6679891	19;24%	
68	104809	5.26	Non-muscle $\alpha$ -actinin 1	Rat	102961	5.23	4210985	25;36%	
70	104809	4.92	Isopeptidase T	Mouse	95834	4.89	7305619	13;23%	
75	102501	6.39	Alix	Mouse	96011	6.15	6755002	36;40%	
75	109260	5.31	$\alpha$ -Actinin 4 / Non-muscle $\alpha$ -actinin 1	Rat / Rat	104787 / 102961	5.24 / 5.23	6636119 / 4210985	47;50%	20;26%
81	99504	7.15	Elongation factor 2	Rat	95285	6.41	119176	24;31%	
88	99679	5.2	Transitional endoplasmic reticulum ATPase	Rat	89349	5.14	1174636	30;47%	
92	94474	6.5	Lysyl hydroxylase isoform 2	Mouse	84527	6.34	6755108	17;24%	
112	92040	5.67	Murine gelsolin protein	Mouse	80878	5.53	6754078	17;20%	
120	88201	7.29	DEAD box protein RB	Human	82945	7.97	539572	9;20%	
125	86088	5.2	Eukaryotic polypeptide chain release factor 3 ?	Oryctolagus Cuniculus	64477	5.41	7077174	17;33%	
130	84708	6.0	Elongation factor G	Rat	83771	6.95	585084	16;28%	
149	81062	6.17	CDC47	Mouse	81211	5.98	10242373	16;28%	
161	78807	6.44	Radixin ?	Mouse	68452	5.81	131820	8;15%	
187	68832	7.38	??						
209	71346	5.96	Glycl-tRNA synthetase	Human	77513	5.88	493066	21;31%	
232	68929	7.01	Lamin A	Rat	74324	6.54	1346413	23;45%	
236	60178	5.97	Leukotriene A4 hydrolase	Rat	69176	5.69	266480	11;23%	
239	68141	5.9	Lysyl-tRNA synthetase	Cricetulus Longicaudatus	67991	5.84	586059	15;21%	



251	66472	5.45	Annexin VI	Rat	75755	5.39	1351943	31;61%	
282	63172	6.37	Lamin A / Neural specific protein CRMP-2	Rat / Bos Taurus	71642 / 62278	6.2 / 5.95	453180 / 3122018	25;41%	9;22%
295	63742	6.07	TOAD-64 (TUC-2)	Rat	62278	5.95	1351260	13;30%	
309	61908	6.9	Lamin A	Mouse	74210	6.54	1346412	28;51%	
340	61124	5.85	$\gamma$ -Butyrobetaine, 2-oxoglutarate dioxygenase	Rat	60925	5.7	1709530	21;34%	
352	60276	5.54	Copine I ?	Human	59059	5.52	4503013	7;10%	
380	58656	6.99	Tyrosyl-tRNA synthetase	Human	59144	6.61	4507947	24;38%	
408	54970	7.28	IMP dehydrogenase	Mouse	55785	6.84	124427	24;44%	
448	53548	6.49	Aspartyl-tRNA synthetase	Rat	57127	6.02	135099	27;54%	
454	46916	4.89	ATP synthase $\beta$ subunit / Class I $\beta$ -tubulin	Rat / Rat	51203 / 49671	4.91 / 4.78	1374715 / 135471	29;53%	9;36%
503	50222	5.59	Eukaryotic polypeptide chain release factor 1 / Probable ATP-dependent RNA helicase p47	Oryctolagus Cuniculus / Rat	49031 / 48972	5.51 / 5.51	4759034 / 4758112	14;36%	13;25%
547	44443	5.34	Translation initiation factor eIF-4A / Actin $\beta$	Mouse / Rat	44493 / 41751	5.4 / 5.29	72888 / 71620	33;72%	10;32%
559	46471	5.62	???						
639	42919	7.01	Cytosolic NADP-dependent isocitrate dehydrogenase	Rat	46735	6.53	1170478	23;62%	
667	39256	5.69	??						
693	38467	5.18	Guanine nucleotide-binding protein $\alpha$ subunit	Rat	40499	5.28	121025	11;39%	
740	38566	6.02	??						
853	34374	6.17	Cytosolic malate dehydrogenase	Rat	36483	6.16	3747085	11;31%	
854	34256	7.19	???						
883	31214	5.72	???						
889	30933	5.75	PA28 $\gamma$ subunit	Mouse	29506	5.69	6755214	7;36%	
1009	21696	5.03	Mitotic checkpoint component Mad2	Mouse	23564	5.16	9506869	7;42%	
1060	24095	5.83	Heat shock protein	Rat	22893	6.12	1170367	13;68%	

<sup>a</sup> calculated from protein markers; <sup>b</sup> calculated from protein data base sequence; <sup>c</sup> number of matched peptides and their combined sequence coverage for the identified protein of the 1<sup>st</sup> search; <sup>d</sup> number of matched peptides and their combined sequence coverage for the identified protein of the 2<sup>nd</sup> search; <sup>e</sup> positive IDs only for Keratins (predominantly Keratin 1, 2, 9, 10 and 16); / separates 1<sup>st</sup> ID from 2<sup>nd</sup> ID; ? marks putative ID; ?? denote good ion signal intensity, but no positive ID; ??? denote poor ion signal intensity.



**Supplementary Information II, Table 5:** Nano-HPLC-ESI-MS/MS protein identification by Mascot database searching (tsa)

Gel Spot #	Gel Spot Mass <sup>a</sup>	Gel Spot pI <sup>a</sup>	Protein Name (# of matching peptides)	Species	Protein Mass <sup>b</sup>	NCBI Acc. #
50	126542	5.49	Alanyl-tRNA synthetase (9) / Heat shock protein 105 kDa (3)	Human / Mouse	107488 / 92726	4501841 / 1001011
61	113722	5.8				
68	104809	5.26				
70	104809	4.92				
75	102501	6.39				
75	109260	5.31	No MS/MS			
81	99504	7.15				
88	99679	5.2				
92	94474	6.5				
112	92040	5.67	Gelsolin (4) & Gelsolin (4)	Human & Mouse	86050 & 81117	4504165 & 6754078
120	88201	7.29				
125	86088	5.2	Eukaryotic polypeptide chain release factor 3 (6)	Oryctolagus Cuniculus	65132	7077174
130	84708	6.0				
149	81062	6.17	CDC47p (1)	Xenopus Laevis	82782	2231177
161	78807	6.44	Radixin (8)	Mouse	68601	6677699
187	68832	7.38	No significant hits			
209	71346	5.96				
232	68929	7.01				
236	60178	5.97	No significant hits			
239	68141	5.9	Lysyl-tRNA synthetase (10)	Cricetulus	68469	586059



				Longicaudatus		
251	66472	5.45				
282	63172	6.37				
295	63742	6.07				
309	61908	6.9				
340	61124	5.85				
352	60276	5.54	Copine I (11) / Chaperonin subunit 8 (θ) (9) / T-complex protein 1, ε subunit (5) / κ-B motif-binding phosphoprotein (2)	Human / Mouse / Human / Mouse	59660 / 60098 / 60097 / 51300	4503013 / 6753328 / 1351211 / 1083569
380	58656	6.99				
408	54970	7.28				
448	53548	6.49				
454	68832	7.38	ATP synthetase β subunit (24) / Tubulin, β 5 (12) / Tubulin, β 2 (13) / Vimentin (4)	Rat / Mouse / Human / Rat	51171 / 50103 / 50263 / 53758	1374715 / 7106439 / 5174735 / 401365
503	50222	5.59				
547	44443	5.34				
559	46471	5.62	No significant hits <sup>c</sup>			
639	42919	7.01				
667	39256	5.69	Serine proteinase inhibitor mBM2A (6) / Serine protease inhibitor 14 (1) / Serine protease inhibitor 6 (1)	Mouse / Mouse / Mouse	21571 / 45863 / 42754	2104737 / 7649264 / 6678101
693	38467	5.18				
740	38566	6.02	No MS/MS			
853	34374	6.17				

854	34256	7.19	LIM protein (2)	Rat	35967	8393153
883	31214	5.72	Hypothetical protein (2)	Human	27318	8923710
889	30933	5.75				
1009	21696	5.03				
1060	24095	5.83	Heat shock 27 protein (4)	Rat	22866	478327

<sup>a</sup> calculated from protein markers; <sup>b</sup> calculated from protein data base sequence; <sup>c</sup> significant hits only for Keratins (predominantly Keratin 1, 2, 9, 10 and 16), Trypsin, and/or standards through crossover contamination; / separates different hits; & indicates different homologues sharing a real subset of matched peptides.



**Supplementary Information II, Table 6:** Amino acid sequences of peptides analysed by nano-HPLC-ESI-MS/MS and Mascot database searching (tsa)

Gel Spot #	Amino Acid Sequence
50	SIDTGMGLER AVFDETYPDVR DIINEEEVQFLK GLVVDMDGFEEER VDDSSSEDKTEFTVK TITVALADGGRPDNTGR NSSHAGAFVIVTEREAIK VLGTAFDPFLGGK NAVEECVYEFR DVSTTLNADEAVAR
61	
68	
70	
75	
75	
81	
88	
92	
112	HVVPNEVVVQR AGALNSNDAFVLK DSQEEEEKTEALTSK QTQVSVLPEGGETPLFK SEDCFILDHGR
120	
125	HLIVLINK KGEFETGFVK HFTILDAPGHK TFDAQIVIIHK KDIHFMPCSGLTGANLK HNVEVLGILSDDVETDSVAPGENLK
130	
149	TVEQNIQLPAALLSR
161	ALELEQER QRIDEFEAM EDSMMEYLK APDFVIFYAPR QLQALSSELAQAR NQEQLAAELAEFTAK IAQDLEMYGVNYFEIK VTTMDAELEFAIQPNTTGK
187	
209	
232	

236

239

LIFYDLR  
MLVVGGIDR  
QLFEEQAK  
FELFVMKK  
ISMVEELEK  
VTMFLTDSNNIK  
LPETSLFETEETR  
LPETSLFETEETRK

251

282

295

309

340

352

QALPQVR  
VVTMEVEAR  
FGIYDIDNK  
DIVQFVPYR  
GTITVSAQELK  
SDPFLEFFR  
DIVQFVPYRR  
LYGPTNFAPIINHVAR  
YNIMLVR  
AVDDGVNTFK  
LATNAAVTVLR  
GEENLMDAQVK  
HFSGLEEAVYR  
AIAGTGANVIVTGGK  
LVPGGGATEIELAK  
GSTDNLMDDIER  
MIIEEAKR  
IADGYEQAAR  
HKLDVTSVEDYK  
MMVDKDGDVTVTNDGATILSMMDVDHQIAK  
FSELTSEK  
IDEPLEGSEDR  
TDYNASVSPDSSGPER

380

408

448

454

VLDSGAPIK  
IGLFGGAGVGK  
IPVGPETLGR  
LVPLKETIK  
VVDLLAPYAK  
TIAMDGTGLVR  
IMNVIGEPIDER  
AHGGYSVFAGVGER  
FTQAGSEVSALLGR  
VALTGLTVAEYFR



	TVLIMELINNVAK
	VALVYGQMNEPPGAR
	LVLEVAQHLGESTVR
	IMDPNIVGSEHYDVAR
	VLDSGAPIKIPVGPETLGR
	FLSQPFQVAEVFTGHMGK
	EGNDLYHEMIESGVINLK
	TAVCDIPPR
	YLTVAAVFR
	NMMAACDPR
	IREEYPDR
	FPGQLNADLR
	LAVNMVPFPR
	ISEQFTAMFR
	ISVYYNEATGGK
	IMNTFSVVPSPK
	EVDEQMLNVQNK
	GHYTEGAELVDSVLDVVR
	FADLSEAANR
	EYQDLLNVK
	ILLAELEQLK
	EEAESTLQSFR
503	
547	
559	
639	
667	VESNLTFEK
	NTNVEVFLPK
	LGIVDVFQEAK
	LQEDYDMESVFQR
	FKLQEDYDMESVFQR
	LVLVNAIYFK
	IPELLSGGSVDSETR
693	
740	
853	
854	SAMPFTASPAPGTR
	VTPPEGYDVVTVFPK
883	EVDYSGLR
	KTPQGPPEIYSDTQFPSLQSTAK
889	
1009	
1060	QLSSGVSEIR
	AQIGGPESEQSGAK
	VSLDVNHFAPEELTVK
	KYTLPPGVDPTLVSSSLSPGTLTVEAPLPK
1069	

# Identification of novel candidates for replicative senescence by functional proteomics

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To identify the underlying mechanisms that limit the mitotic potential of normal somatic cells, we have undertaken a high resolution differential proteomic analysis aimed at identifying proteins that were differentially expressed upon replicative senescence. Since replicative senescence in heterogenous primary fibroblast cultures is asynchronous, we analysed a group of conditionally immortalized rat embryo fibroblast cell lines that have previously been shown to undergo synchronous senescence upon inactivation of SV40 tsA58 T antigen. This identified 43 spots that were differentially expressed in these cell lines. Comparison of the identity of these features with those identified in a complimentary independent differential proteomic analysis of replicative senescence, directly in primary rat embryo fibroblasts upon serial passaging, identified nine features that were in common between the two studies even though they had been conducted entirely separately. None of these proteins have previously been recognized to be involved with replicative senescence. Thus, they represent novel starting points for elucidating the underlying mechanism that regulates the finite mitotic life span of somatic cells and how it can be overcome in cancer cells.

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**Keywords:** replicative senescence; proteomics; gene expression; mass spectrometry

## Introduction

Normal cells undergo a finite number of divisions and then cease dividing whereas cancer cells are able to proliferate indefinitely. The acquisition of an unlimited proliferative potential has thus been proposed to be one of the critical steps in cancer, which arises as a consequence of the accumulation of multiple independent mutations in genes that regulate cell proliferation

and survival (Hanahan and Weinberg, 2000). When normal cells cease dividing they undergo replicative senescence and even though this has been extensively studied, the underlying mechanism that limits the mitotic potential and how this is subverted in cancer cells is not known. The lack of progress has mainly been due to the absence of suitable systems for its study and to the asynchrony, as well as the complexity, of this process in heterogenous cells populations (Campisi *et al.*, 1996; Campisi, 1996).

Replicative senescence is commonly studied by the serial cultivation of primary cells. Under the appropriate culture conditions these cells initially proliferate exponentially but cease dividing after some passages. The loss of proliferative potential in such heterogenous primary cultures is asynchronous and occurs after differing numbers of passages depending upon the cell type and the donor species (Stanulis-Praeger, 1987; Cristofalo and Pignolo, 1993). When the cells have reached the end of their *in vitro* life span, they can be maintained, remain metabolically active, but cannot be induced to undergo new rounds of cell division (Darmon and Jat, 2000). In the serial cultivation of primary mammalian fibroblasts the culture as a whole divides initially and undergoes growth arrest towards the end. However, there can be some growth arrested cells in the early passages and dividing cells towards the later passages.

The discovery that certain viral oncogenes have the capacity to confer indefinite growth in various cell types has allowed us to develop a rodent system in which senescence can be induced synchronously by altering the growth temperature, in an analogous way to the use of conditionally lethal mutants in the characterization of many complex processes in prokaryotes and yeast.

Introduction of Simian Virus 40 large tumour (T) antigen alone into rodent fibroblasts gives rise to cells that can proliferate indefinitely but are dependent upon it for maintenance of their growth once the normal mitotic life span has elapsed (Conzen and Cole, 1995; Darmon and Jat, 2000; Jat and Sharp, 1989). We showed by using the thermolabile SV40 tsA58 T antigen that inactivation of T antigen in these immortalized cells causes a rapid and irreversible cessation of growth and synchronous entry into senescence (Gonos *et al.*, 1996). This was done by

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using the thermolabile SV40 tsA58 T antigen to isolate conditionally immortal cell lines derived from primary rat embryo fibroblasts (REFs). These tsa cell lines grow continuously at 33°C, the permissive temperature, where the large T antigen is active, but rapidly undergo growth arrest upon shift up to 39.5°C, the non-permissive temperature, due to inactivation of the tsA58 T antigen. Analysis of these conditionally immortalized tsa cell lines showed that upon shift up to the non-permissive temperature they show a loss of proliferative potential that has characteristics very similar to replicative senescence of normal REFs upon serial cultivation (Gonos *et al.*, 1996). However, in contrast to replicative senescence upon serial passaging of primary REFs, senescence in these tsa cell lines occurs synchronously within 72 h (one or two generations) (Gonos *et al.*, 1996).

To determine when cells became dependent upon the SV40 large T antigen to continue dividing, the H-2K<sup>b</sup>tsA58 strain of transgenic mice was developed (Jat *et al.*, 1991). These mice harbour the tsA58 T antigen under the control of the  $\gamma$ -interferon inducible H-2K<sup>b</sup> class I promoter, and allow the preparation of cell populations in which the expression of T antigen can be induced or suppressed in every cell by manipulating the growth conditions (Jat *et al.*, 1991). Analysis of embryonic fibroblasts prepared from these mice has demonstrated that cells become dependent upon T antigen for maintaining growth only when their normal mitotic life span has elapsed and that the biological clock that limits the mitotic potential continues to function normally, even in the presence of the immortalizing gene (Ikram *et al.*, 1994). Thus cell division in rodent cells is not regulated by random accumulation of cellular damage and neither is it simply a culture shock but involves a genetic program comprising two components: a counting mechanism that measures the finite mitotic life span, and a process of entry into the post-mitotic state. The counting mechanism in mouse cells is not dependent upon shortening of telomeres (Wright and Shay, 2000), as has been proposed for human cells (Blasco *et al.*, 1997; Serrano and Blasco, 2001), but is temperature sensitive (Ikram *et al.*, 1994). SV40 T antigen blocks the entry into the post-mitotic state and it is for this reason that the cells undergo synchronous senescence upon its inactivation. Therefore the tsa cell lines should represent an excellent model system for identifying the changes that are critical for the entry into senescence.

We have therefore undertaken a differential proteomic analysis of the loss of proliferative potential in these tsa cell lines. Moreover we have chosen to analyse three tsa cell lines (tsa4, 8 and 12) and only characterize changes that were commonly observed in all the lines. Since this model requires that cells be shifted to a higher growth temperature and genes could be differentially regulated by the increase in the temperature, we have complimented our studies by analysing SV2 and SV4 cell lines (Jat and Sharp, 1989). These two cell lines had been derived from the same

batch of Fischer REFs, at the same time using the same protocol, except that they were isolated after infection with a recombinant retrovirus that transduced the wild type SV40 T antigen and thus grow at both temperatures. To ensure that the changes in expression were not specific to a particular genetic background, we analysed also tsa129, another tsa cell line, that was derived from Sprague–Dawley REFs but using the same thermolabile tsA58 T antigen (Mazars and Jat, 1997). Changes in protein expression profiles were monitored by high-resolution 2-D polyacrylamide gel electrophoresis. Features that were commonly differentially expressed between all the tsa cell lines, and not in the SV cell lines were excised and identified by peptide mass mapping by matrix-assisted laser desorption/ionization (MALDI-MS) and nano-HPLC electrospray ionization tandem mass spectrometry (ESI-MS/MS). This analysis identified 43 spots whose expression changed more than 1.5-fold upon loss of proliferative potential. The study presented here has been carried out in parallel with another study where we have also used differential proteomics to directly examine replicative senescence in asynchronous cultures of serially passaged Sprague–Dawley REFs (Benvenuti *et al.*, 2002).

## Results

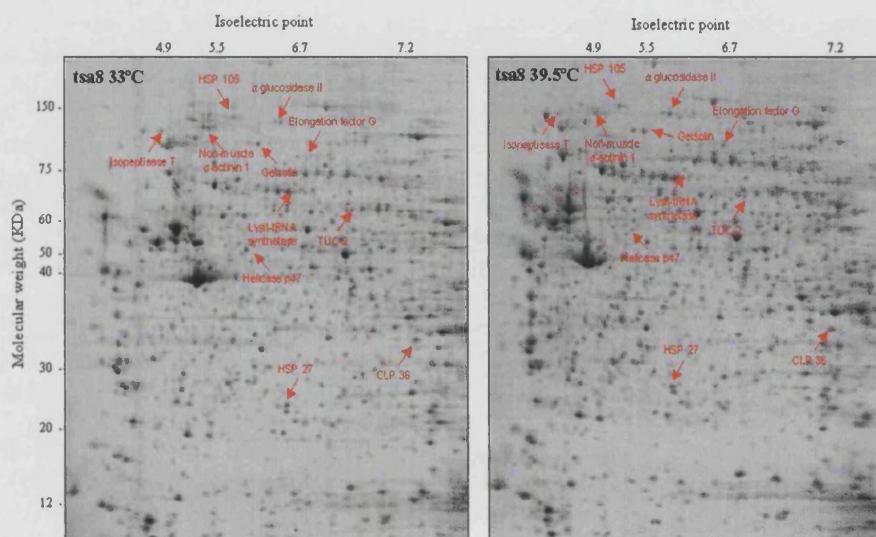
### 2-D gel analysis

Triplicate independent total protein extracts prepared from four different clonal conditionally immortalized cell lines (tsa4, 8, 12 and 129) grown at 33°C, or after shift up to 39.5°C, for 72 h were fractionated on 2-D gels, to visualize changes in their proteome. To eliminate any changes in protein expression due to the shift up to 39.5°C for 72 h, extracts prepared from the two SV (SV2 and SV4) cell lines were analysed as a control. Since SV2 and SV4 were derived by immortalization of REFs with wild-type SV40 T antigen, they proliferate at both 33°C and 39.5°C and permit elimination of changes that are not related to loss of proliferative potential but are the consequence of the temperature shift. Moreover, to ensure that only changes in expression due to senescence were being examined, extracts were prepared from sub-confluent cultures in which the culture media had been changed the day before the extraction, and thus should not contain quiescent cells.

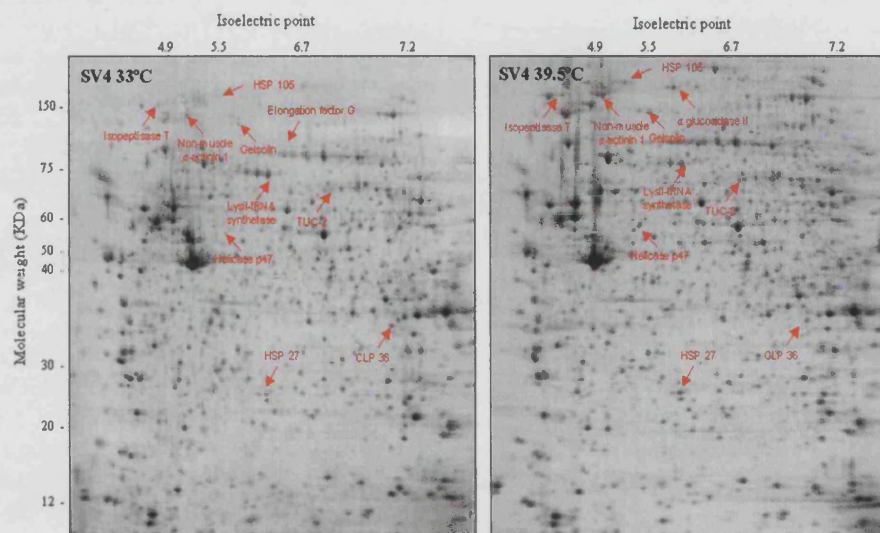
Three independent extracts were prepared for each cell line at 33°C and 39.5°C, and run as three independent gels. The fractionated proteins were detected by staining the gels with the fluorescent dye OGT MP17, followed by scanning at a detection level of less than 1 ng of protein (Page *et al.*, 1999). Representative 2-D gels for tsa8 and SV4, at 33°C and 39.5°C are shown in Figure 1. The primary images were processed with a customized version of Melanie II. Individually resolved protein features were enumerated and quantified on the basis of fluorescence signal intensity. Intensities were measured as percentage of



## A) Representative tsa gels



## B) Representative SV gels



**Figure 1** Representative 2-D gel images of tsa8 (a) and SV4 (b) cells obtained upon fractionation of 150  $\mu$ g of total cell extract

volume, corresponding to pixel intensities integrated over the area of each spot and divided by the sum over all spots in the gel. The intensity of each spot was then corrected by subtracting a background intensity of equal area. The isoelectric point (pI) and molecular weight of each spot was calculated by bilinear interpolation between landmark spots on each image that had previously been calibrated with respect to *Escherichia coli* proteins. Staining and scanning of the gels were by previously published procedures.

A triplicate gel curation was performed for each triplicate. Within each triplicate the gel with the most detected features (spots) using the Melanie II software was chosen and designated as the Reference gel; the other two gels, inside each triplicate, were subsequently designated gel I and gel II. Gel I was first curated against the Reference gel and then gel II was independently curated against the Reference gel. Only

in the last step gel I and gel II were curated against each other. For the 'transitive rule' gel I and gel II were expected to contain exactly the same features. If some sort of mismatch was found in the final comparison between gel I and gel II, the Reference gel was utilized to clarify the problem. Triplicate curation is a time consuming procedure but it allows a differential analysis using only the Reference gels. However it is necessary, in a second step of the analysis, to check that all the features found to be differentially expressed in the Reference gels also occur in gel I and gel II within each triplicate. This was done to ensure that only relevant changes were identified, and to ensure that the features found to be differentially expressed in Reference gels were also present in the other two gels and that the change occurred in the same direction and was at least of the same magnitude.

After curation, each gel was found to comprise over 1200 spots. Moreover, the gels were highly reproducible amongst each triplicate (shown in Table 1). The triplicate gels of tsa4, tsa129, SV2 and SV4, at both temperatures, 33 and 39.5°C, showed a percentage of homology above 80%, while tsa8 and tsa12 gels showed a percentage of homology above 66% when compared with the Reference gel.

The differentially expressed spots were then submitted to CHIMAP, a newly developed program that gives a visual representation of the differentials across a set of matched spots in many different gels with respect to a reference gel (Harris, 2002). Only protein spot changes that were greater than 1.5-fold in magnitude and observed in all the three gels in each triplicate, were considered. It should be noted that for changes that are designated as n.c., it does not imply that there was no change, but that it was less than 1.5-fold in magnitude.

After curation of all the gels and comparison of the proteome of each cell line at 33°C *versus* 39.5°C, we proceeded to determine if the different cell lines showed the same changes and if the common changes were of a similar magnitude. We considered relevant all changes that were observed in the four tsa cell lines but not in the controls. We did not consider those changes that occurred in both the tsa cell lines as well as in the SV controls, because they were most likely to be due to the temperature shift, and not to cellular senescence *per se*. We also considered those changes that occurred in the SV controls but not in the tsa cell lines, hypothesizing that an opposite change was occurring in the cell lines to balance the change observed in the controls due to the temperature shift.

This analysis identified a total of 43 spots that represented proteins differentially expressed in the conditionally immortalized tsa cell lines upon shift up to 39.5°C and thus, in accordance with our model system, represent features that are differentially expressed upon replicative senescence. These differentials were grouped into five different sub-groups, according to how well they fit with the rationalization presented above.

Table 1

Per cent homology between the triplicate gels

	Reference gel	Gel I	Gel II
33°C			
tsa8	100	86.9	87.5
tsa12	100	84.9	76.8
tsa4	100	91.1	88.3
tsa129	100	82.3	94.3
SV4	100	84.8	80.3
SV2	100	89.4	87.0
39.5°C			
tsa8	100	66.0	87.1
tsa12	100	79.0	73.4
tsa4	100	97.8	94.0
tsa129	100	96.0	96.5
SV4	100	92.3	87.4
SV2	100	97.0	93.1

Of the 43 spots, 17 followed exactly the criteria that we used for the differential analysis. Spot 309 and 639 increased in all the tsa cell lines upon shift up from 33 to 39.5°C, and did not change in the two control SV cell lines. Spot 408 behaved in an analogous way except that it decreased in one of the controls (SV2) upon shift up from 33 to 39.5°C. Spot 295 (presented in Figure 3) and 340 decreased in all the tsa cell lines upon shift up from 33 to 39.5°C, and did not change in the two control SV cell lines. Spot 854 was found to remain flat in all the tsa cell lines upon transfer from 33 to 39.5°C, and to decrease from 33 to 39.5°C in the two SV control cell lines. All the other 11 spots remain unchanged in all the tsa cell lines at both temperatures but increased in the control SV cell lines upon shift up from 33 to 39.5°C. We designated these 17 changes the ‘perfect matches’ (Table 2A and Figure 2).

We also decided to consider relevant those changes that did not fit exactly the above criteria and occurred in some of the cell lines, but not in all of them. This was done because we thought that it might be possible that the level of ‘agreement’ could be influenced by the degree of conditionality of the tsa cell lines, because the reduction in the proliferative potential upon shift up to the non-permissive temperature varies between the cell lines.

We identified 13 spots that fit our criteria in all except one cell line (Table 2B). We next identified four changes that were mismatched in two of the cell lines (Table 2C) and six that were mismatched in more than two cell lines (Table 2D). We also identified a further three spots that showed a shift in their migration at the different temperatures. Spot 883 and 889 (shown in Figure 2) showed a shift in their migration in the two control SV cell lines, while they migrated at the same place in gels from all four tsa cell lines. Interestingly spot 853 showed a shift in its migration in the tsa cell lines but not in the control SV cell lines (Table 2E).

It was particularly interesting to note that the tsa cell line that had the lowest number mismatches with our rationale was tsa8 (no mismatches), while tsa12 had a single mismatch, and tsa129 and tsa4 had three and five mismatches respectively. This correlates exactly with the degree of conditionality of the cell lines; tsa8 is the most conditional cell line, followed by tsa12 and tsa4 and 129 are the least conditional in this group of tsa cell lines.

#### Identification by mass spectrometry

The differentially regulated excised spots were first subjected to MALDI-MS peptide mass mapping. The results are presented in Table 3 and in greater details in Supplementary information Tables 1–3. For some of the spots where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous, ESI-MS/MS was performed. In addition, a few samples with sufficient MALDI-MS data for protein identification were also analysed by ESI-MS/MS to verify the MALDI-MS

Table 2 Differentially expressed features

ID	MW	pI	tsa4	tsa8	tsa12	tsa129	SV2	SV4
(A) All cell lines								
61	113722	5.80	n.c.	n.c.	n.c.	n.c.	+2.5	+4.1
92	94474	6.50	n.c.	n.c.	n.c.	n.c.	+2.1	+1.5
112	92040	5.67	n.c.	n.c.	n.c.	n.c.	+1.8	+2.2
161	78807	6.44	n.c.	n.c.	n.c.	n.c.	+1.5	+1.8
209	71346	5.96	n.c.	n.c.	n.c.	-4.9	+1.5	+1.6
236	60178	5.97	n.c.	n.c.	n.c.	n.c.	+2.1	+3.5
239	68141	5.90	n.c.	n.c.	n.c.	n.c.	+1.6	+2.3
295	63742	6.07	-1.5	-1.7	-1.5	-1.6	n.c.	n.c.
309	61908	6.90	+1.5	+1.5	+1.8	+1.8	n.c.	n.c.
340	61124	5.85	-1.5	-1.5	-1.5	-1.5	n.c.	n.c.
352	60276	5.54	n.c.	-1.5	n.c.	n.c.	+2.3	+1.5
408	54970	7.28	+2.1	+1.5	+1.9	+1.6	-1.5	n.c.
448	53548	6.49	n.c.	6.49	n.c.	n.c.	+2.0	+2.0
547	44443	5.34	n.c.	-2.0	n.c.	-1.5	+1.9	+2.6
639	42919	7.01	+1.7	+1.5	+1.6	+1.5	n.c.	n.c.
854	34256	7.19	n.c.	n.c.	+1.8	n.c.	-1.5	-1.5
1009	21696	5.03	n.c.	-1.8	n.c.	n.c.	+2.0	+1.3
(B) Five cell lines								
50	126542	5.49	n.c.	n.c.	-4.3	+1.7	+1.7	+2.8
70	104809	4.92	n.c.	n.c.	n.c.	+1.5	+2.1	+1.7
75	109260	5.31	n.c.	-2.0	+2.0	n.c.	+4.5	+4.3
88	99679	5.20	n.c.	n.c.	+1.6	n.c.	+8.3	+5.5
125	86088	5.20	+1.5	n.c.	n.c.	n.c.	+1.9	+1.5
130	84708	6.00	n.c.	n.c.	n.c.	n.c.	+1.5	n.c.
232	68929	7.01	+2.1	+1.7	+2.1	n.c.	n.c.	n.c.
282	63172	6.37	n.c.	-1.8	n.c.	n.c.	n.c.	+2.9
454	46916	4.89	n.c.	n.c.	n.c.	-2.2	+1.9	n.c.
503	50222	5.59	+2.1	-2.0	n.c.	n.c.	+4.0	+2.5
559	46471	5.62	+1.6	n.c.	n.c.	n.c.	+2.2	+1.8
667	39256	5.69	+1.9	n.c.	n.c.	n.c.	+1.6	+1.7
1060	240995	5.83	+1.5	-1.5	-1.5	-1.5	n.c.	n.c.
(C) Four cell lines								
68	104809	5.26	-1.6	-1.9	+2.1	+2.2	+7.5	+1.6
81	99504	7.15	n.c.	n.c.	+2.1	n.c.	n.c.	+1.9
693	38467	5.18	+2.5	-3.3	+1.5	n.c.	n.c.	n.c.
740	38566	6.02	n.c.	-1.7	-1.7	n.c.	n.c.	n.c.
(D) Three cell lines								
75	102501	6.39	n.c.	+1.8	n.c.	n.c.	n.c.	n.c.
120	88201	7.29	n.c.	+1.8	+3.3	n.c.	-1.6	n.c.
149	81062	6.17	n.c.	n.c.	+1.5	-1.7	n.c.	+1.8
187	68832	7.38	+1.5	+2.0	n.c.	n.c.	-1.8	n.c.
251	66472	5.45	n.c.	n.c.	-2.3	-1.5	+2.8	n.c.
380	58656	6.99	n.c.	n.c.	+1.9	n.c.	n.c.	n.c.
(E) Shift in the migration								
853	34374	6.17	SHIFT	SHIFT	SHIFT	n.c.	n.c.	SHIFT
883	31214	5.72	n.c.	n.c.	n.c.	n.c.	SHIFT	SHIFT
889	30933	5.75	n.c.	n.c.	n.c.	n.c.	SHIFT	SHIFT

n.c.: no changes

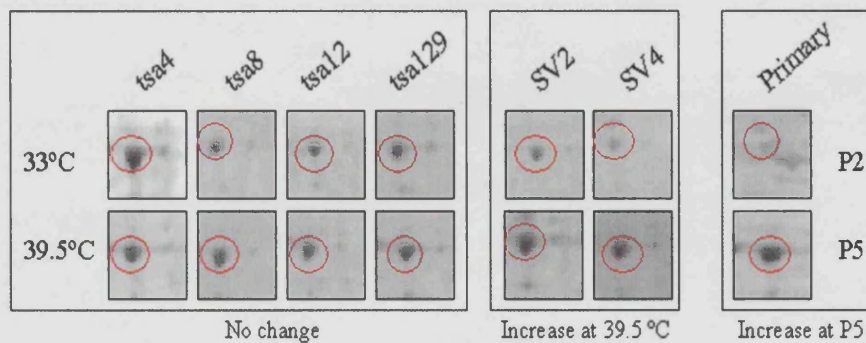
peptide mass mapping. These results are also summarized in Table 3. Positive protein identification by MALDI-MS was confirmed in all cases where ESI-MS/MS was employed and all putatively identified proteins were confirmed.

*Differentially expressed features that are common between cellular senescence in the tsa cell lines and replicative senescence in primary REFs*

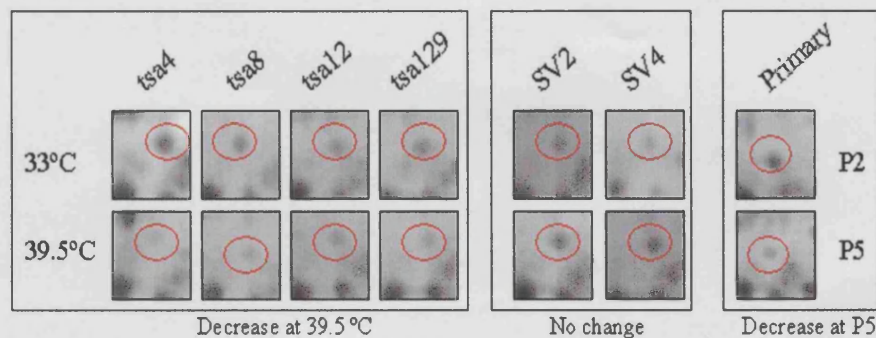
The list of proteins that we found to be differentially expressed upon loss of proliferative potential in the conditionally immortalized tsa cell lines was then

compared with a similar differential proteome study conducted on replicative senescence of primary REFs upon serial passaging (Benvenuti *et al.*, 2002). This identified nine differentially expressed features, shown in Table 4, that were in common between the two studies even though they had been conducted entirely separately. It was possible to match and subsequently compare the tsa gels versus the REF gels because the isoelectric point (pI) and molecular weight of each spot was calculated by bilinear interpolation between landmark spots on each image that had previously been calibrated with respect to the same *Escherichia coli* proteins.

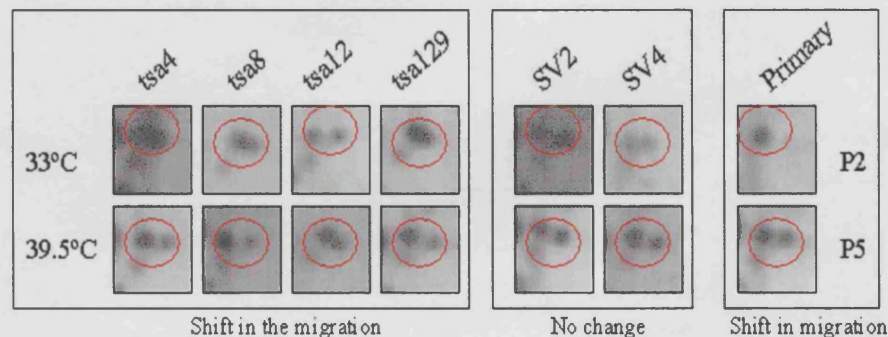
## ID 112



## ID 295



## ID 883 and 889



**Figure 2** Representative features. 2-D gels images showing selected differentially expressed features

The differentially expressed spots were identified independently by mass spectrometry from both cell systems, (they have been excized and analysed twice (once from a REF 2D gel and once from a tsa gel)). From the nine features that were in common between the tsa cell lines and the primary REFs, shown in Table 4, seven pairs of spots identified the same protein from the two systems; tsa spot 50 and REF spot 102 was alanyl-tRNA synthetase; tsa spot 61 and REF spot 127 was  $\alpha$ -glucosidase II; tsa spot 112 and REF spot 190 was gelsolin; tsa spot 295 and REF spot 304 was TUC-2; tsa spot 503 and REF spot 677 identified eukaryotic polypeptide chain factor 1+ probable ATP-dependent RNA helicase p47; tsa spot 1060 and REF spot 1302

was hsp27; tsa spot 854 was identified as a LIM domain containing protein whereas REF spot 1076 was identified more specifically as CLP36 which contains a LIM domain.

One spot (tsa spot 309 which matched with REF spot 504) yielded a different identity when analysed from the two different systems. It was identified as Lamin A from the tsa spot whereas from the REF spot it was identified as MPAST and Lamin A or C. The last spot did not yield any protein identity in the REF sample (tsa spot 239 which matched with REF spot 369) while it was identified as Lysyl-tRNA synthetase from the tsa spot.

One possibility for the lack of correlation between the identities for spot 309/504 could be the presence of



**Table 3** Identification of the differentially expressed features by Mass Spectrometry

ID	MW	pI	MALDI-MS spot identity	ESI spot identity
50	126542	5.49	Alanyl-tRNA synthetase	Alanyl-tRNA synthetase + Heat shock protein 105 kDa $\alpha$ 1B
61	113722	5.8	$\alpha$ -glucosidase II	
68	104809	5.26	Non-muscle $\alpha$ -actinin 1	
70	104809	4.92	Isopeptidase T	
75	102501	6.39	Alix	
75	109260	5.31	$\alpha$ -Actinin 4 + Non-muscle $\alpha$ -actinin 1	No MS/MS
81	99504	7.15	Elongation factor 2	
88	99679	5.2	Transitional endoplasmic reticulum ATPase	
92	94474	6.5	Lysyl hydroxylase isoform 2	
112	92040	5.67	Gelsolin	
120	88201	7.29	DEAD box protein RB	Eukaryotic polypeptide chain release factor 3
125	86088	5.2	Eukaryotic polypeptide chain release factor 3	
130	84708	6.0	Elongation factor G	
149	81062	6.17	CDC47	
161	78807	6.44	Radixin	
187	68832	7.38		*
209	71346	5.96	Glycyl-tRNA synthetase	Lysyl-tRNA synthetase
232	68929	7.01	Lamin A	
236	60178	5.97	Leukotriene A4 hydrolase	
239	68141	5.9	Lysyl-tRNA synthetase	
251	66472	5.45	Annexin VI	
282	63172	6.37	Lamin A + Neural specific protein CRMP-2	
295	63742	6.07	TUC-2	
309	61908	6.9	Lamin A	
340	61124	5.85	$\gamma$ -Butyrobetaine, 2-oxoglutarate dioxygenase	
352	60276	5.54	Copine I	
380	58656	6.99	Tyrosyl-tRNA synthetase	Copine I(11) + Chaperonin subunit 8( $\theta$ ) + T-complex protein 1, $\epsilon$ subunit + $\kappa$ -B motif-binding phosphoprotein
408	54970	7.28	IMP dehydrogenase	
448	53548	6.49	Aspartyl-tRNA synthetase	
454	46916	4.89	ATP synthase $\beta$ subunit + Class I $\beta$ -tubulin	
503	50222	5.59	Eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47	
547	44443	5.34	Translation initiation factor eIF-4A + Actin $\beta$	ATP synthetase $\beta$ subunit + Tubulin, $\beta$ 5 + tubulin, $\beta$ 2 + vimentin
559	46471	5.62		
639	42919	7.01	Cytosolic NADP-dependent isocitrate dehydrogenase	
667	39256	5.69		
693	38467	5.18	Guanine nucleotide-binding protein $\alpha$ subunit	
740	38566	6.02		LIM protein Hypothetical protein†
853	34374	6.17	Cytosolic malate dehydrogenase	
854	34256	7.19		
883	31214	5.72		
889	30933	5.75	PA28 $\gamma$ subunit	
1009	21696	5.03	Mitotic checkpoint component Mad2	Heat shock 27 protein
1060	24095	5.83	Heat shock protein	

\*: no significant hits. †Accession number 8923710

multiple proteins and the relative abundance of each component of that spot being slightly different in the two different cell models, and only the most abundant protein being identified by mass spectrometry. Since we have hypothesized that the tsa cell lines are a model for studying the transition to the post-mitotic state of senescence, it indicates that the features found to be differential in both different model systems are likely to correspond to proteins that are involved in this step.

## Discussion

Here we have presented a high resolution differential proteomic analysis aimed at identifying proteins that were differentially expressed when REF cell lines conditionally immortalized with the thermolabile

SV40 tsA58 T antigen undergo replicative senescence upon its inactivation. This identified 43 differentially expressed features that were subsequently identified by mass spectrometry. Comparison of these differentially expressed features with those identified in a complementary differential proteomics study of replicative senescence upon serial passaging of primary REFs identified nine features that were in common between the two systems. We have previously shown that the conditionally immortalized cell lines are committed to undergo senescence but are prevented from undergoing this process by the presence of the tsA58 T antigen and therefore are a model for studying the entry into cellular senescence upon its inactivation. Therefore these common features are likely to correspond to proteins that are either involved in or are markers for the entry into the senescent state.

**Table 4** Differential features that are common between replicative senescence in primary REFs and the tsa cell lines

SD ID	MW	pI	SD identity	tsa ID	MW	pI	tsa identity
102	125386	5.48	Alanyl-tRNA synthetase	50	125642	5.49	Alanyl-tRNA synthetase + heat shock protein 105 kDa $\alpha/\beta$
127	113345	5.82	$\alpha$ glucosidase II, $\alpha$ subunit (m)	61	113722	5.8	$\alpha$ -glucosidase II
190	91608	5.66	Gelsolin + myosin heavy chain	112	92040	5.67	Gelsolin
304	63329	6.09	TUC-2	295	63742	6.07	TUC-2
369	67881	5.90		239	68141	5.9	Lysyl-tRNA synthetase
504	60158	6.94	MPAST + Lanmin A and C	309	61908	6.9	Lamin A
677	47888	5.57	Eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47	503	50222	5.59	Eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47
1076	33986	7.2	CLP36 (rat LIM domain containing protein)	854	34256	7.19	LIM protein
1302	24177	5.58	Heat shock protein 27	1060	24095	5.83	Heat shock protein 27

The proteins that were found to be differentially expressed in the tsa cell lines when they ceased proliferating upon inactivation of tsA58 T antigen comprised a variety of cytoskeletal, heat shock and metabolic proteins as well as proteins involved in trafficking, differentiation and protein synthesis, turnover and modification. Comparison of these differential proteins with those that were found to be differential in a complimentary functional proteomic study of replicative senescence upon serial passaging of primary REFs identified nine common features; three of these features fit perfectly the criteria for potential candidates for entry into replicative senescence.

These three features comprised tsa spot 295 that was identified as TUC-2. It was down-regulated in all four tsa cell lines and remained unchanged in the control SV cell lines upon shift up; it was also down-regulated in REFs upon serial passaging. Moreover the down-regulation of the TUC-2 protein in senescent REFs was associated with down-regulation at the RNA level. Since we have found that ectopic expression of TUC2 alone was not sufficient for immortalization of REFs (unpublished data) it suggests that loss of expression TUC-2 was not the cause of replicative senescence but TUC-2 may be an excellent marker for this process. TUC-2 belongs to the TUC (TOAD-64/Ulip/CRMP) family of intracellular phosphoproteins implicated in axon guidance and outgrowth and thereby regulation of neuronal differentiation (Wang and Strittmatter, 1996; Byk *et al.*, 1998; Quinn *et al.*, 1999). This was further supported by their homology to *unc-33*, a *Caenorhabditis elegans* gene that is required for normal axon outgrowth and guidance. TUC-2 is expressed by both neurons and their progenitors and is also slightly up-regulated upon treatment of PC12 cells with NGF. Recently TUC-2 was found to be associated with microtubule bundles at the mitotic spindle and proposed to be involved in regulating microtubule dynamics (Gu and Ihara, 2000). Since our results suggest that TUC-2 may be a very good marker for senescence, it will be very informative to determine whether the down-regulation at the RNA level is due to decreased transcription or changes in RNA stability and identify the factors that are the cause of these changes. In addition to this down-regulation of TUC-2, we have found that there was a loss of TUC-2

phosphorylation upon both replicative senescence of REFs and premature senescence of REF52 cells. A similar loss of phosphorylated TUC-2 was found in the tsa cells upon shift up (S Benvenuti, data not shown). Interestingly, Ihara and colleagues have previously shown that in Alzheimer's disease (AD) a phosphorylated form of TUC-2 was present within neurofibrillary tangles and some plaque neurites and was significantly increased within the soluble fraction of AD brain extracts (Gu *et al.*, 2000). They further showed that the phosphorylation occurred at serine and threonine residues within the carboxy-terminal basic region and proposed that it may play a role in regulating its activity. It will therefore be very interesting to determine in growing cells, the sites of TUC-2 phosphorylation, identify the kinase responsible and finally determine whether the loss of phosphorylation has a role in cellular senescence.

Another feature that fit our criteria was tsa spot 309. It was up-regulated in all the tsa cell lines and remained unchanged in the control SV cell lines upon shift up and was up-regulated in senescent REFs. This feature was identified in the tsa gels as Lamin A whereas in the REF gels it was identified as MPAST and Lamin A or C. Further work is thus necessary to determine which of these candidate proteins is truly differentially expressed, whether the up-regulation is due to up-regulation at the RNA level and what is the cause of this up-regulation. Lamin A is a member of a protein family that includes three known mouse A-type lamins (A, C and C2), encoded within a single genomic locus (Nakajima and Abe, 1995). They are the major components of the nuclear lamina, a two-dimensional filamentous network at the periphery of the nucleus in higher eukaryotes, directly underlying the inner nuclear membrane (Gotzmann and Foisner, 1999). Lamins as well as lamin-binding proteins appear to be important for various steps of post-mitotic nuclear reassembly, including cross-linking of chromatids, nuclear membrane targeting, nuclear lamina assembly, and the formation of a replication-competent nucleus, however their specific role is unknown (Gotzmann and Foisner, 1999).

Another feature that showed a perfect fit with our criteria was tsa spot 854. It remained unchanged in three of the tsa cell lines and was up-regulated in

tsa12 cells. Interestingly it was down-regulated in the control SV cells indicating that it had to have been up-regulated upon senescence to compensate for the down-regulation due to temperature shift. This up-regulation was in accordance with the finding that it was up-regulated in senescent REFs. This feature was identified to be CLP36, a LIM domain containing protein. CLP36 was originally identified as a gene down-regulated by chemical hypoxia in a subtractive screen of normoxic *versus* hypoxic rat hepatocytes. It encodes a 327 aa protein that contains a conserved LIM domain, a Cystine-rich domain initially identified in homeodomain proteins, Lin-11 (Freyd *et al.*, 1990) Isl-1 (Karlsson *et al.*, 1990) and Mec-3 (Way and Chalfie, 1988; Wang *et al.*, 1995). The LIM domain is a zinc finger motif present in several types of proteins, including homeodomain transcription factors and kinases. Proteins containing LIM domains have been discovered to play important roles in a variety of fundamental biological processes including cytoskeleton organization, cell lineage specification and organ development, but may also be involved in oncogenesis. The LIM domain is a protein-protein interaction motif critical for these processes (Bach, 2000). CLP36 has been shown to bind, via  $\alpha$ -actinin-1, to actin filaments and stress fibers in activated human platelets and endothelial cells (Bauer *et al.*, 2000). In the rat, CLP36 mRNA is mainly expressed in heart, lung and liver, less in spleen and skeletal muscle, and at extremely low levels in testis and brain tissues. Further experiments are now necessary to determine the cause of the up-regulation of CLP36 and whether the up-regulation contributes towards replicative senescence.

There were three other features that were commonly differentially expressed but did not fully fit our criteria. tsa spot 112 identified as gelsolin was one of these. Gelsolin mediates the rapid remodeling of cortical actin filaments and has a role in cell functions that are dependent upon stress fibers. It has been proposed to be a candidate tumor suppressor gene for breast cancer because its expression is either partially or completely lost in the majority of breast cancers of diverse aetiologies (Asch *et al.*, 1996). It is also down-regulated in transformed cells as well as other tumour types (Lee *et al.*, 1999; Shieh *et al.*, 1999; Dong *et al.*, 1999). Our previous finding that gelsolin was up-regulated upon replicative senescence in both rat and mouse embryo fibroblasts were consistent with these observations. However the results presented here show that gelsolin remained unchanged in the tsa cells upon shift up but it was up-regulated in the control SV cells suggesting that either the tsa cells do not respond to the temperature shift or there was a compensatory down-regulation. Since we have previously shown that tsa cells respond to heat shock, the most likely cause of our results is that there is a compensatory down-regulation of the gelsolin protein upon shift up. This was not in accordance with our previous finding that gelsolin levels were increased in senescent cells and this increase was due to protein stability. It will therefore

be intriguing to determine how and why this protein becomes stabilised in senescent cells and why this does not occur when the tsa cells cease dividing. It is interesting to note that Asch *et al.* (1996) have shown that in cancer cells both gelsolin protein and its mRNA were down-regulated, suggesting that an alteration in the rate of transcription was the cause of the dysfunction.

Another commonly differential spot that did not fully fit our criteria was tsa spot 61, identified to be  $\alpha$ -glucosidase II. The  $\alpha$ -glucosidases are intimately involved in quality control in the endoplasmic reticulum (ER), a process that ensures proper folding of newly formed polypeptide chains leading to retention and/or degradation of incorrectly folded proteins (Herscovics, 1999). Interestingly we have previously found that  $\alpha$ -glucosidase was up-regulated upon replicative senescence in both rat and mouse embryo fibroblasts and premature senescence of REF52 cells upon ectopic expression of activated H-ras. Here we have shown that it was differentially expressed in the tsa cell lines upon inactivation of T antigen and entry into senescence. As for gelsolin, it was not up-regulated in the tsa cells upon shift up rather it was up-regulated in the control SV cell lines. Interestingly we have shown by RT-PCR analysis of RNA extracted from serially passaged REFs that  $\alpha$ -glucosidase levels decrease rather than increase suggesting that the increase in protein level is most likely to be due to protein stability.

The third feature that comprises this group of common differential features was tsa spot 239 that was identified as lysyl-tRNA synthetase. Unfortunately there was insufficient protein within the REF spot for identification by mass spectrometry. Therefore further work is necessary to determine the identity of the protein that was differential within this spot.

Our analysis identified three other features that were common between the two systems, tsa spots 50 (alanyl-tRNA synthetase + heat shock protein 105 KDa  $\alpha/\beta$ ), 503 (Eukaryotic polypeptide chain release factor 1 + probable ATP-dependent RNA helicase p47) and 1060 (heat shock protein 27). Unfortunately these three spots were not differential in all the tsa cell lines and were thus not considered as prime candidates. Interestingly two of them, spots 50 and 503 behave as spots whose expression was up-regulated only in the control SV cells. The data for spot 1060 was rather confusing because even though it is a heat shock protein, it remained unchanged in the control SV cells upon temperature shift, was down-regulated in tsa8, 12 and 129 and up-regulated in tsa4 cells upon shift up.

The differential proteomic analysis presented here and elsewhere has allowed us to identify some candidate proteins that have previously not been linked with replicative senescence. Even though it remains to be unequivocally demonstrated that any of these differentially expressed proteins either singly or in combination are the cause of replicative senescence, they represent important starting points for determining the activities critical for the observed changes in

expression, whether they occur at the level of mRNA, or protein or by post-translational modification and whether they are involved in replicative senescence of human cells.

## Materials and methods

### Cell cultures

The conditionally immortalized cell lines, tsa4, 8, 14 and 129, as well as the control cell lines, SV2 and SV4, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All of these cell lines were propagated at 33°C, the permissive temperature and cultures were shifted up to 39.5°C for 72 h prior to preparation of cell lysates. All media and components were obtained from Invitrogen Life Technologies.

### Sample preparation for 2-D gels

The cell extracts used for the 2-D gels were prepared by adding a solution containing 4% wt/vol 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, 5 M urea, 2 M thiourea, 65 mM DTT, 0.8% wt/vol Resolytes 3-10 (Bio-Rad), and trace bromophenol blue to a frozen cell pellet to yield approximately 300 µg protein in a final volume of 925 µl. This was vortexed, left to stand for 5 min, vortexed again, then centrifuged at 13 000 g for 5 min at 15°C. Samples were prepared as triplicate independent samples.

### 2D-gel electrophoresis

Immobilized pH gradient (IPG) gels (Immobiline DryStrip 3-10 NL, Amersham Pharmacia Biotech) were rehydrated with

370 µl of solubilized sample and focused overnight (70 kVh, 20°C) according to Sanchez (Sanchez *et al.*, 1997). Immediately after focussing, IPG gels were equilibrated in 6 M urea, 2% wt/vol SDS, 2% wt/vol DTT, 50 mM Tris.HCl, pH 6.8 and 30% vol/vol glycerol for 15 min before running in the second dimension on 9–16% T, 2.7% C gels, cast with the gel bound to one of the glass plates, in an electrophoresis tank similar to that described by Amess and Tolkovsky (1995) at 30 mA per gel and 20°C. Immediately after electrophoresis, gels were fixed in 40% vol/vol ethanol:10% vol/vol acetic acid and stained with the fluorescent dye OGT MP17 and 16-bit monochrome fluorescence images at 200 µm resolution were obtained by scanning the gels with an Apollo II linear fluorescence scanner (Oxford GlycoSciences, UK). Two gels were run for each sample.

### Protein identification by mass spectrometry

Identifications of all differentially expressed proteins utilized a standard approach using MALDI-MS and if necessary ESI-MS/MS. Differential spots were excised from one of the triplicate gels, which showed the highest expression level for each spot. Prior to mass spectrometry, tryptic in-gel digests were carried out on all samples using a protocol similar to already published procedures (e.g., <http://donatello.ucsf.edu/ingel.html>). Details of the procedures for peptide mass mapping by matrix-assisted laser desorption/ionization and nano-HPLC electrospray ionization tandem mass spectrometry have been previously published (Benvenuti *et al.*, 2002).

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# Differential Proteome Analysis of Replicative Senescence in Rat Embryo Fibroblasts\*<sup>§</sup>

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Normal somatic cells undergo a finite number of divisions and then cease dividing whereas cancer cells are able to proliferate indefinitely. To identify the underlying mechanisms that limit the mitotic potential, a two-dimensional differential proteome analysis of replicative senescence in serially passaged rat embryo fibroblasts was undertaken. Triplicate independent two-dimensional gels containing over 1200 spots each were run, curated, and analyzed. This revealed 49 spots whose expression was altered more than 2-fold. Of these, 42 spots yielded positive protein identification by mass spectrometry comprising a variety of cytoskeletal, heat shock, and metabolic proteins, as well as proteins involved in trafficking, differentiation, and protein synthesis, turnover, and modification. These included gelsolin, a candidate tumor suppressor for breast cancer, and  $\alpha$ -glucosidase II, a member of the family of glucosidases that includes *klotho*; a defect in *klotho* expression in mice results in a syndrome that resembles human aging. Changes in expression of TUC-1, -2, -4, and -4 $\beta$ , members of the TUC family critical for neuronal differentiation, were also identified. Some of the identified changes were also shown to occur in two other models of senescence, premature senescence of REF52 cells and replicative senescence of mouse embryo fibroblasts. The majority of these candidate proteins were unrecognized previously in replicative senescence. They are now implicated in a new role. *Molecular & Cellular Proteomics* 1:280–292, 2002.

Cancer arises as a consequence of the accumulation of multiple independent mutations in genes that regulate cell proliferation and survival (1). The acquisition of an unlimited proliferative potential has been proposed to be one of the critical steps in this process, because normal cells can only

undergo a finite number of divisions when cultured *in vitro* before undergoing replicative senescence (2). Even though replicative senescence has been studied extensively and can be overcome by immortalizing genes, the underlying molecular basis is still not understood fully.

In human somatic cells telomere shortening is a critical component of the machinery that counts the number of cell divisions and therefore entry into senescence. It was proposed initially that reconstitution of telomerase activity resulting in maintenance of telomeres was sufficient for immortalization of human somatic cells, but others have found that this is not sufficient and requires additional activities such as those that can be provided by the SV40 (simian virus 40) large T antigen or inactivation of the pRB/p16 INK4 pathway (3–5). Further studies have now shown that in freshly isolated human mammary fibroblasts and endothelial cells, reconstituted telomerase activity was sufficient neither for immortalization nor maintenance of the immortal state in cell lines that had been immortalized with a combination of the SV40 T antigen and the catalytic subunit of telomerase (6). Inactivation of SV40 T antigen in these cells resulted in a rapid and irreversible cessation of cell growth and entry into senescence.

Even though telomere shortening cannot be demonstrated in rodent cells, they, too, have a finite life span. In contrast to human cells this can be overcome readily in rodent cells by either the exogenous introduction of any member of the family of viral and cellular immortalizing genes, such as SV40 T antigen, or even by spontaneous mutation. Interestingly like the human cells immortalized with SV40 T antigen and hTERT, rodent cells expressing SV40 T antigen proliferate indefinitely and are absolutely dependent upon its continued expression for maintenance of growth (7). Inactivation also results in a rapid and irreversible cessation of growth and entry into senescence (8). Moreover, we have shown that primary mouse embryo fibroblasts are able to measure their proliferative life span even in the presence of SV40 T antigen at the normal rate (9). Taken together these results have raised the possibility that the non-telomere shortening-dependent regulatory components of the finite proliferative life span may be conserved between human and rodent cells and that human cells may have acquired telomere shortening as a further control mechanism.

Replicative senescence is an asynchronous process

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whereby a growing culture gives rise to an irreversibly arrested culture. The model systems that are commonly used for its study involve the isolation and serial *in vitro* cultivation of primary fibroblasts. Initially these cells proliferate exponentially but cease dividing after some passages, at which point the cell numbers no longer increase. The loss of proliferative potential in such heterogeneous cultures of primary cells is asynchronous. When these cells have reached the end of their *in vitro* mitotic lifespan, they can be maintained and remain metabolically active but cannot be induced to undergo new rounds of cell division (10). In such model systems, the culture as a whole divides initially and undergoes growth arrest toward the end; however there can be growth-arrested cells in the early passages and dividing cells toward the later passages.

The senescent phenotype is dominant as fusions of senescent cells with dividing cells give rise to senescent cells (11). Even though a variety of traditional approaches have been utilized to try to identify the underlying changes that are the cause of senescence, this process is still not understood fully, probably because these procedures were insufficient to analyze comprehensively such a complex process. New approaches to address complex biological systems include DNA microarrays that monitor global changes in mRNA expression (12). However studies in *Saccharomyces cerevisiae* and human liver have suggested that mRNA levels may correlate poorly with corresponding protein levels (13, 14), and mRNA-based assays are unable to detect changes in protein level because of stability and changes in post-translational modifications. Another approach would be to analyze changes in protein expression that have the potential for resolution of all expressed proteins in a cell (proteome), which can then be identified by mass spectrometry.

Even though not all proteins may be resolved by two-dimensional (2-D)<sup>1</sup> gels and also may not be identified by mass spectrometry, we have initiated a differential proteomic approach to study replicative senescence. This was done, because it has the potential for identifying changes in protein expression, post-translational modification, stability, and even changes in cellular localization. Furthermore, we have chosen to study the protein expression profiles of serially passaged rat embryo fibroblasts (REFs), rather than primary human fibroblasts, to minimize differences because of epigenetic variation between cells obtained from different donors and also, because human fibroblasts have much longer finite proliferative lifespan *in vitro*. Human fibroblasts are capable of undergoing 50–60 divisions before undergoing replicative senescence, in contrast to 20–30 divisions for rodent embryo

fibroblasts. The issue of epigenetic variation was critical, because we needed to be able to go back and repeat the passaging with freshly isolated identical cells to prepare protein extracts for validation of the proteome analysis and also extraction of RNA for determining whether changes at the protein level correlate with changes at the RNA level. Cells that are cultivated serially after freezing exhibit an altered finite mitotic life span.

Changes in protein profiles upon cellular senescence were monitored by high resolution 2-D polyacrylamide gel electrophoresis, and differentially expressed protein spots were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nano-HPLC electrospray ionization tandem mass spectrometry (ESI-MS/MS). This analysis identified 49 spots whose expression changed more than 2-fold upon replicative senescence; 32 of these spots were up-regulated, 12 were down-regulated, and five displayed an altered migration pattern. The majority of these proteins were unrecognized previously in replicative senescence. They are now implicated in a new role.

#### EXPERIMENTAL PROCEDURES

**Cell Cultures**—REFs were prepared from 12–13-day-old Sprague-Dawley rat embryos, cultured, and passaged serially. All cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. REF52 cells obtained from Scott Lowe were propagated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. All media and components were obtained from Invitrogen.

**2-D Polyacrylamide Gel Electrophoresis**—2-D gels were prepared and run as described previously (15). Staining, scanning, and curation of primary images with subsequent analysis to identify differential spots were all carried out by previously published procedures (15).

**Protein Identification by Mass Spectrometry**—Identifications of all differentially expressed proteins utilized a standard approach using MALDI-MS and if necessary ESI-MS/MS. Differential spots were excised from one of the triplicate gels, which showed the highest expression level for each spot. Prior to mass spectrometry, tryptic in-gel digests were carried out on all samples using a protocol similar to already published procedures (e.g. donatello.ucsf.edu/ingel.html; see Ref. 16). Details of the procedures for peptide mass mapping by matrix-assisted laser desorption/ionization mass spectrometry and peptide sequencing by nano-HPLC electrospray ionization tandem mass spectrometry are provided as Supplementary Material.

**1-D Western Blotting**—Cell lysates for immunoblotting were prepared in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0). Protein concentrations were determined using the Bradford reagent (Bio-Rad). 30 µg of each protein lysate was fractionated on an SDS polyacrylamide gel, transferred to Hybond C membrane, and probed with the following antibodies: α-glucosidase (StressGene), HSP27 Ab-1 (NeoMarkers), HSP70 W27 (Santa Cruz Biotechnology), CDC47 (NeoMarkers), gel-solin (kindly provided by Helen Yin), cyclin A Ab-4 (Oncogene Sciences), and p19<sup>ARF</sup> (Abcam).

**Semiquantitative RT-PCR Analysis**—First strand cDNA was prepared from 2 µg of total RNA using SuperScript™ II Moloney murine leukemia virus RNaseH<sup>-</sup> reverse transcriptase from Invitrogen according to the manufacturer's instructions. RNA was denatured

<sup>1</sup> The abbreviations used are: 2-D, two-dimensional; REF, rat embryo fibroblast; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; HPLC, high pressure liquid chromatography; ESI-MS/MS, electrospray ionization tandem mass spectrometry; RT, reverse transcriptase; HSP, heat shock protein; 1-D, one-dimensional.

(65 °C for 5 min) in the presence of an oligo(dT) primer (0.5 µg; Promega) and dNTPs (0.5 mM; Promega) and quickly chilled on ice. Ribonuclease inhibitor (RNasin; Promega) and dithiothreitol (100 mM) were then added, together with 1× first strand buffer, and incubated at 42 °C for 2 min. Moloney murine leukemia virus RNaseH<sup>-</sup> reverse transcriptase (200 units) was added, and reactions were incubated at 42 °C for 50 min, followed by heat inactivation at 70 °C for 15 min.

For each gene, an optimal cycle number was established that enabled the bands to be visible on a gel but did not result in saturation of the amplification procedure. This was done partially by confirming that the same relative intensities were obtained when the cycle number was increased by two cycles. The sequences of the primers used for PCRs, along with the product size, cycle numbers, and annealing temperatures, are provided as Supplementary Material. All PCR reactions were carried out in 50 µl and contained 1 µl of first strand cDNA. They also contained 0.5 µg of each oligonucleotide primer, 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase (Promega), 1× PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% (w/v) Triton X-100 (10× PCR buffer provided by Promega)), 0.5 mM dNTPs (Promega), and 1.5–2.5 mM MgCl<sub>2</sub> (Promega). The optimum MgCl<sub>2</sub> concentration was determined for each primer set and is also provided as Supplementary Material.

A 5-min 95 °C denaturation step was used prior to amplification. The amplification parameters were denaturation at 94 °C for 1 min, annealing at the specific temperature for each primer pair for 1 min, and extension at 72 °C for 1 min, with a final extension of 5 min at 72 °C after the last cycle. For each PCR, appropriate controls were carried out to check for nonspecific amplification. All PCR primers were designed using Primer3 software ([www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)).

**Premature Senescence of REF52 Cells**—BOSC23 cells ( $1.75 \times 10^6$ ) were plated in a 6-cm dish and transfected 24 h later with 3 µg of either pBabePuroEJras or pBabePuro using FuGENE (Roche Molecular Biochemicals). 48 h after transfection, the virus-containing medium was removed, filtered (through a 0.45 µm filter), and used to infect REF52 cells in the presence of 8 µg/ml polybrene (Aldrich). REF52 were plated at  $8 \times 10^5$  cells per 10-cm dish and incubated overnight prior to infection. For the infection, the culture medium was replaced by 2 ml of virus-containing medium for 2 h. The infection was repeated a second time 6 h later. Sixteen h later, the infected cultures were subjected to 2 µg/ml puromycin selection. The medium was changed on the third day, and the infected cells were extracted with RIPA buffer after 7 days.

## RESULTS AND DISCUSSION

### 2-D Gel Analysis

REFs were prepared from 12–13-day-old Sprague-Dawley rat embryos and passaged using the 3T3 passaging regime of Todaro and Green (17). Cells were passaged serially until they ceased to divide and cell numbers no longer increased, which takes about five passages. Cultures were continually examined microscopically to ensure that there were no mitotic cells visible when passaging was ceased. This involved plating  $2.6 \times 10^6$  cells per 15-cm dish. Cells were plated on day 0 and allowed to adhere, the medium was changed on day 1, and the cells passaged on day 3. For preparation of cell lysates, cells were harvested on day 2. In this way all lysates were prepared from cultures that were in fresh medium and subconfluent and thus not quiescent. This was done to ensure that only changes in expression because of senescence were being examined.

Triplicate independent total protein extracts prepared from three serially passaged independent dishes of REFs at P2 (proliferating cells), P3, P4, and P5 (senescent cells) were fractionated on 2-D gels to visualize changes in the proteome. The fractionated proteins were detected by staining the gels with the fluorescent dye OGT MP17, followed by scanning at a detection level of less than 1 ng of protein. A representative 2-D gel for P2 and P5 senescent REFs is shown in Fig. 1. The triplicate scanned gels were then subjected to triplicate curation in which the gel with the most spots (detected using MELANIE II software) was chosen as the reference gel, and the two other gels were called gel II and III, respectively. First gel II was curated against the reference gel and then gel III was curated against it. Next gel II was curated against gel III. Although triplicate curation is a time-consuming procedure, it allows identification of differential features using only the reference gels. However it is necessary as a follow-up to check manually that all features expressed differentially in the reference gel are also differential in the other two gels within the triplicate. Only protein spots that changed more than 2-fold in magnitude, in the same direction (i.e. up or down), and were observed in all three gels were considered. The pI and molecular weight of each spot was calculated by bilinear interpolation between landmark spots on each image that had been calibrated previously with respect to *Escherichia coli* proteins. After curation, each gel was found to comprise over 1200 spots. Moreover, the gels were very reproducible among each triplicate (the % of homology among the gels was always above 90%, shown in Table I).

The detected spots were then submitted to CHIMAP to identify the differentials. CHIMAP is a newly developed program that calculates a differential value between a large series of matched features either as a percentage change or a -fold change and represents them graphically (18). It generally uses the Ward's minimum variance method although other agglomeration methods can be selected within the program. The comparison of growing *versus* senescent REFs identified 49 spots that were reproducibly regulated differentially, of which 32 were up-regulated, 12 were down-regulated, and five shifted in their position of migration (shown in Table II). Representative features that correspond to each type of differential are shown in Fig. 2. We also examined expression of the differential spots, in P3 and P4 gels, to determine whether the changes were also observed at these passages and whether they occurred stepwise or processively (shown in Supplementary Material, Table I).

### Protein Identification by Mass Spectrometry

**Peptide Mass Mapping by MALDI-MS**—Analysis of the differentially regulated spots by MALDI-MS yielded 24 single positive identifications, two double positive identifications, six single putative identifications, one double putative identification, and one double mixed (positive and putative) identifica-

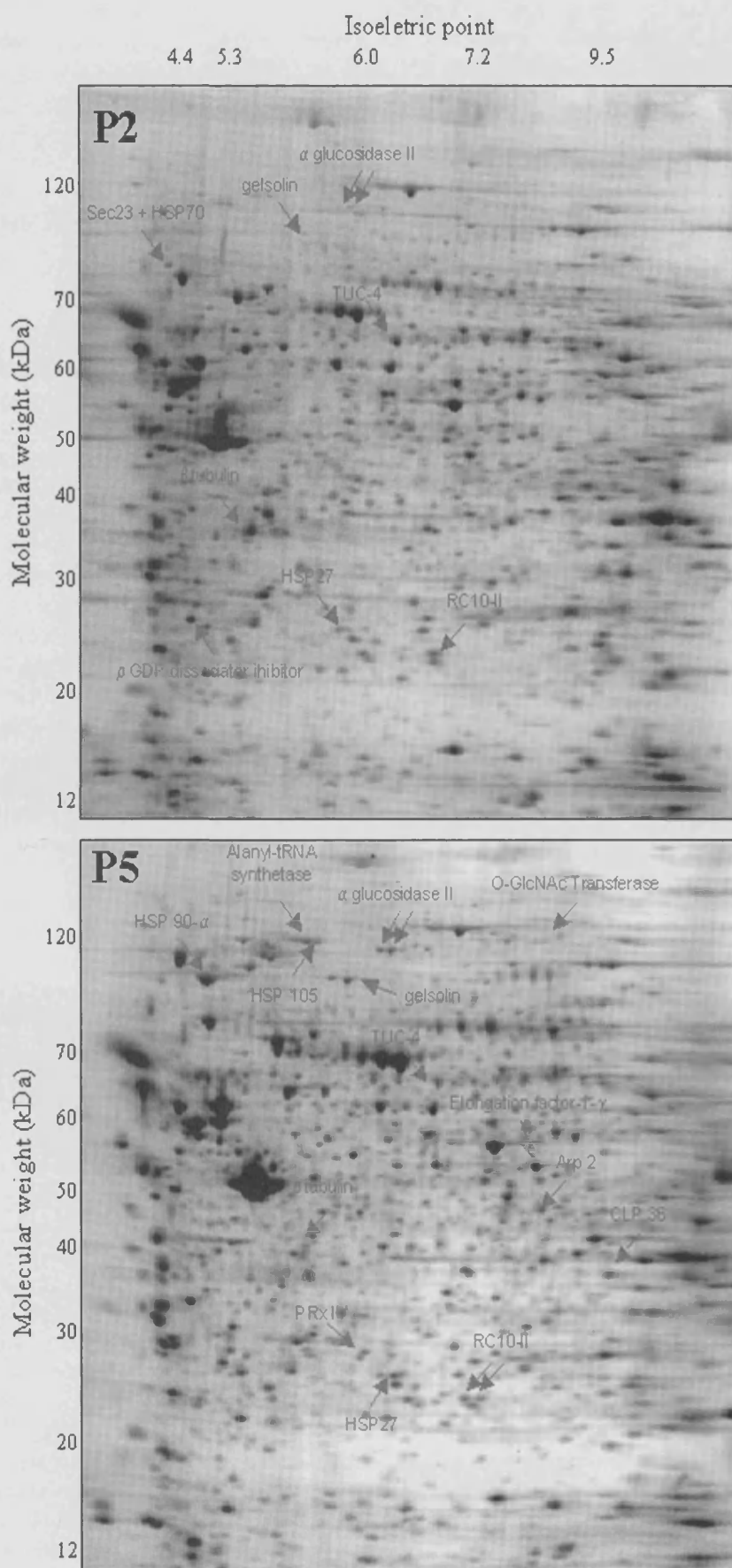


FIG. 1. A representative 2-D gel image of P2 and P5 cells obtained upon fractionation of 150  $\mu$ g of total cell extract.

TABLE I

Percent homology between the triplicate gels

Triplicate 2-D gels were run, curated, and compared.

	Reference gel	Gel II	Gel III
P2	100	98.6	97.1
P3	100	98.0	99.0
P4	100	95.0	93.0
P5	100	96.0	96.1

tion (shown in Table II, and presented in greater detail as Supplemental Material, Table II). Five spots yielded only keratin, and in four cases, more than 20 peptide ion signals were obtained, but database searches with these peptide mass lists did not identify any proteins. For the remaining six of the 49 differential spots, the mass spectra recorded exhibited not more than 10 peptides; these data were classified as insufficient for peptide mass mapping. From all identified proteins only 18 proteins were identified as rat proteins, nine were mouse, nine were human, and one was bovine. Interestingly, five proteins identified as non-rat proteins have rat homologues in the NCBI protein database suggesting either extensive nucleotide polymorphisms or incorrect database entries for the rat protein sequences.

**Protein Identification by ESI-MS/MS**—ESI-MS/MS analysis was performed in all cases where peptide mass mapping by MALDI-MS was unsuccessful or ambiguous. In addition, a few spots with sufficient MALDI-MS data for protein identification were analyzed by ESI-MS/MS to verify the identification by MALDI-MS peptide mass mapping. Almost two-thirds of the excised spots were analyzed by nano-HPLC ESI-MS/MS. The results for the protein identification by ESI-MS/MS (false positives excluded) are also summarized in Table II and presented in greater detail as Supplemental Material, Tables III and IV. From all proteins identified by searching the NCBI protein database only 15 proteins were identified as rat proteins, whereas three were from mouse, one was from chicken, one was from Chinese hamster, and 10 were human. MS/MS data from two spots identified human proteins with some peptides giving matches to the mouse and bovine homologues that did not match the human protein. Similarly, MS/MS data from one other spot resulted in peptide matches not matching the rat protein but rather the Chinese hamster homologue. The chicken myosin heavy chain identified in spot 190 was quite unusual, because a very close rat homologue exists in the NCBI protein database. However, the peptides for which the MS/MS data were obtained cover regions with little homology suggesting that the rat homologue remains to be identified.

Positive protein identifications obtained by MALDI-MS peptide mass mapping were confirmed in all cases where ESI-MS/MS was employed. All putatively identified proteins by MALDI-MS peptide mass mapping were also confirmed by ESI-MS/MS whenever sufficient data were obtained. In addition to these confirmations three more proteins were identified, t-complex polypeptide in sample 497, lamin A or C2 in

sample 504, and heat shock protein 27 in sample 1295. For five previously unidentified spots, positive protein identification was obtained. The comparison of the results from the MALDI-MS and ESI-MS/MS data demonstrated that both techniques show comparable sensitivity in protein identification. Furthermore, this comparison also showed that the criteria applied for protein identification were reliable and gave virtually no false identifications, with a minimal loss in analytical sensitivity.

### Description of Differentially Expressed Proteins

The differentially expressed proteins comprised a variety of cytoskeletal, heat shock, and metabolic proteins, as well as proteins involved in trafficking, differentiation, and protein synthesis, turnover, and modification. For the purposes of the discussion below, we have assumed that all proteins identified by mass spectrometry for each spot were expressed differentially. However this may clearly not be the case for spots that yielded multiple proteins; for these spots Western blot analysis using specific antibodies will be required to determine the identity of the differential protein.

**Cytoskeletal Proteins**—A number of the proteins identified, like gelsolin,  $\beta$ -tubulin, and Arp2 (actin-related protein 2), have been shown previously to be involved in cytoskeletal scaffolding. Most of these proteins were up-regulated during senescence; however  $\beta$ -tubulin was down-regulated. It is perhaps not surprising that proteins involved in the cytoskeleton show changes in expression upon replicative senescence, because senescent cells are very different morphologically from growing cells; they have the classic fried egg morphology of senescent cells. They are much larger, have lots of stress fibers, and overexpress many extracellular matrix components such as collagenases, collagen, stromelysin, and fibronectin.

Gelsolin is involved in nucleation of actin filaments and is responsible for regulating the growth rate of these filaments and therefore has a role in cell motility (19). It mediates the rapid remodeling of cortical actin filaments and has a role in stress fiber-dependent cell function (20). Gelsolin is also a caspase 3 substrate and is cleaved during Fas-mediated apoptosis (21) and gives rise to a fragment that can depolymerize actin filaments and either promote or inhibit apoptosis depending on the cell type (22).

Here we have found that gelsolin (spot 190) was up-regulated 4.8-fold upon replicative senescence; growing cells have low levels of gelsolin whereas post-mitotic senescent cells have high levels of gelsolin. This was consistent with previous observations that gelsolin was down-regulated in several types of transformed cells and tumors (23–25). The partial or total loss of gelsolin expression in a majority of breast cancers of diverse aetiologies has led to the proposal that gelsolin is a candidate tumor suppressor gene for breast cancer (26). Both gelsolin protein and its mRNA have been shown to be down-regulated in cancer cells, suggesting that

TABLE II  
Protein annotations

ID, identity number; F.C., -fold change; h, human; m, mouse; r, rat; c, chicken; b, bovine. \*, only contaminants such as human keratin were identified.

Up-regulated features				MALDI-MS spot identification	ESI spot identification
ID	MW	pI	F.C. P5 vs. P2		
102	125386	5.48	+3.2	Alanyl-tRNA synthetase (h)	
104	125386	6.37	+2.3		2-Oxoglutarate dehydrogenase precursor (h)
108	124240	6.53	>10	*	O-GlcNAc transferase p110 subunit (r)
110	123105	5.53	>10	105-kDa heat shock protein (m)	
122	114390	5.57	>10	$\alpha$ -Glucosidase II, $\alpha$ subunit (m)	
127	113345	5.82	>10	$\alpha$ -Glucosidase II, $\alpha$ subunit (m)	$\alpha$ -Glucosidase II, $\alpha$ subunit (m)
158	98305	5.01	+2.3		
177	933348	6.52	+2.2	Lysyl hydroxylase isoform 2 (m)	
185	91608	4.96	>10	Heat shock protein 90- $\alpha$ (h)	
190	91608	5.66	+4.8		Gelsolin (h and m) + myosin heavy chain (c)
223	83776	5.31	>10		
248	81062	7.08	>10		
290	74833	6.64	+2.0	Transferrin (b)	
369	67881	5.90	>10		
426	64712	6.19	+2.9	Vesicle transport-related protein (RA410) (r)	
493	60767	5.76	+2.0	2-Oxoglutarate dioxygenase $\gamma$ -butyrobetaine (r)	
495	60767	6.47	+3.3	Chaperonin-containing TCP-1, $\gamma$ -subunit (m) + TUC-4 (m)	
497	60462	6.06	+2.0	Seryl-tRNA synthetase (h)	Seryl-tRNA synthetase (h) + T complex protein 1, $\alpha$ (r)
504	60158	6.94	+2.2	MPAST1 (m)	MPAST (h) + lamin A or C (r)
677	47888	5.57	+3.0	ERF1 (h) + probable ATP-dependent RNA helicase p47 (r)	
713	45110	5.14	+3.0	Similar to cdc37 (r)	
773	45386	6.54	+3.1	Elongation factor-1- $\gamma$ (h)	Elongation factor-1- $\gamma$ (h)
902	40126	6.58	+2.0	Arp2 (h)	Arp2 (h) + hnRNP-E2 (h)
907	40010	5.71	+2.1	26 S proteasome subunit p40.5 (m)	26 S proteasome subunit p40.5 (h)
1075	47888	5.79	+2.0	Isopentenyl diphosphate dimethylallyl diphosphate isomerase (r)	
1076	33986	7.22	2.0	CLP36 (r)	
1093	23343	5.84	>10	Thiol-specific antioxidant (r)	
1095	33385	6.42	+2.0		
1124	32329	5.34	+2.0	*	
1264	25789	6.05	>10	*	
1302	24177	5.58	+3.0	Heat shock 27 protein (r)	
1319	23370	5.57	>10	*	
Down-regulated features				MALDI-MS spot identification	ESI spot identification
ID	MW	pI	F.C. P2 vs. P5		
15	158986	6.38	+2.0	*	
153	79874	4.76	+2.5	Sec23 protein (h) + ischemia-responsive 94-kDa protein (r)	Sec23 protein (h) + ischemia-responsive 94-kDa protein (h) + HSP70 (h)
278	64858	5.09	+3.2	Preimmunoglobulin heavy chain binding protein (r)	
304	63329	6.09	+2.4	TUC-2 (r)	
460	53263	7.39	+2.0	IMP dehydrogenase (m)	
736	39212	6.13	+2.6	30-kDa protein (h)	
863	34304	5.41	+4.7	Tubulin $\beta$ chain 15 (r)	
882	34172	5.57	+3.9	Transitional endoplasmic reticulum ATPase (r)	
1054	25301	5.01	+2.0	$\rho$ GDP dissociation inhibitor (b)	$\rho$ GDP dissociation inhibitor (h and b)
1239	14189	5.66	+2.2	Proteasome subunit RC10-II (r)	Proteasome subunit RC10-II (r) + PRx III (r)
1295	24425	5.87	+2.0	PRx IV (r)	
1299	24549	5.78	+2.1		
Features that shift in their migration			pI	MALDI-MS spot identification	ESI spot identification
ID	MW				
331	71199		6.67	Moesin (r)	
332	71199		6.56	Moesin (r) + guanosine 5'-monophosphate synthetase ? (h)	
1117	22014		6.19		
1348	22134		6.20	Proteasome subunit RC10-II (r)	Proteasome subunit RC10-II (r)
1351	22134		6.10		PRx III (r)



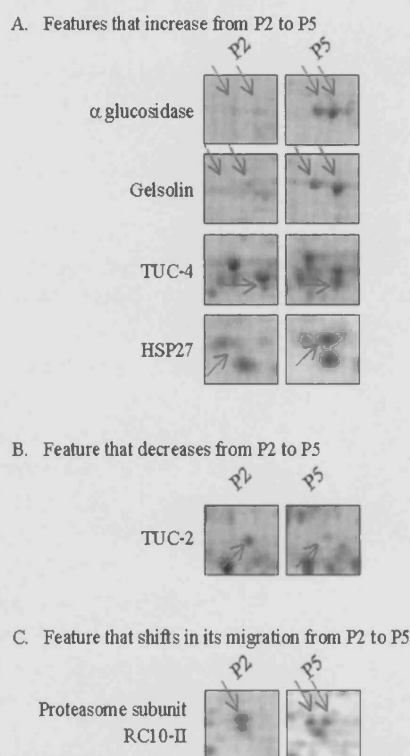


FIG. 2. 2-D gel images showing selected up-regulated features (panel A), down-regulated features (panel B), and a feature with an altered migration (panel C).

an alteration in the rate of transcription underlies the dysfunction of this gene (26). The decrease at the transcriptional level is most likely to be because of loss of p53, because it has been found recently that gelsolin is up-regulated transcriptionally by p53 (27).

Moesin, radixin, and ezrin, members of the ERM family of proteins, are general cross-linkers between cortical actin filaments and plasma membranes. They are involved in the formation of microvilli, cell adhesion sites, cleavage furrows, and membrane ruffling (28). It is interesting to note that moesin was identified as an up-regulated (spot 331), as well as a down-regulated (spot 332) protein, and thus represents a feature that shifts in its migration. Because these spots migrate at the same molecular weight but different pI, it suggests that it is not the level of the protein that changes but rather the modification. Because the feature that is up-regulated has a higher pI, it suggests that moesin is most likely undergoing phosphorylation upon senescence.

Spot 902 that was up-regulated 2-fold in senescent REFs was found to comprise Arp2 and hnRNP-E2. The Arp2/3 protein is ubiquitous and an essential component of the actin cytoskeleton in eukaryotic cells. It nucleates actin filaments, caps their pointed ends, and cross-links them into orthogonal networks (29, 30). Arp2/3 proteins are activated by the GTPase Cdc42, together with the WASP family of proteins (31). They are expressed differentially between human breast myoepithelial and luminal epithelial cells (15).

**Heat Shock Proteins**—Four HSPs were identified during this study. HSP105 (spot 110), HSP90α (spot 185), and HSP27 (spot 1302) were up-regulated, and HSP70, identified in spot 153, together with Sec23 and ischemia-responsive 94-kDa protein, was down-regulated. HSPs are a group of proteins that are highly conserved from bacteria to mammals and classified into different families according to their size as follows: HSP110/105, 90, 70, 60, 40, and 27. They were identified initially by virtue of their rapid induction under stress conditions and proposed to play a critical role in protection from hypothermia and other types of stress (32–34). However they are now known to play essential roles under normal physiological conditions such as assisting the folding of newly synthesized proteins, protein translocation across organelle membranes, promoting assembly or disassembly of oligomeric proteins, and facilitating protein degradation of incorrectly folded or denatured proteins. They have also been shown to localize to the centrosome, but the significance of this localization is not clear (35). The mammalian HSP110/105 may exist as two forms, α and β. Even though both forms are induced upon stress, the α form is also expressed constitutively (36). Both forms associate with HSP70 and have been proposed to regulate negatively its chaperone activity. They are also induced upon adipocyte differentiation (37). Our finding that HSP27 was up-regulated upon senescence is in accordance with the observation that overexpression of HSP27 in bovine pulmonary endothelial cells stimulates their growth rate and accelerates the rate at which the cultures reach senescence (38). HSP27 is also expressed differentially between human breast luminal and myoepithelial cells (15).

The finding that heat shock proteins were expressed differentially may be particularly relevant, because it has been proposed by some that replicative senescence in rodent cells may be caused by the stress of *in vitro* tissue culture (62, 63). It has been observed that increased stress such as freeze-thawing and growth at lower temperatures can shorten the finite proliferative life span (9). Increases in resistance to stress have been shown to increase chronological lifespan in *Drosophila melanogaster* and *Caenorhabditis elegans* by acting on post-mitotic cells rather than affecting replicative potential (64). Stress resistance could be increased by reducing expression of HSP90, which can down-regulate heat shock transcription factor HS1 (64). Induction of HSP70, the most abundant and most evolutionarily conserved, by stress is significantly lower in late passage senescent fibroblasts, probably because of reduced levels of HSF1 that are required for transcriptional up-regulation of HSP70 (65). Recently two mortalin genes (Mot1 and Mot2), members of the HSP70 family that are derived from two distinct genes, were identified. They encode proteins that differ by only two amino acids but exhibit different subcellular localizations and have contrasting activities. Mot1 induces senescence in NIH 3T3 cells whereas Mot2 results in transformation. Mot2 has also been shown to increase proliferative life span and block induction



of senescence-associated- $\beta$ -galactosidase activity in normal human diploid fibroblasts by interfering with p53 activity by blocking nuclear translocation of p53 and down-regulating p53-responsive genes (66, 67). Because up-regulation of HSP90 could account for down-regulation of HSP70 via HSF1, it suggests that it would be very important to determine what is the cause of the up-regulation of HSP90 and if it is because of stress of *in vitro* tissue culture, to determine the nature of this stress. It will also be interesting to determine whether HSP105/110 and 27 are also up-regulated by the same mechanism. One possible mechanism would be via a common transcription factor. It is also important to determine the exact form of HSP70 that we have found to be down-regulated and whether it corresponds to one of the mortalin proteins, particularly Mot2.

**Factors Affecting Protein Synthesis**—Two proteins, ERF1 (eukaryotic releasing factor 1, spot 677) and elongation factor-1- $\gamma$  (spot 773), that may play a role in protein synthesis, were up-regulated upon senescence. ERF has been implicated in translation termination in eukaryotes (39). Although its function in translation termination remains obscure, it has been shown to promote a stop codon and ribosome-dependent hydrolysis of aminoacyl-tRNAs (40) and thus catalyzes the termination of protein synthesis at all three stop codons. It has been conserved remarkably during evolution suggesting that it may have an essential role in the termination of translation (41). Elongation factor-1- $\gamma$ , a subunit of EF1, one of the G-proteins that mediate transport of aminoacyl-tRNA to the 80 S ribosomes during translation, has been found to be overexpressed in colorectal carcinomas and adenomas (42, 43). Therefore it was rather surprising that we found elongation factor-1- $\gamma$  be up-regulated upon replicative senescence.

**Proteins Involved in Differentiation**—Two differentially regulated spots were identified as proteins belonging to the TUC (TOAD-64/Ulip/CRMP) family of intracellular phosphoproteins implicated in axon guidance and outgrowth and thereby regulation of neuronal differentiation (44–46). The TUC family consists of four 64-kDa isoforms known as TUC-1 (CRMP-1/Ulip-3), TUC-2 (CRMP-2/Ulip-2), TUC-3 (CRMP-3/Ulip-4), and TUC-4 (CRMP-4/Ulip-1). In addition, TUC-4 $\beta$  has been identified recently as a 75-kDa variant of TUC-4.<sup>2</sup> The TUC proteins are up-regulated in the rat brain after embryonic day 12, a time that corresponds to the beginning of neurogenesis. TUC-4 is the most abundant member of this family and first detected in new post-mitotic neurons after neuronal birth (47), reaches peak expression levels during neurogenesis but is not expressed by neural progenitor cells. It is down-regulated in the adult brain but is re-expressed if axonal re-growth is triggered. It is also increased upon differentiation of PC12 cells by nerve growth factor and neuroblastoma cells by retinoic acid. In contrast to TUC-4, much less is known about the expression of the other TUC proteins. TUC-2 is expressed by

both neurons and their progenitors. It is also slightly up-regulated upon treatment of PC12 cells with nerve growth factor. Recently TUC-2 was found to be associated with the microtubule bundles at the mitotic spindle and proposed to be involved in regulating microtubule dynamics (48). The TUC proteins are also required for the growth cone collapsing activity of semaphorin-3A, an extracellular guidance cue for axonal outgrowth, suggesting a role in the signal transduction pathway initiated by extracellular stimuli. Their importance in neuronal differentiation is further suggested by their homology to *unc-33*, a *C. elegans* gene that is required for normal axon outgrowth and guidance. They are also highly conserved across species; the rat TUC-2 protein has 98% identity with its chicken ortholog and 89% identity with its *Xenopus laevis* ortholog. The proteins identified by the proteomic analysis correspond to TUC-2 in spot 304 and TUC-4 in spot 495 that also contained the  $\gamma$ -subunit of the TCP-1 chaperone (45). TUC-2 was down-regulated 2.4-fold whereas TUC-4 was up-regulated when REFs underwent senescence, in accordance with the idea that TUC-4 is an early marker of post-mitotic neurons. Interestingly TUC-2 showed an opposite regulation to TUC-4; it was down-regulated in the post-mitotic senescent cells.

**Protein Turnover, Synthesis, and Modification**—Some differential spots were identified as subunits of the proteasome complex, the 26 S proteasome subunit p40.5 and RC10-II. The level of subunit p40.5 (spot 907) increases upon senescence whereas RC10-II both decreases in level (spot 1239) and shifts in its migration (spot 1348). The shift in migration is most likely to be because of changes in post-translational modification such as phosphorylation. The p40.5 subunit is expressed differentially between human luminal and myoepithelial cells (15). In contrast, changes in the migration pattern of the RC10-II have not been observed previously. It would therefore be very interesting to determine the site of phosphorylation, the kinase responsible, and how it is regulated.

Other proteins that could potentially be considered to be members of this family of differentially expressed proteins are O-GlcNAc transferase p110 subunit (spot 108), seryl-tRNA synthetase (spot 497), alanyl-tRNA synthetase (spot 102), and an ATP-dependent RNA helicase p47 (spot 677). All these proteins were identified as up-regulated proteins. O-GlcNAc transferase is capable of glycosylating both serine and threonine residues on nuclear and cytosolic proteins. On several proteins the O-GlcNAc and O-phosphorylation occur on the same or adjacent sites, and thus it is possible that one function of the addition of O-GlcNAc is to block phosphorylation at this site (49). Therefore the O-GlcNAc modification may have an important role in control of intracellular signaling. It is highly conserved, dynamic, and inducible. Disruption of O-GlcNAc transferase in mouse embryonic stem cells is lethal, demonstrating the importance of this modification.

**Protein Trafficking**—SEC23 identified in the down-regulated spot 153 in senescent cells has been proposed to be involved

<sup>2</sup> C. C. Q., unpublished observations.

in protein trafficking. It is a component of COPII, a cytosolic complex that is responsible for the formation of vesicles within the endoplasmic reticulum and involved in the antero-grade transport of proteins to the Golgi (50, 51). In contrast to SEC23, transferrin (spot 290) and RA410, a vesicle transport-related protein (spot 426), both appear to be up-regulated upon senescence. None of these proteins have been found previously to be expressed differentially in senescence or tumorigenesis. Nevertheless, RA410 has been proposed to have a role in post-Golgi transport and participates in the ischemia-related stress response in astrocytes (52).

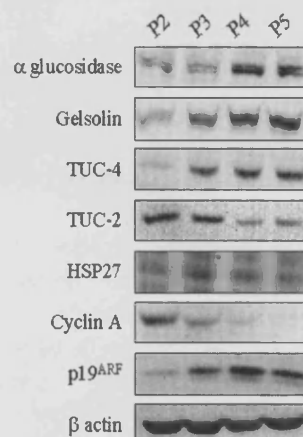
**Metabolic Proteins**—Two up-regulated spots (spots 122 and 127) that were identified to be the  $\alpha$ -subunit of the  $\alpha$ -glucosidase II complex were particularly interesting.  $\alpha$ -Glucosidase II is the soluble form of the enzyme and removes the  $\alpha$ -1,2-glucose and  $\alpha$ -1,3-glucose residues following transfer of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> to nascent polypeptides (53). The  $\alpha$ -glucosidases participate in glycoprotein folding mediated by calnexin and calreticulin by forming the monoglucosylated high mannose oligosaccharides required for interaction with chaperones. In addition to their role in *N*-glycan processing, these enzymes are involved intimately in quality control in the endoplasmic reticulum, a process that ensures proper folding of newly formed polypeptide chains leading to retention and/or degradation of incorrectly folded proteins (53). Interestingly it was shown recently that a 3'-untranslated region of an  $\alpha$ -glucosidase-related mRNA was able to promote colony formation and immortalization in REFs and cooperate with an immortalization-defective mutant of SV40 T antigen to immortalize REFs (54). Moreover, a gene named *klotho* (55) that shares sequence homology to the  $\beta$ -glucosidase family of enzymes and encodes a secreted protein that appears to function outside cells has been identified recently. A defect in its expression in mice results in a syndrome that resembles human aging (short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema) (55).

We also identified other differentially expressed metabolic proteins. Among these, 2-oxoglutarate dehydrogenase precursor (spot 104), 2-oxoglutarate dioxygenase  $\gamma$ -butyrobetaine (spot 493), and isopentenyl diphosphate dimethylallyl diphosphate isomerase (spot 1075) were up-regulated whereas GDP dissociation inhibitor of the Rho protein (spot 1054) was down-regulated. PRx IV (spot 1295), an enzyme with antioxidant function, was down-regulated whereas PRx III was found both to be down-regulated (spot 1239) and to undergo changes in mobility (spot 1351). Guanosine 5'-monophosphate synthetase (spot 332) was identified in a down-regulated spot.

#### Validation of the 2-D Proteome Analysis

To validate the 2-D proteome analysis, REFs were passaged serially again using the strict 3T3 passaging regime to prepare extracts corresponding to P2, P3, P4, and P5 and analyzed for expression by 1-D Western blotting. To ensure

#### A. Validation of 2D gels with one dimensional western blots



#### B. RT-PCR analysis of RNA expression

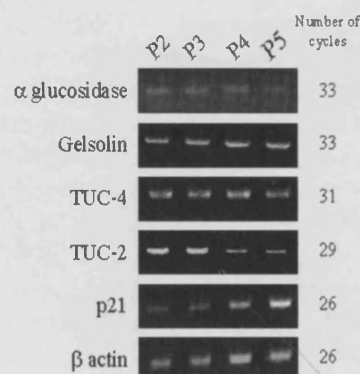
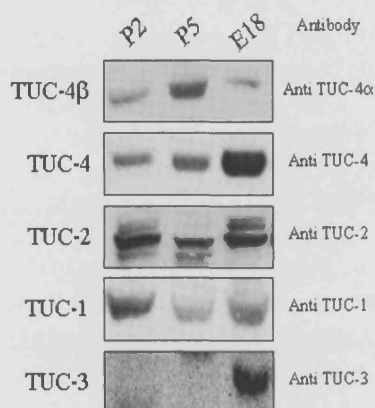


Fig. 3. A, 1-D Western blots using 30  $\mu$ g of total protein per sample.  $\alpha$ -Glucosidase, gelsolin, TUC-4, and TUC-2 show correlation between 2-D and 1-D gels. No correlation was found for HSP27. Cyclin A and p19<sup>ARF</sup>, known to be modulated upon replicative senescence, were used as a positive control;  $\beta$  actin was used to control for equal loading. B, RT-PCR analysis of differentially expressed candidate genes. A direct correlation between protein and mRNA levels was found for TUC-2 but not for  $\alpha$ -glucosidase, TUC-4, and gelsolin. p21<sup>WAF1/CIP1</sup>, a gene known to be up-regulated upon replicative senescence, was used as a positive control;  $\beta$  actin was used as control.

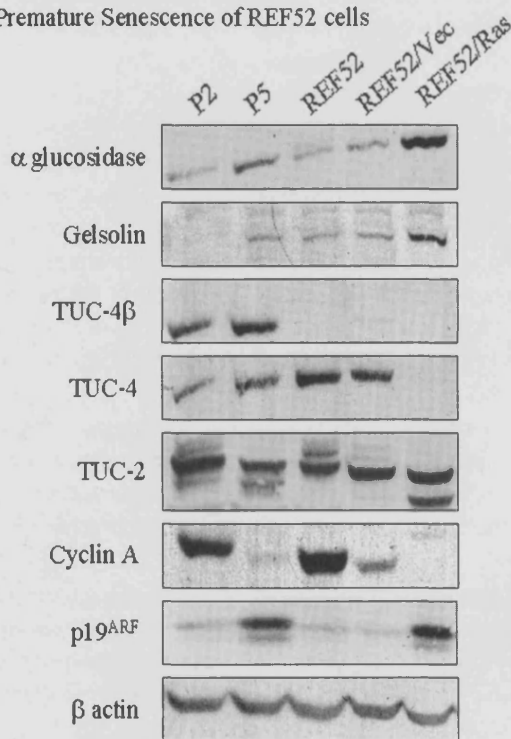
that the extracts exhibited changes in expression of proteins known to be involved in replicative senescence, extracts were first analyzed for expression of cyclin A and p19<sup>ARF</sup>. As expected cyclin A was down-regulated, and p19<sup>ARF</sup> was up-regulated as REFs underwent replicative senescence (Fig. 3A). Expression was then analyzed for some of the identified proteins for which antibodies were available.

Expression analysis of gelsolin,  $\alpha$ -glucosidase, and TUC-4 showed that they were up-regulated whereas TUC-2 was down-regulated in accordance with the proteome analysis (Fig. 3A). In contrast, even though HSP27 had been identified as an up-regulated feature, it was not found to be differential by 1-D Western blot analysis (see Fig. 2A and Fig. 3A). The most likely explanation for this is that HSP27 may have multiple isoforms and therefore be present as multiple spots on

## A. Protein expression analysis of the TUC family of proteins



## B. Premature Senescence of REF52 cells



## C. Phosphatase treatment of total extracts

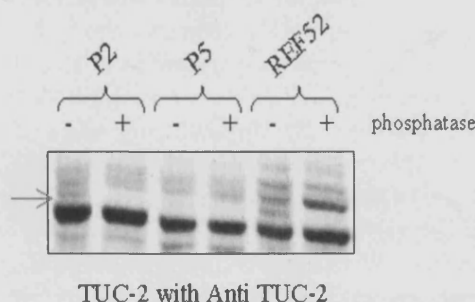


FIG. 4. A, protein expression of the TUC family of proteins was analyzed using specific antisera. An E18 rat brain extract was used as a positive control. B, protein expression in the REF model of the replicative senescence model was compared with the premature

the 2-D gels, and only one of these spots was differential. Thus the identity of the form of HSP27 that is expressed differentially upon replicative senescence remains to be determined. The 1-D Western blot experiments were repeated several times, and as expected each single experiment gave the same result. Further blotting experiments using specific antisera against the remainder of identified proteins are required to validate fully the differential proteome analysis.

We next undertook experiments to determine whether the changes at the protein level that were validated by 1-D Western blotting correlated with changes in mRNA by semiquantitative RT-PCR; the results are presented in Fig. 3B. Once again REFs were passaged serially using the strict 3T3 passaging regime and RNA extracted from cultures of P2, P3, P4, and P5 REFs. For these experiments p21<sup>WAF1/CIP1</sup>, known to be up-regulated upon replicative senescence (56), was used as a positive control. TUC-2 RNA decreased in accordance with the decrease in protein level. The level of TUC-4 and  $\alpha$ -glucosidase RNA did not change whereas gelsolin RNA showed a slight increase. These results are consistent with the studies in *S. cerevisiae* and human liver, which have suggested that mRNA levels may correlate poorly with corresponding protein levels (13, 14).

Because TUC-4 and TUC-2 are two members of the same family of proteins but showed opposite regulation at the protein level, we analyzed expression of other members of this family. The results presented in Fig. 4A show that TUC-4 $\beta$ , a variant of TUC-4, was up-regulated, and TUC-1 was down-regulated, whereas TUC-3 was not present in the REFs. TUC-4 $\beta$  has been found previously to only be expressed in neural cells and cell lines of neural origin such as PC12, N1E-115, and neuro-2a cells. Interestingly both TUC-4 and TUC-4 $\beta$  were detected in all three of these cell lines, as well as in brain and REF extracts. The significance of the changes in expression of this family of proteins is not yet understood. Neither is it clear what is their function and whether they have overlapping functions. Nevertheless it is highly intriguing that the different family members show divergent but identical regulation in both neural differentiation and in fibroblasts upon replicative senescence.

We next examined whether some of the changes observed in replicative senescence also occur in oncogene-induced premature senescence, another commonly used system for studying senescence. In this model, REF52 cells, from an immortal rat cell line, are induced to senescence prematurely by ectopic expression of the activated Ha-Ras oncogene (57).

senescence model (49) in which REF52 are induced to undergo senescence upon ectopic expression of activated Ras. Cyclin A and p19<sup>ARF</sup>, known to be expressed differentially in both model systems, were used as a positive control. A direct correlation between the two models was found for  $\alpha$ -glucosidase, gelsolin, and TUC-2. C, 20  $\mu$ g of total cell lysates per sample were treated with Lambda protein phosphatase (BioLabs) prior to Western blotting.



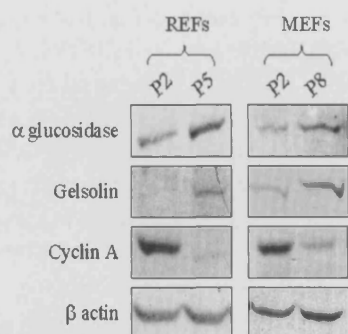


FIG. 5. Protein expression in P2 and P5 REFs was compared with P2 and P8 mouse embryo fibroblasts (MEFs). Cyclin A expression was analyzed as a positive control.

The results in Fig. 4B show that as observed previously, cyclin A was down-regulated whereas p19<sup>ARF</sup> was up-regulated upon premature senescence. In accordance with replicative senescence, both  $\alpha$ -glucosidase and gelsolin were up-regulated whereas TUC-2 was down-regulated upon premature senescence. In addition to the major TUC-2 band, minor bands that migrated more slowly were detected in P2 REFs and growing REF52 cells whereas they were absent in senescent REFs or prematurely senescent REF52 cells, but a more prominent faster migrating band was observed. Phosphatase treatment of extracts before Western blotting indicated that at least one of the slower migrating bands observed in the dividing REFs, as well as REF52 cells, but not in the growth-arrested cells was due to phosphorylation (Fig. 4C). TUC-4 $\beta$  splice variant was not detected in REF52 cells, and rather surprisingly TUC-4 showed the opposite regulation upon premature senescence. These experiments have been repeated several times, and each experiment yielded the same result. Gelsolin and  $\alpha$ -glucosidase were also found to be up-regulated when mouse embryo fibroblasts were passaged serially *in vitro*, and cultures ceased dividing and underwent replicative senescence (Fig. 5). Unfortunately these were the only two candidates for which the antibodies were capable of detecting the mouse protein. As more mouse specific antibodies become available it will be very interesting to validate the differential proteome analysis in the mouse system.

#### SUMMARY

Here we have presented a 2-D differential proteome analysis of replicative senescence in serially passaged rat embryo fibroblasts. Triplicate independent 2-D gels containing over 1200 spots each were run, curated, and analyzed. This revealed 49 spots, whose expression was altered more than 2-fold. Mass spectrometry yielded positive protein identification for 39 of these spots, comprising a variety of cytoskeletal, heat shock, and metabolic proteins, as well as proteins involved in trafficking, differentiation, and protein synthesis, turnover, and modification. Because proteomic studies do identify occasionally false positives, we validated this study by 1-D Western blotting using antisera where available. We

next showed that some of the candidates were also expressed differentially in two other models of senescence, Ras-induced premature senescence of REF52 cells and replicative senescence of mouse embryo fibroblasts.

2-D gels have been used previously to compare the patterns of protein expression between normal and transformed cell lines. In studies where 400–1000 proteins were compared, it was reported that transformation caused a significant change in expression for 10–30% of the proteins (58, 59). In addition a number of marker proteins such as the tropomyosin family of cytoskeletal proteins and the proliferation sensitive nuclear antigen for this process were identified (58, 60). These studies were extended by Garrels and Franzen (58, 59) to generate a 2-D gel database for REF52 cells under a variety of growth states such as quiescence, rapidly dividing, and upon transformation by DNA and RNA tumor viruses. Moreover for a number of these 2-D spots, they determined the identity, the subcellular localization, and the post-translational modifications. Replicative senescence has not been analyzed previously by 2-D gels, and our study is one of the first such studies. In contrast to transformation, where 10–30% of the spots were differential, we found that only 49 spots of ~1200 were altered more than 2-fold (~4%). Because we have used triplicate curation to maximize reproducibility and eliminate false positives, it is possible that 4% differentials might be a slight underestimate for the proportion of differential spots, but it is considerably lower than the 10–30% differentials found in the REF52 studies. None of the differential spots that we have identified correspond to those identified previously by Garrels and Franzen (58). This is perhaps not surprising, because REF52 cells are an immortal rat cell line, and the aim of our experiments was to identify changes that are the cause of the senescent phenotype, and these changes should not occur in REF52 cells when the immortal state is unaffected such as in quiescence, rapid cell division, and transformation.

Even though the proteins that we have identified have not been directly associated with replicative senescence previously, some of them, for example gelsolin,  $\alpha$ -glucosidase, TUC, and heat shock proteins, have been linked to cell proliferation. Gelsolin has been proposed to be a tumor suppressor for breast cancer. A 3'-untranslated region of an  $\alpha$ -glucosidase-related mRNA has been shown to be able to promote colony formation and immortalization in REFs and cooperate with an immortalization-defective mutant of SV40 T antigen to immortalize REFs (54). The TUC proteins are critical for neuronal differentiation, and it has been proposed by some that replicative senescence reflects a state of terminal differentiation (61) and that senescent fibroblasts are the equivalent of terminally differentiated end stage cells. However, the mechanisms by which these proteins modulate cell growth are unknown.

The finding that TUC genes are expressed differentially upon replicative senescence suggests another family of proteins that may be involved in this process. Treatment of neu-

roblastoma cells with retinoic acid results in differential expression of the same TUC genes that we have observed. This treatment also affects expression of the p73 family of proteins, and p73 knock-out mice have a neuronal differentiation defect. p73 proteins are one of the two recently discovered members of the family of p53 related genes. We have already shown that p63 proteins, derived from the other member of this p53 family, are expressed differentially in replicative senescence and proposed that they may have a role in modulating p53 activity during replicative senescence (68). Moreover because the TUC-2 gene is down-regulated at the level of mRNA, it will be very interesting to determine whether this occurs at the level of transcription or stability and to determine the cause.

Therefore further experiments are necessary to determine whether any of the differentially expressed proteins, either singly or in combination, have a role in replicative senescence. The differentially expressed proteins also represent important starting points for determining the activities critical for the observed changes in expression whether they occur at the level of mRNA or protein or by post-translational modification and whether they are involved in replicative senescence of human cells. They may provide new markers for cancer diagnosis and therapeutics.

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