Plasticity of the Differentiated State in Adult Newt Cardiomyocytes

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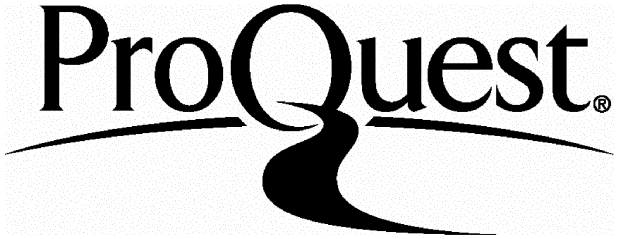

Abstract

The repair of cardiac lesions in adult mammals is limited to compensatory hypertrophy and fibrosis, because cardiomyocytes withdraw from the cell cycle soon after birth. In contrast, adult amphibian ventricular cardiomyocytes, such as those from the newt, proliferate locally after direct injury to the ventricle. This is paralleled by other newt differentiated cell types, which contribute to the regeneration of structures such as limbs and lens, by cell cycle re-entry and reversal of differentiation.

I have established and characterised a system for primary culture of adult newt ventricular cardiomyocytes. The plasticity of the adult newt cardiomyocyte, defined here as the ability to enter into S phase and divide, was maintained in these cultures. Foetal bovine serum promoted newt cardiomyocyte entry into S phase.

To address the cellular regulation of newt cardiomyocyte plasticity, I quantitatively characterised the proliferative potential of these cells at the single cell level by lineage tracing and time-lapse video microscopy. These experiments showed that 75% of adult newt ventricular cardiomyocytes enter S phase, 60% of the total undergo mitosis and approximately half of these undergo cytokinesis, during the first 18 days in culture. This ability to divide is clearly distinct from that of adult mammalian ventricular cardiomyocytes, which have rarely been observed to undergo mitosis, and never to undergo cytokinesis.

Other experiments showed that inactivation of pRb-family proteins is an endpoint of the serum stimulation pathway in newt cardiomyocytes. These results suggest that regulation of these proteins is important for the plasticity of urodele differentiated cells.

to my family

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NEB	Nuclear envelope breakdown
p15	p15INK4b
p16	p16INK4a
p18	p18INK4c
p19	p19INK4d
p21	p21 CIP
p27	p27KIP
p57	p57KIP
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PEC	Pigmented epithelial cell
PH	Partial hepatectomy
pRb	Retinoblastoma protein
Px	Post-natal day x
RNA	Ribonucleic acid
SM	Smooth muscle
T3	triiodothyronine
TGF	Transforming growth factor
TH	Thyroid hormone
TNF-α	Tumour necrosis factor alpha
TSP1	Thrombospondin-1
uPA	Urokinase-type plasminogen activator
VSMCs	Vascular smooth muscle cells
WE	Wound epidermis

List of acronyms

A1 cells	Myogenic cell line derived from a newt ankle explant
Ab	Antibody
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
APC	Anaphase promoting complex
app	Approximately
ATP	Adenosine triphosphate
BMP	Bone morphogenic proteins
BrdU	5-bromo-2-deoxyuridine
C	Haploid DNA content
C/EBP	CCAAT/ enhancer-binding protein
CDI	Cyclin dependent kinase inhibitor
CDK	Cyclin dependent kinase
d	days
DNA	Deoxyribonucleic acid
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GFP	Green Fluorescence protein
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
MMP	Matrix metaloprotease
MPF	Mitosis promoting factor
MyBP	Myosin binding protein
MyHC	Myosin heavy chain

-Chapter 1-

INTRODUCTION

1.1 INTRODUCTION

During the embryonic development of most multicellular organisms, cells become progressively committed to specific differentiated lineages. This is a consequence of the interaction of cell intrinsic factors and the extracellular signals to which each cell is exposed during its ontogenetic history. Once differentiated, cells are resistant to respecification and in many cases lose the ability to proliferate. This stability of the differentiated state is very important to maintain the complex architecture of tissues. Most tissues are an intricate mixture of cell types that must remain different from one another while coexisting in the same microenvironment.

The stability of the differentiated state impinges on the regenerative capabilities of many tissues. Tissues that require constant renewal, such as the immune system, epithelia and skeletal muscle, maintain a pool of less differentiated cells (somatic stem cells or progenitors) as they develop. These cells can divide and differentiate, and are responsible for replacing cells lost by normal turnover or injury (Stocum, 1998). Many other tissues do not have progenitor cells capable of repair; in most of these cases the integrity of the tissue is restored with a fibrous scar. This is the case in cardiac muscle. The substitution of the muscle tissue by a scar after injury leads to loss of contractile performance and ultimately failure of the heart (Stocum, 1998; Sun and Weber, 2000; Weber et al., 1995).

How is the stability of the differentiated state achieved? Since the 1950s, several nuclear transplantation experiments in amphibians have shown that cellular differentiation is not necessarily accompanied by inactivation or loss of genes (Briggs and King, 1952; Briggs and King, 1960; DiBerardino, 1988; Gurdon, 1999). For example, a nucleus derived from a post-mitotic red blood cell from

juvenile frogs could generate feeding tadpoles after transplantation into an activated oocyte (DiBerardino, 1988; DiBerardino et al., 1986). The capacity to give rise to several types of cells was also observed using the same experimental approach with nuclei from other types of differentiated cells, such as keratinocytes or myotomal myofibres (Gurdon, 1999; Gurdon et al., 1984; Gurdon et al., 1975). More recently, adult sheep, pig and mice were derived from adult somatic cell nuclei transplanted to enucleated oocytes (Campbell, 1999; Polejaeva et al., 2000; Wakayama et al., 1998; Wilmut et al., 1997). A variety of factors may be important in nuclear reprogramming since the efficiency of these nuclear transplantation experiments (assessed as the proportion of eggs or oocytes that develop to term) is still very low (lower than 10%, and in most cases approximately 1-2%; Colman and Kind, 2000; Okada, 1991).

In the experiments described above, nuclear reprogramming was achieved in the context of normal development. Could the reactivation of dormant genes depend upon exposure of the nuclei to a sequence of cues that accompanies the progression from undifferentiated zygote to specialized tissue? Experiments with somatic cell hybrids or heterokaryons (two distinct somatic cells fused to form a single cell that contains nuclei from both parental cells) suggested that the differentiated state of a cell could be altered without recourse to the regulatory hierarchy characteristic of development (Blau and Blakely, 1999; Kikyo and Wolffe, 2000). For example, fusion of muscle cells with human amniotic fibroblasts induced muscle gene expression in the latter (Blau et al., 1983). The fact that adult nuclei can be reprogrammed in a different cellular environment, in an oocyte or a heterokaryon, has shown that cell differentiation is not intrinsically irreversible, at least at the nuclear level.

The results discussed above contrast with the stability of the differentiated state as generally observed in animal cells. Can the stability of the differentiated state be challenged by changes in the extracellular environment, without

experimental manipulation of the cytoplasm? The potential to reverse the differentiated state and re-enter the cell cycle and/or differentiate according to necessity should provide the animal with the ability to repair tissues. This is the case for smooth muscle and liver, where injury triggers the proliferation of smooth muscle cells and hepatocytes. In these cases, changes in the extracellular matrix or exposure to certain growth factors seem to play an important role in destabilising the differentiated state (reviewed in: Fausto, 2000; Michalopoulos and DeFrances, 1997; Sanz-Gonzalez et al., 2000). On the other hand, there may exist intrinsic differences in the regulation and plasticity of the differentiated state among different cell types. Quite strikingly, the same tissue can display a different regulation of the differentiated state depending on phylogeny. Several cases of tissues that display an increased plasticity are found in urodele amphibians, as compared to their mammalian counterparts. Urodele amphibians are able to regenerate various parts of their body, such as limbs, tail, lens, and sections of the heart (Brockes, 1997; Goss, 1969). The recruitment of differentiated cells by reversal of differentiation is thought to be an important mechanism to generate progenitor cells for regeneration (Brockes, 1997; Brockes, 1998a). One tissue that displays increased plasticity in urodele amphibians is the heart muscle.

In contrast to their mammalian counterparts, adult amphibian ventricular cardiac muscle cells (cardiomyocytes), such as those from the newt and the frog, have been shown to proliferate locally, after direct injury to the ventricle (Becker et al., 1974; Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995; Rumyantsev, 1973; Rumyantsev, 1991). This response of newt cardiomyocytes provides us with a framework in which to study how the plasticity of the differentiated state is regulated, and is also a cellular model enabling comparative analysis of the different regenerative ability of urodeles and mammals.

In order to address the problem of the plasticity of the differentiated state in adult newt cardiomyocytes, I begin by reviewing the theme of cellular plasticity

and what is understood about its cellular and molecular basis. I then describe our current knowledge on the establishment and stability of the differentiated state in the cardiomyocyte. Finally, the evidence for plasticity in the amphibian cardiomyocyte is presented and the focus for this thesis is discussed.

1.2 Regeneration And Plasticity of The Differentiated State

1.2.1 Introduction

There are two main aspects of the stability of the differentiated state in adult tissues: withdrawal from the cell cycle and maintenance of the differentiated phenotype. As such, several scenarios of flexibility/plasticity of the differentiated state can be envisaged: the cell can go back into the cell cycle, called cell cycle re-entry from the differentiated state; the cell can lose characteristics of its phenotype, here called dedifferentiation; the cell, which has already expressed specific differentiated traits, switches into another cell type with different phenotypic characteristics, called transdifferentiation (Okada, 1991).

Examples of plasticity of the differentiated state in adult organisms have long been recognised and studied. Pathological cases resulting from deregulation of the stability of the differentiated state are common. One example is heterotopic ossification in muscle, an ectopic bone growth that develops as a consequence of direct muscle injury (DiMaio and Francis, 2001; Okada, 1991). The regeneration field shows a variety of scenarios of cellular plasticity, including dedifferentiation, transdifferentiation and cell cycle re-entry from the differentiated state. Transdifferentiation of iris-pigmented epithelium in lens regeneration, was first experimentally investigated by G. Wolff, at the end of the nineteenth century (Okada, 1991; Wolff, 1895).

The ability to regenerate large sections of the body plan, after transection or amputation is widely distributed among invertebrates (Goss, 1969; Thouveny and Tassava, 1998). Some species are even able to regenerate in a bidirectional fashion; after transection, both fragments regenerate the missing structures, reconstituting the whole organism (Fig. 1.2.1). One extreme example of regenerative abilities, extensively studied by Morgan 100 years ago, is the planarian, able to regenerate an entire organism from a mere 1/279th of its body

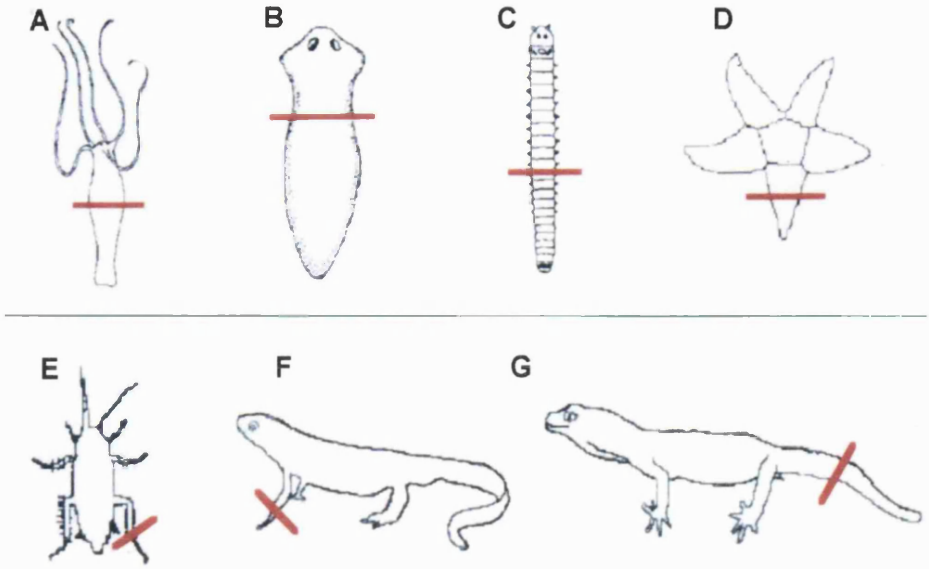

Figure 1.2.1 Regeneration is widespread among metazoans. (A through D) Bidirectional regeneration. These animals can respond to transection by regeneration from both fragments. (A) *Hydra*, a cnidarian. (B) *Dugesia*, a planarian worm. (C) *Nereis*, an annelid worm. (D) *Linckia*, an echinoderm. (E through G) Unidirectional regeneration of appendages. (E) Cockroach limb. (F) Newt limb. (G) Lizard tail. The amputation or transection plane is represented by a red line. From Brockes (1997).

(Alvarado, 2000; Goss, 1969). One of the striking aspects of bi-directional regeneration in invertebrates is that it is indistinguishable from their asexual mode of reproduction (budding in hydra, and fission in planarians, for example) (Alvarado, 2000; Brockes et al., 2001). Regeneration in these animals might therefore have co-opted asexual reproductive mechanisms (Alvarado, 2000; Brockes et al., 2001).

The ability to regenerate missing parts is more limited in vertebrates, and among these, urodele amphibians (which retain the tail after metamorphosis) are exceptional in their regenerative capabilities. The distribution of regenerative capacities in phylogeny is a mystery: on the one hand, almost every phylum possesses one or several species capable of regenerating missing body parts; on the other, closely related species are found which have very different regenerative abilities (Brockes et al., 2001).

In the search for unitary mechanisms of regeneration, it has to be taken into account that regeneration is a combination of complex phenomena, such as wound healing, formation of progenitor cells and morphogenesis. The last clearly involves convergence with ontogeny, since there is reactivation of developmental programs, and there is extensive evidence about unitary mechanisms of development throughout phylogeny (reviewed in: Alvarado, 2000; Brockes, 1997; Brockes et al., 2001). The search for unitary mechanisms for the generation of progenitor cells is a more complex task. There are two distinct modes of regeneration in what refers to the origin of the cells: reorganisation of the cells into the new tissues in the absence of cell division (called morphallaxis), and regeneration involving proliferation of cells (Alvarado, 2000; Goss, 1969). Proliferating cells may be recruited by mobilisation of a reserve population, by reversal of differentiation of cells at the site of injury, or by both mechanisms. The contribution of neoblasts to planarian regeneration is an example of the importance of stem cells (Agata and Watanabe, 1999; Newmark and Sanchez Alvarado, 2000), while urodele regeneration appears to depend largely or entirely on local reversals of differentiation (Brockes, 1997). Regeneration may proceed in the presence or absence of a blastema, a mesenchymal growth zone containing the proliferating, undifferentiated cells. Liver regeneration in mammals, and lens and heart regeneration in urodeles, are examples of non-blastemal regeneration. Blastema-based regeneration is found among planarians, molluscs, echinoderms, urochordates, and limb and tail regeneration in vertebrates (Alvarado, 2000). A discussion and further references on the problem of the evolution of regenerative mechanisms can be found in Alvarado (2000), Brockes et al. (2001), Goss (1969) and Goss (1992).

In the following pages, I will focus on examples of cellular plasticity during regeneration and the signals that may be involved in triggering the plastic response. These particular examples were chosen either because they have

contributed significantly to our current knowledge in this field, or because they have direct relevance to the plasticity of the adult newt cardiomyocyte.

1.2.2 Liver Regeneration

The mammalian liver has an amazing regenerative capacity. It is able to regenerate after several types of injury such as partial amputation or the use of hepatic toxins. After partial hepatectomy (PH), an experimental protocol that involves removal of intact liver lobes totalling two-thirds of the liver mass, the residual lobes enlarge to make up for the missing mass. The whole process lasts 5 to 7 days in the rat. This process of liver regeneration can be repeated several times in experimental animals (Michalopoulos and DeFrances, 1997; Thorgeirsson, 1996).

The size of the regenerated liver seems to be regulated by the size of the organism; upon transplantation of livers from large animals into smaller animals and vice-versa, the size of the liver gradually decreases or increases until the size of the organ becomes proportional to the new body size (Kawasaki et al., 1992; Starzl et al., 1993).

New hepatocytes arise through two different mechanisms: proliferation of differentiated cells or recruitment of stem-like cells. After PH, liver regeneration is carried out by proliferation of all the existing mature cellular populations composing the intact organ, including hepatocytes (Michalopoulos and DeFrances, 1997). If however, hepatocytes are prevented from proliferating, by administration of hepatocyte-toxic substances in the diet, progenitor cells, called oval cells, are activated and can differentiate into several cell lineages, including hepatocytes (Thorgeirsson, 1996). It is currently thought that oval cells may originate from stem cells present in the liver and in the bone marrow (Petersen et al., 1999a; Sigal et al., 1992; Thorgeirsson, 1996). I will focus hereafter on the plasticity of the differentiated state of hepatocytes.

1.2.2.1 Hepatocyte Differentiation and Polyploidization

Early foetal hepatocytes or hepatoblasts differentiate from the endoderm. It is thought that the mesoderm supports continued growth and proliferation of the hepatic endoderm through the action of factors such as hepatocyte growth factor (HGF; Carlson, 1999; Thorgeirsson, 1996). Hepatoblasts are initially progenitors for both adult hepatocytes and bile epithelial cells, but by embryonic day (E) 15 in the rat most hepatoblasts are committed to the hepatocyte lineage (Brill et al., 1995; Sigal et al., 1995). As the liver develops, hepatocytes acquire the capacity to perform the biochemical functions of the liver, such as production of the plasma protein albumin, synthesis and storage of glycogen, synthesis of urea from nitrogenous metabolites and production of bile through the breakdown of hemoglobin (Brill et al., 1995; Carlson, 1999; Sigal et al., 1995).

Polyploidization is a characteristic feature of the post-natal growth of liver in mammals, although the extent of polyploidization can vary through a large range in different species (Table 1.2.1; Brodsky and Uryvaeva, 1977; Gerlyng et al., 1993; Brodsky and Uryvaeva, 1985). Polyploidization results in an increase in cell mass, without a corresponding increase in the number of cells (Brodsky and Uryvaeva, 1977). Cells with a 2C DNA complement (C represents an haploid DNA content) may undergo acytokinetic mitosis generating binucleate cells, with diploid nuclei (2Cx 2). These in turn generate 4C cells, which replicate to give rise to 4Cx2 cells (Fig. 1.2.2; Brodsky and Uryvaeva, 1977; Carriere, 1967; Carriere, 1969; Nadal and Zajdela, 1966; Brodsky and Uryvaeva, 1985). The existence of other minor mechanisms of polyploidization should not be excluded (Gupta, 2000). After polyploidization, liver cells also undergo extensive protein synthesis, so that their mass increases without cell division (Brodsky and Delone, 1990). This process is called cellular hypertrophy.

Table 1.2.1 Variation in the ploidy of hepatocytes (%) in different mammalian species. 2C (C represents haploid DNA content), mononucleate cell with diploid nucleus; 2Cx 2 (the number after x represents the number of nuclei in the cell), binucleate cell with diploid nuclei. Adapted from Brodsky and Uryvaeva (1985). See for original references Brodsky and Uryvaeva (1985).

Order	Species	2C	2cx2	4C	4Cx2	8C	8Cx2
Rodentia	*Rattus rattus*	6-17	13-27	53-75	1-2	-	-
Carnivora	*Mus musculus*	0.1-4	10-30	25-40	30-45	3-9	2-5
	Mustela vison	78-96	4-21	0-1			
Primates	*Homo sapiens*	83-91	6-13	1-7	0-1	0-0.2	
	Gorilla gorilla	81.3	12.2	5.6	0.6	0.3	

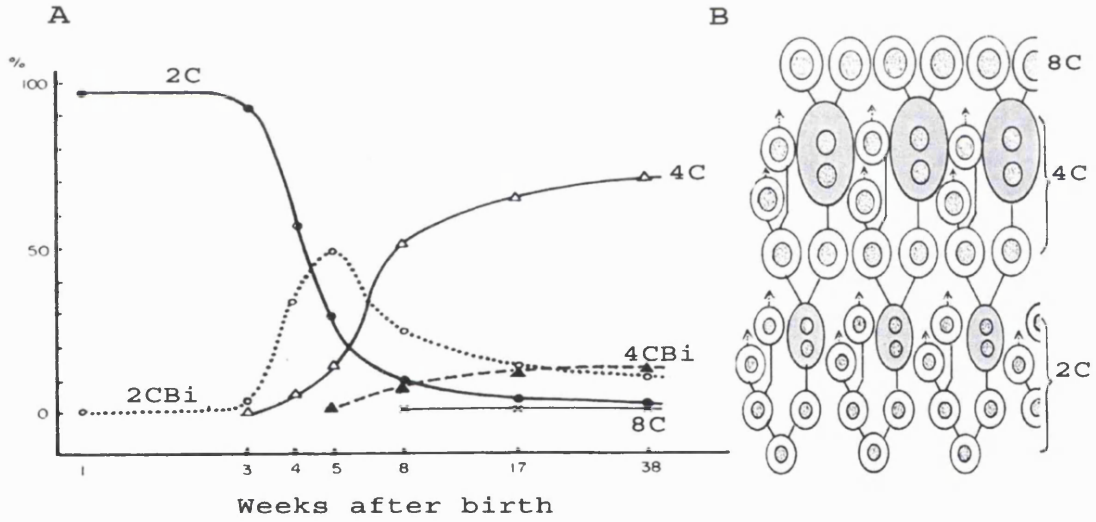

Figure 1.2.2 Formation of polyploid hepatocytes in rats. (A) Appearance of the different populations of hepatocytes in rats after birth. Bi-indicates binucleate cell. (B) Model of polyploidization of hepatocytes. The numbers on the right indicate the ploidy of nuclei within each cell. Adapted from Nadal (1966).

Changes in gene expression and cellular morphology may also occur after birth, including an increase in granularity, autofluorescence, and number of lysosomes (Brill et al., 1995; Sigal et al., 1995). DNA synthesis is practically

undetectable in hepatocytes of adult healthy animals (Brill et al., 1995; Sigal et al., 1995).

1.2.2.2 Reactivation Of Cell Division In Differentiated Hepatocytes

After PH, most rat hepatocytes start synthesizing DNA at 12-13 hr post-operation, irrespective of cellular ploidy (Brodsky and Uryvaeva, 1977; Gerlyng et al., 1993). Most of the hepatocytes (95% in young and 75% in very old rats) in the residual lobes participate in one or two proliferative events (Gerlyng et al., 1993; Michalopoulos and DeFrances, 1997; Brodsky and Uryvaeva, 1985). This is achieved while simultaneously performing all essential functions for homeostasis, such as glucose regulation, synthesis of many blood proteins, secretion of bile and biodegradation of toxic compounds (Michalopoulos and DeFrances, 1997).

Recent studies *in vivo* and *in vitro* have shown that the hepatocyte has a very large clonogenic potential. Hepatic transplantation experiments showed that hepatocytes can undergo more than 10 cell doublings (Fausto, 2000; Overturf et al., 1999; Rhim et al., 1994; Rhim et al., 1995). When hepatocytes are stimulated to proliferate in culture, they also undergo several rounds of division (Block et al., 1996). Interestingly, under these conditions, there might be some dedifferentiation of the cells, with the loss of the expression of hepatocyte-specific genes, such as albumin and cytochrome P450 IIB1 (Block et al., 1996). In the presence of the adequate extracellular matrix (ECM), these cells can re-differentiate to form mature hepatocytes (Block et al., 1996). It has been suggested that proliferation of adult hepatocytes may involve cell division of diploid hepatocytes without polyploidization and further polyploidization of the polyploid cells (Gerlyng et al., 1993).

1.2.2.3 Factors Involved in Triggering Hepatocyte Proliferation in Liver Regeneration

Several extracellular factors are important for hepatocyte proliferation during regeneration and in culture, such as transforming growth factor-alpha (TGF-α), HGF, epidermal growth factor (EGF), norepinephrine, the thyroid hormone triiodothyronine (T_3), and insulin (Block et al., 1996; Fausto, 2000; Michalopoulos and DeFrances, 1997). Cytokines such as tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) seem to be necessary for priming the hepatocytes before they can fully respond to the growth factors (Fausto, 2000). DNA synthesis in liver regeneration was found to be suppressed in knock-out mice for IL-6 (Cressman et al., 1996) and for TNF- α receptor I (Yamada et al., 1997).

Additionally, proteolysis of the extracellular matrix is important in the regulation of hepatocyte proliferation. After PH, there is increased proteolysis of some components of the hepatic extracellular matrix, (Michalopoulos and DeFrances, 1997). The urokinase-type plasminogen activator (uPA) receptor is translocated to the plasma membrane in the hepatocyte and urokinase activity increases within 1-5 minutes of hepatectomy (Mars et al., 1995). uPA is involved in activation of a proteolytic cascade involving conversion of plasminogen to plasmin, which will activate matrix-degrading metalloproteases (Blasi, 1993). Consistent with an important role for the extracellular matrix, the proliferation of hepatocytes, after injection of HGF or TGF-α in the circulation, is dramatically increased if the injection is preceded by infusion of a small amount of collagenase (which alone has no effect; Liu et al., 1994).

One of the important consequences of matrix degradation might be the release of HGF since the matrix contains large amounts of HGF. In addition, uPA may be important for the conversion of inactive, matrix-binding, single-chain HGF to its active, receptor-binding, two-chain form (Michalopoulos and DeFrances, 1997).

1.2.3 Smooth Muscle Regeneration

Vascular smooth muscle cells (VSMC) are responsible for modulating the arterial tone. They arrest in the G0/G1 phase of the cell cycle during postnatal development and differentiate, expressing a set of genes encoding myofibrillar and cytoskeletal smooth-muscle-proteins (Parmacek, 2001). After vessel injury, VSMCs are able to increase smooth muscle mass through cellular proliferation, cellular hypertrophy and production of extracellular matrix proteins. Although this plasticity is important to repair vessel injuries, the overgrowth of VSMCs is at the origin of pathological lesions, such as atherosclerosis (Parmacek, 2001; Sanz-Gonzalez et al., 2000).

1.2.3.1 Differentiation of Smooth Muscle

VSMCs arise from two distinct embryological origins: lateral mesenchyme (mesoderm) and neural crest (ectoderm). Recently, it was proposed that they can also arise from endothelial cells and bone marrow mesenchymal stem cells (Conway et al., 2001). Embryonic VSMCs proliferate and express low levels of smooth muscle (SM) specific markers, such as smooth muscle myosin heavy chain (SM-MyHC) (Parmacek, 2001). In the early neonatal period, VSMC proliferation decreases and vessel wall growth continues through VSMC hypertrophy, and production of extracellular matrix (ECM). Vessel wall growth slows down at approximately 3 months of age in the mouse (Parmacek, 2001).

1.2.3.2 Plasticity of the Differentiated State in Smooth Muscle Cells

VSMCs are able to modulate their phenotype during postnatal development. Based on *in vitro* and *in vivo* studies, these cells can be classified into two extreme phenotypes: a "contractile phenotype" vs. a "synthetic phenotype". The "contractile" cell is the one observed in healthy adult vessels. The "synthetic phenotype" is associated with vessel wall lesions (Li et al., 1999). A synthetic VSMC is similar to embryonic VSMCs at the level of proliferation, migration,

myofibrillar content, and ECM production (Kenneth Walsh, 1999; Parmacek, 2001). It is still unclear whether VSMC populations are heterogeneous, therefore whether this phenotypic conversion may be more typical of less differentiated VSMCs (Holifield et al., 1996; Li et al., 1999).

1.2.3.3 Factors Involved in Triggering the Phenotypic Conversion

Uninjured vessels display very low proliferation of VSMCs. Vessel injury may result in denudation and inflammation of the endothelium. The cells involved in the inflammatory response, such as subendothelial T cells and macrophages, secrete mitogens. Within hours, VSMCs in the vicinity of the lesion re-enter the cell cycle and convert to a synthetic phenotype (Kenneth Walsh, 1999).

An interesting characteristic of VSMC plasticity is that the growth response depends on the stimuli. In cultured rat aortic VSMCs, Angiotensin II (Ang II) and thrombin induce cellular hypertrophy, whereas platelet derived growth factor-BB (PDGF-BB) can cause a strong hyperplastic response (Servant et al., 2000). The molecular regulation of this response will be further discussed (1.3.3).

The extracellular matrix seems to play a role in the regulation of VSMC proliferation. For example, matrix-degrading metalloproteases (MMP) expression is induced within atherosclerotic plaques (Sanz-Gonzalez et al., 2000). Moreover, MMP inhibitors repressed VSMC proliferation *in vitro* and after angioplasty *in vivo* (Sanz-Gonzalez et al., 2000). Changes in collagen content have also been documented in different animal models of atherosclerosis (Sanz-Gonzalez et al., 2000). Additionally, mitogen-stimulated VSMCs proliferate in culture dishes coated with monomeric collagen that may mimic the ECM within atherosclerotic plaques, but are arrested in G1 when grown on polymerised collagen, which may resemble a normal artery (Koyama et al., 1996). Another component of the extracellular matrix that may be important in triggering the response is thrombospondin 1 (TSP1). TSP-1 is synthesized and secreted by activated platelets and a variety of cell types including endothelial cells, macrophages, fibroblasts

and VSMCs. TSP1 promotes VSMC proliferation and migration (Majack et al., 1986; Sanz-Gonzalez et al., 2000).

1.2.4 Plasticity of the Differentiated State in the Jellyfish *Podocoryne carnea*

1.2.4.1 Transdifferentiation of Skeletal Muscle into Smooth Muscle and Nerve Cells

The jellyfish *Podocoryne carnea* is an interesting example of the role of cell-substrate interactions in maintaining the stability of the differentiated state. Jellyfish are marine medusoid forms composed of a swimming organ (the bell), a feeding organ (or manubrium) and tentacles, where gametogenesis and fertilization occur. The bell of the medusa is composed of three tissue layers; the exumbrellar tissue, the subumbrellar plate endoderm and the innermost layer, which is composed of mononucleate-striated muscle cells that are not cycling. The tissue layers are separated by ECM (mesoglea), which gives the animal its consistency (Schmid, 1992; Schmid et al., 1999).

The striated muscle layer can be isolated in small patches and cultured, maintaining the association between muscle and the ECM. In this case, there is stable maintenance of the striated phenotype and of the post-mitotic state. However, if the tissue is isolated with protease treatment, disrupting the muscle-ECM association, the muscle cells shrink and form aggregates. After 24 hours, DNA synthesis is detected in the isolated muscle. Two days after isolation, the striated muscle gives rise to smooth muscle cells, and four to five days later, it gives rise to nerve cells. These smooth muscle cells are similar to the ones in the manubrium, and can be identified by flagella formation and antigenic markers. The nerve cells show flagella, and stain positive for FMRF-amide, typical of cells from the nervous system. The smooth muscle cells continue to divide and give rise to more muscle cells and nerve cells, but the nerve cells do not divide further (Alder and Schmid, 1987; Schmid, 1992; Schmid and Alder, 1984). Amazingly, a whole new manubrium can occasionally be regenerated in vitro from the striated

muscle cells. In these cases, striated muscle cells transdifferentiate into several non-muscle types (Schmid, 1992).

The evidence for the occurrence of transdifferentiation from striated muscle to smooth muscle and nerve cells in these experiments was indisputable, since the initial striated muscle population was very homogeneous and the new smooth muscle and nerve cells had phenotypic remnants from the parental cell, such as bundles of striated myofibrils (Schmid and Alder, 1984). It is interesting that the phenotypic conversion to smooth muscle cells was independent of DNA synthesis, whilst the generation of nerve cells was completely prevented by inhibition of DNA synthesis (Schmid, 1992; Schmid and Alder, 1984).

1.2.4.2 Factors Involved in Destabilising the Differentiated State

Several experiments have shown the importance of the extracellular matrix and cellular shape in the maintenance of a stable differentiated state in striated muscle cells from the jellyfish. If the muscle is isolated without enzyme treatment, and grafted on cell-free ECM (Fig. 1.2.3 (a)), 60-80% of the cells migrate from their native matrix to the cell free one (Fig. 1.2.3 (d)). DNA synthesis and transdifferentiation are mostly inhibited in the peripheral portions of the grafted matrix, where the cells are more stretched. The percentage of cells undergoing DNA synthesis and transdifferentiation is highly increased in the contact zone between the two matrices, called the gap zone. Cells in this zone are not stretched. In this region, proteolytic activity degrades both types of ECM underneath the cells. If the isolated muscle is grafted onto ECM, but not stretched, then the muscle cells leave their native ECM and cover the grafted one, forming floating grafts. Both matrices are gradually degraded by the cells, and after three days, there is DNA replication and transdifferentiation of the skeletal muscle cells into nerve cells (Fig. 1.2.3 (b) and (c); Schmid et al., 1992).

If the muscle is isolated with enzyme treatment and grafted on top of large ECM that is permanently stretched, DNA synthesis and transdifferentiation are

inhibited in comparison to muscle that was not grafted (Schmid et al., 1992). If cells are grafted after being activated, between the first and second cell cycles, subsequent cell cycles are reduced but the cells can still transdifferentiate into neurons (Schmid et al., 1992).

These results have shown that only stretched extracellular matrix can inhibit DNA synthesis. Stretched ECM did not inhibit transdifferentiation of the skeletal muscle cells into neurons (which requires DNA synthesis). These experiments established the important role of cell configuration and the ECM in maintaining the stability of the differentiated state in the skeletal muscle cells from the jellyfish.

The role of the ECM and cell shape in the regulation of the differentiated state was further investigated. First, oxidation of the carbohydrates of the ECM with sodium-metaperiodate and the use of a monoclonal antibody that recognises a carbohydrate epitope in the medusa ECM, abolished cell-matrix adhesion of grafted isolates (Reber-Muller et al., 1994; Schmid, 1992; Schmid et al., 1999). These experiments showed that carbohydrate mediated cell-ECM interactions are necessary for cell adhesion and spreading, and consequently for the stability of the differentiated state. Second, according to the hypothesis that the shape of the cells is important for the stability of the differentiated state, that stability can be challenged by drugs that act upon the cytoskeleton. The events of transdifferentiation in mechanically isolated muscle could be triggered by activators of protein kinase C, as well as by disassembly of actin filaments induced by dihydrocytochalasin B (Schmid, 1992; Schmid and Reber-Muller, 1995).

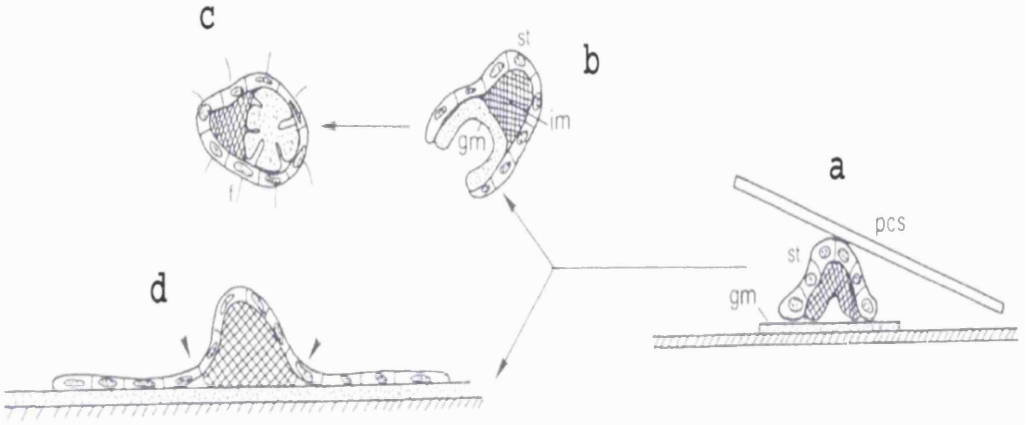

Figure 1.2.3 Schematic drawing summarizing the combination of isolated ECMs with isolated striated muscle tissue from *Podocoryne carnea*. Isolated striated muscle tissue was grafted onto cell-free ECMs, isolated from polyps and medusae, with the help of a piece of coverslip (a). For floating cultures, the ECMs together with the adherent and spreading tissue, were removed from the coverslip (b, c); cultures with permanent stretched ECMs remained unchanged (d). Arrowheads mark the position of gap cells. Stippled areas-grafted ECMs from medusae or polyp; hatched areas-inner ECM to which the muscle tissue adheres; Pcs-pieces of broken glass; gm-grafted ECM from polyp or medusae; st-striated muscle; im-inner ECM; f- flagellum. See text for explanations. Adapted from Schmid et al. (1992).

1.2.5 Plasticity of the Differentiated State in Urodele Cells

As first described by Spallanzani in his *Prodromo* (1768), urodele amphibians, such as the newt and axolotl, are remarkable in their regenerative abilities, both as larvae and adults. Newts can regenerate their limbs, tails, jaws, lens, iris, and retina, as well as various internal organs, including the central nervous system and large sections of the heart (Fig. 1.2.4; Brockes, 1997; Eguchi, 1998; Ghosh et al., 1994; Oberpriller et al., 1995).

A critical aspect of the regenerative ability is reflected in the origin of the precursor cells. Urodeles can effect local reversals in the differentiated state of tissues in response to amputation or tissue removal. For example, in both lens and limb regeneration, differentiated post-mitotic cells of the iris epithelium or the

Figure 1.2.4 Adult newts can regenerate several parts of their body, such as limbs, tail, lens, jaws and large portions of internal organs, such as the heart.

limb mesenchyme are able to re-enter the cell cycle and lose their differentiated character. Such dedifferentiation is reversible, in that after several rounds of division, the ocular precursor cells or limb blastema cells arrest and undergo differentiation into lens or limb mesenchyme respectively.

1.2.5.1 Lens Regeneration in Newts

After removal of a newt lens, regeneration proceeds from the iris pigmented epithelial cells (PEC) existent in the dorsal margin of the iris. These cells enter the cell cycle and dedifferentiate, losing their pigment granules. Some of the dedifferentiated cells subsequently transdifferentiate into lens, while others reconstruct the local architecture of the iris epithelium. Interestingly, the new lens is never observed to arise from the ventral iris (Eguchi, 1998; Kodama and Eguchi, 1995). The conversion of newt iris PECs into lens cells was established by critical experiments with clonal cell culture of pigmented cells, when at least 15% of the clonal colonies examined underwent definitive lens differentiation (Eguchi et al., 1974).

Newts are the only adult vertebrates that are able to regenerate the lens. In contrast, the ability of cultured PECs of iris or retina to dedifferentiate and transdifferentiate into lens cells is widespread in culture, under appropriate

conditions. Chick and even human PECs transdifferentiate to form lens cells in culture in the presence of phenylthiourea, which inhibits melanogenesis, and fibroblast growth factor two (FGF-2), which promotes proliferation and transdifferentiation (Hyuga et al., 1993; Itoh and Eguchi, 1986; Kodama and Eguchi, 1995; Yasuda et al., 1978).

Although FGF-1 and its receptors, FGFR-2 and FGFR-3 might be expressed in newts during lens regeneration (Del Rio-Tsonis et al., 1997), no difference was found when comparing dorsal to ventral iris. A direct role for this factor in lens regeneration has yet to be shown. Results from Dr. Yutaka Imokawa in the laboratory show that thrombin proteolytic activity is localised to the dorsal iris after ablation of the lens (unpublished results). This will be further discussed below in the light of the plasticity of skeletal muscle cells in the newt.

The ECM might also have an important role in transdifferentiation of PECs, since collagen represses the transdifferentiation of retinal PECs from chick embryos (Eguchi, 1998; Yasuda et al., 1978).

1.2.5.2 Limb Regeneration in Newts

Regeneration of the jaws and appendages is an epimorphic phenomenon proceeding by local formation of a blastema at the plane of amputation (Goss, 1969; Wallace, 1981). The events of limb regeneration in the adult newt are shown in the series of photographs of Figure 1.2.5. After amputation of a urodele limb, the wound surface is covered within 12 h by epithelial cells that migrate from the edge of the dermis, forming the wound epidermis (WE; Repesh and Oberpriller, 1978; Repesh and Oberpriller, 1980; Schmidt, 1968). The blastema is formed beneath the WE. The blastemal cells proliferate to produce a conical mound of cells, then progressively exit from the cell cycle and differentiate into the cartilage, connective tissue, and muscle of the regenerate in a proximal (shoulder) to distal (hand) direction. Amazingly, the blastema gives rise to the structure appropriate for its site of origin- a wrist blastema gives rise to a hand, whereas a shoulder

blastema makes an entire arm (Fig. 1.2.5). The blastema has considerable morphogenetic autonomy, giving rise to the appropriate structure, even after transplantation into neutral territories, such as the ocular globe and the dorsal fin (Stocum, 1968; Stocum, 1984). It is possible that after amputation at a particular proximo-distal location, cells arise with the appropriate level-specific identity and then generate more distal identities by successive local interactions (for a discussion and further references on positional identity see Brockes, 1997).

1.2.5.2.1 Origin of the Progenitor Cells During Regeneration

The critical processes for blastema formation are dedifferentiation of stump tissues and proliferation of the dedifferentiated cells. Blastemal cells arise locally, within 1-2 mm of the plane of amputation (Wallace, 1981). Although early anatomical descriptions of regeneration are consistent with dedifferentiation of mesenchymal tissues in the limb (Chalkley, 1954; Hay, 1959), direct evidence for dedifferentiation of local tissues has come from implantation studies.

In an early example, ^{3}H-thymidine-labelled cartilage was implanted into normal blastemas; careful analysis established the purity of the donor population as differentiated chondrocytes (Steen, 1968). These gave rise to labelled cartilage, bone and connective tissue, but not to muscle. Other studies have underlined the importance of the contribution to the blastema from dermal fibroblasts; it was proposed that 43% of the limb blastema cells derive from dermis (Gardiner et al., 1986; Muneoka et al., 1986).

Muscle is a particularly interesting case because the myofibre is a prototypical example of a differentiated cell. The transition from mononucleate myoblasts to a tube-like syncitium (the myotube) after fusion is clear cytological evidence of differentiation. Additional evidence for terminal differentiation is the post-mitotic arrest of the myotube (Okazaki and Holtzer, 1966). A newt myogenic cell line (termed A1) was isolated from an explant of ankle level mesenchyme

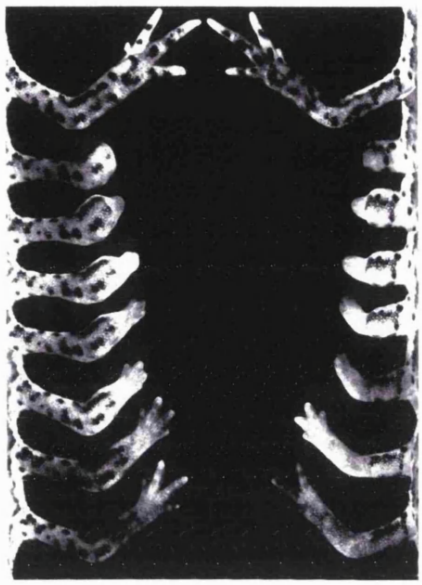

Figure 1.2.5 Regeneration of the forelimb in a red-spotted newt (Notophtalmus viridescens) after amputation at distal (mid-radius and ulna;shown at left) or proximal (mid-humerus; shown at right) sites. The original limb is shown at the top and the regenerated limb at the bottom of the image. The photographs were taken at 7, 21, 25, 28, 32, 42, and 70 days after amputation. Note that the blastema gives rise to structures distal to its level of origin. From (Goss, 1969).

(Ferretti and Brockes, 1988). When the serum concentration in the medium is lowered to 0.5% for several days, these mononucleate cells fuse to form contractile multinucleate myotubes expressing sarcomeric myosin heavy chain, and the muscle specific antigen 12/101 (Lo et al., 1993; Tanaka et al., 1997). The myotubes also enter a state of post mitotic arrest, such that they are completely refractory to several mitogenic growth factors, for example PDGF, which act on the mononucleate A1 cells (Lo et al., 1993; Tanaka et al., 1999; Tanaka et al., 1997).

After differentiation in culture, A1 myotubes labelled in different ways (lineage tracer; expression of a reporter gene, or an integrated retrovirus marker) can be implanted into regenerating limb blastemas. A direct contribution of cells of muscle origin to the limb blastema is observed, as mononucleate labelled cells are recovered after one week (Kumar et al., 2000; Lo et al., 1993; Velloso et al., 2000). *In vivo* labelling of single resident muscle fibres in the tail blastema of the axolotl larva also shows that these cells can dedifferentiate after amputation,

giving rise to mononucleate cells (Echeverri et al., 2001). Furthermore, approximately 16% of the blastemal cells are estimated to be derived from muscle in that context (Echeverri et al., 2001).

One striking feature of myofibre nuclei close to the amputation plane is that they re-enter the cell cycle and synthesise DNA (Hay, 1959; Kumar et al., 2000). It seems, therefore, that both indices of myogenic differentiation - the change in cytology upon fusion, and the post-mitotic arrest - are reversed after amputation. It is not clear whether S-phase entry prior to mononucleate cell formation is a normal step of the dedifferentiation process since mononucleate formation can occur when DNA synthesis is blocked (Velloso et al., 2000).

1.2.5.2.2 Regulatory Events in Dedifferentiation, Cell Cycle Re-entry and Proliferation

Which signals could be triggering dedifferentiation and cell cycle entry? The identities of the signals that provoke re-entry and reversal after amputation are not yet known. Complete amputation is not a prerequisite to initiate these events in urodeles. For example, flank wounds evoke the expression of several markers of blastemal cells, and in conjunction with nerve deflection can lead to formation of a limb (Egar, 1988; Gordon and Brockes, 1988). Because the myotube is a prototypical example of a differentiated cell, new data on the regulatory events of plasticity has been produced by the study of these cells *in vitro* and *in vivo*.

Although newt myotubes dedifferentiate *in vivo*, they do not seem to dedifferentiate *in vitro* (Tanaka et al., 1997; C. Velloso, personal communication). In contrast to their mammalian counterparts, cultured newt myotubes re-enter the cell cycle in response to foetal bovine or calf serum (FBS, FCS; Fig. 1.2.6; Tanaka et al., 1997). Newt myotubes must lose contact inhibition in order to enter the cell cycle. This has led to a model (Fig. 1.2.6) where there are two classes of factors: a stimulatory soluble activity that is present in serum; and a dominant inhibitory

factor on the surface of other cells, resulting in contact inhibition of DNA synthesis.

A dual-signal system might also operate to regulate dedifferentiation of muscle cells *in vivo*. The labelling of a single myofibre in the larval axolotl tail has allowed the study of factors that promote dedifferentiation of muscle (Fig. 1.2.6; Echeverri et al., 2001). Damage to the muscle fibre is required but not sufficient to induce dedifferentiation. The signal that induces dedifferentiation is only produced after severe tissue damage, even though complete amputation is not necessary, suggesting that, as observed for DNA synthesis *in vitro*, at least two signals are required to initiate the process of dedifferentiation (Fig. 1.2.6). These signals are direct cell injury and tissue damage (Echeverri et al., 2001). It is an attractive hypothesis that fibre damage *in vivo* relieves the inhibition of cell–cell contact, thus allowing muscle fibres and other differentiated cells at the amputation plane to respond locally to soluble serum factors released by tissue damage.

Could the signals for dedifferentiation and cell cycle re-entry be the same? The identity of the signal for dedifferentiation is unknown. Several studies have addressed the identity of the factor that induces cell cycle re-entry of myotubes *in vitro*. What could be the signal in FCS and FBS that induces cell cycle re-entry? As referred to previously, the cultured newt myotubes are refractory to mitogenic growth factors from a variety of families, such as PDGF, EGF, or FGF, which are active on their mononucleate precursors. This aspect of the post mitotic arrest is intact and is equivalent to that in mammalian myotubes (Tanaka et al., 1999; Tanaka et al., 1997).

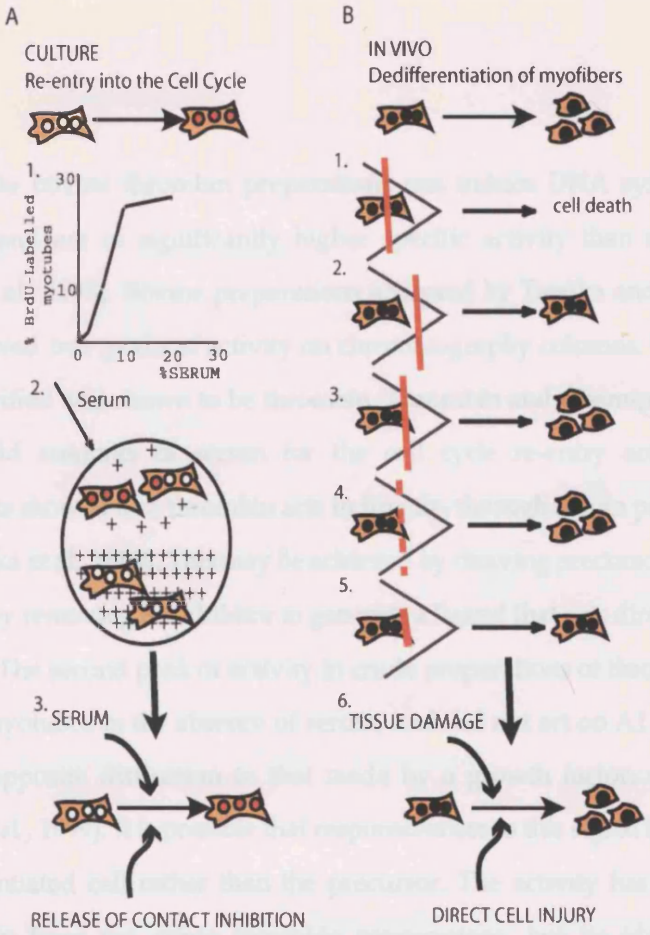

Figure 1.2.6 Signal model for the plasticity of newt skeletal muscle cells. (A) Plasticity of the differentiated state of newt skeletal myotubes in culture. 1. Newt myotubes re-enter the cell cycle in response to serum. 2. Myotubes plated in the same plate, surrounded by different amounts of mononucleate cells ("+" represents these cells) have been shown to respond differently to serum. 3. Model - Re-entry into the cell cycle depends on the presence of serum (nuclei in S phase represented in red) and on the release of contact inhibition. (B) Plasticity of the differentiated state of skeletal myofibres *in vivo*. The cone represents the tip of the tail. Single myofibres were injected with a lineage tracer in the tail of larval axolotls. The reaction of the myofibres to specific conditions of amputation was analysed. 1. Amputation results in severe damage to the fibre; the fibre retracts and disappears. 2. Amputation is close to the fibre, but does not damage it. There is no dedifferentiation. 3. Amputation results in gentle clipping of the fibre. In this situation, mononucleate cells are generated in 25% of cases. 4. The tail is cut halfway through, leaving the distal portion attached. A blastema-like structure forms and the parts heal back together. 40% of the myofibres dedifferentiate. 5. No dedifferentiation is observed if there is clipping of the end of the myofibres with only a small tear in the tissue. 6. These experiments suggest that at least two signals are necessary to initiate the process of dedifferentiation: direct cell injury and tissue damage. See text for references.

Crude bovine thrombin preparations can induce DNA synthesis by the myotubes and are of significantly higher specific activity than serum sources (Tanaka et al., 1999). Bovine preparations analysed by Tanaka and collaborators (1999) showed two peaks of activity on chromatography columns. One peak was further purified and shown to be thrombin. Thrombin and plasmin required low, subthreshold amounts of serum for the cell cycle re-entry activity. Further experiments showed that thrombin acts indirectly, through serum proteolysis (Fig. 1.2.7; Tanaka et al., 1999). This may be achieved by cleaving precursor protein (s) in serum, or by removing an inhibitor to generate a ligand that acts directly on the A1 myotubes. The second peak of activity in crude preparations of thrombin acted on the newt myotubes in the absence of serum, and did not act on A1 mononucleate cells, the opposite distinction to that made by a growth factor, such as PDGF (Tanaka et al., 1999). It is possible that responsiveness to this signal is a property of the differentiated cell rather than the precursor. The activity has been purified significantly from the crude thrombin preparations, but its identity remains unknown. It is striking that cultured mammalian C2C12 myotubes are completely refractory to this activity, thus providing a possible clue to the restriction of regenerative ability in mammals.

This mechanism for activating cell cycle re-entry by a post mitotic cell may be relevant for the initiation of regeneration. When frozen sections of a regenerating newt limb blastema were overlaid with a membrane impregnated with a fluorogenic thrombin substrate, a local maximum of thrombin activity was readily detected, in the mesenchyme underlying the WE. This activity was only detected in the first three weeks after amputation, a time where the blastemal cells are known to arise (Tanaka et al., 1999). Results from Dr. Yutaka Imokawa in the laboratory

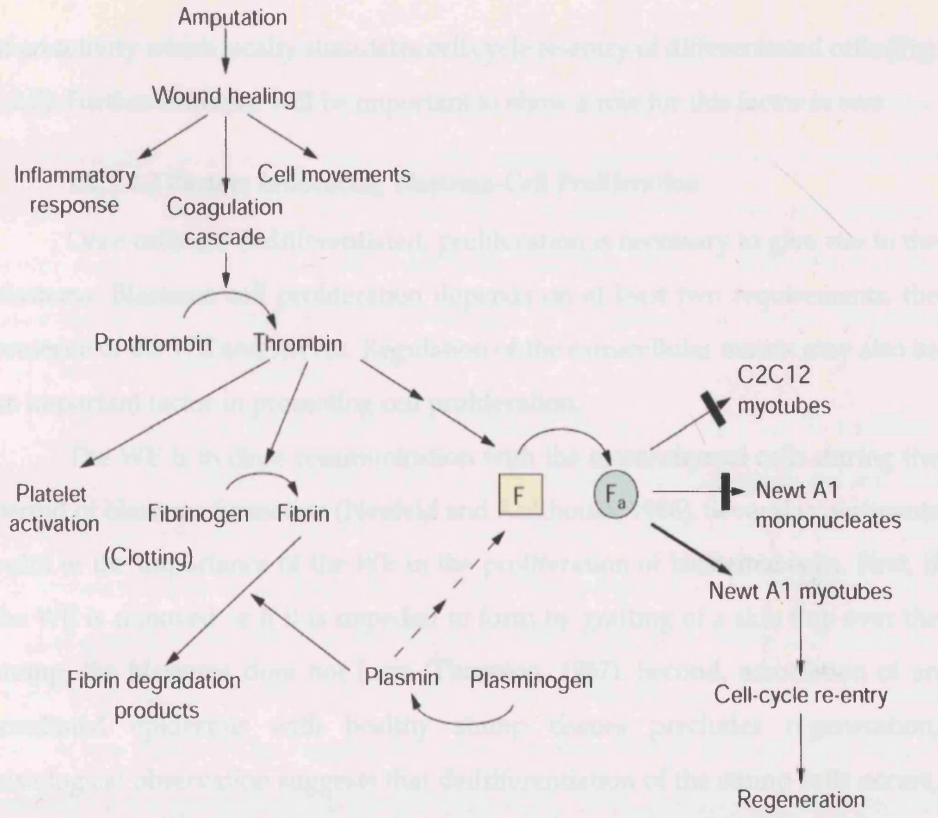

Figure 1.2.7 Schematic diagram of the activation of S phase re-entry by newt myotubes in the context of the wound-healing responses that lead to regeneration. Amputation of the limb triggers multiple responses such as inflammation and cell migration. A major aspect of wound healing is activation of the coagulation cascade, resulting in the conversion of prothrombin to thrombin. In mammals, this induces the conversion of fibrinogen to fibrin polymers and formation of a clot. In newts, thrombin activation, in addition, leads to cell cycle re-entry from the differentiated state. This involves the conversion of a latent activity (F to Fa) within serum which can selectively stimulate newt myotubes, but not their mononucleate precursors, to undergo S phase. Mouse myotubes of the myogenic cell line C2C12 were refractory to this activity. From Tanaka et al. (1999).

suggest that thrombin proteolysis may be involved in lens regeneration as well. Thrombin proteolytic activity is localised to the dorsal iris after ablation of the lens, which is the first experimental difference found between the dorsal iris, which supports regeneration, and the ventral iris.

It seems plausible that the events of wound healing and coagulation in vertebrates lead to activation of thrombin and plasmin, and hence to the generation

of an activity which locally stimulates cell cycle re-entry of differentiated cells (Fig. 1.2.7). Further evidence will be important to show a role for this factor *in vivo*.

1.2.5.2.3 Factors Influencing Blastema-Cell Proliferation

Once cells are dedifferentiated, proliferation is necessary to give rise to the blastema. Blastema cell proliferation depends on at least two requirements, the presence of the WE and nerves. Regulation of the extracellular matrix may also be an important factor in promoting cell proliferation.

The WE is in close communication with the mesenchymal cells during the period of blastema formation (Neufeld and Aulthouse, 1986). Several experiments point to the importance of the WE in the proliferation of blastemal cells. First, if the WE is removed or if it is impeded to form by grafting of a skin flap over the stump, the blastema does not form (Thornton, 1957). Second, association of an irradiated epidermis with healthy stump tissues precludes regeneration; histological observation suggests that dedifferentiation of the stump cells occurs, but no proliferation is observed (Lheureux and Carey, 1988). Third, displacement of the wound epithelium to an ectopic site leads to ectopic blastema formation underneath (Thornton, 1960). The role of the WE in promoting proliferation might be due to secretion of mitogenic factors. It has been observed that a glycoprotein synthesised in the WE is transported to the blastema (Chapron, 1974). Additionally, extracts of the WE can sustain proliferation of blastemal cells in culture (Boilly et al., 1991; Globus et al., 1980). The identity of those factors is still unclear.

A requirement for nerves in limb regeneration has been recognised since the experiments of Todd in the 19th century (Todd, 1823), involving denervation of regenerating limbs. Denervation at the time of the amputation does not prevent epithelial closure of the wound, inflammation, or the onset of histolysis, which precede blastema formation. It does prevent the proliferation of the dedifferentiating cells, and hence the formation of a blastema (Mesher, 1998).

Marcus Singer and collaborators demonstrated that the trophic effect of nerves depends on a threshold level of innervation. This effect is independent of the type of innervation and of connection to the central nervous system (Kamrin and Singer, 1959; Singer, 1952; Singer et al., 1967; Thornton, 1956). The only instance where regeneration has been observed in the complete absence of nerves is in the aneurogenic limb, a limb that has developed in the absence of nerves (Thornton and Thornton, 1970; Yntema, 1959).

The molecular basis of nerve dependence has proven difficult to elucidate (see for review Mesher, 1998). According to Singer's neurotrophic theory, the nerve fibres exert their action by releasing a factor that promotes blastema cell proliferation. Various candidate factors have been proposed to play that role, such as glial growth factor (Brockes and Kintner, 1986; Wang et al., 2000) and members of the FGF family (Mesher, 1998; Mullen et al., 1996). The nerves may also exert their action indirectly by causing the release of growth factors from their sites of binding on the extracellular matrix (Zenjari et al., 1997). Additionally, denervated Schwann cells may release an inhibitor of proliferation, for example a member of the TGFβ family, which would be turned off after re-innervation by regenerating axons (Ferretti and Brockes, 1991).

The remodelling of the ECM in the blastema may also regulate cell proliferation. The ECM of the early blastema is characterised by the presence of molecules that may favour cell proliferation and migration, and by the absence of those which may inhibit these processes. In the early blastema, there is degradation of collagen (Grillo et al., 1968) and the synthesis of hyaluronate and fibronectin are intensified (Gulati et al., 1983; Mescher and Cox, 1988; Repesh et al., 1982). When differentiation begins, fibronectin and tenascin are downregulated (Gulati et al., 1983; Onda et al., 1990; Onda et al., 1991). Conversely, collagen and laminin reappear (Gulati et al., 1983; Mailman and Dresden, 1976). In chick myoblast cultures, differentiation is inhibited on cells

cultured on hyaluronate, whereas it is promoted in cells cultured on collagen (Kujawa et al., 1986; Kujawa and Tepperman, 1983). In myoblasts of the cell line C2C12, laminin binding may be sufficient to override the mitogenic stimulus of soluble growth factors since activation of a laminin receptor results in differentiation, even in the presence of mitogens (Sastry et al., 1999).

1.2.5.2.4 Proliferative Potential of the Progenitor Cells

When newt blastemal cells are propagated in culture, they are able to divide for at least two hundred generations without evidence of crisis or senescence (Ferretti and Brockes, 1988). This may be related to the fact that it is possible to amputate a newt limb at least 20 times, and to observe regeneration with essentially the same time course on each occasion (Brockes, 1998b).

1.2.5.2.5 Redifferentiation/Transdifferentiation of the Progenitor Cells

Upon implantation of cultured labelled myotubes into a limb blastema, a small number of cases were reported of clones of 2 to 4 labelled cells in cartilage nodules in the regenerate (Lo et al., 1993). Therefore, transdifferentiation from muscle to cartilage might occur at a low level. An earlier study showed that labelled chondrocytes implanted into the blastema can give rise to labelled cartilage, bone and connective tissue, but not to muscle (Steen, 1968).

1.2.6 Discussion

I have reviewed several examples where the loss of differentiated characteristics occurs in the context of regeneration, in vertebrates and invertebrates. It is amazing that the control of the differentiated state allows such plasticity in the described cases. In jellyfish the plasticity may be related to the important role of asexual reproduction and regeneration and it could be argued that such plasticity would not be compatible with the existence of more complex tissues in vertebrates. However, where regeneration does occur in vertebrates, the plasticity of cells seems to be extremely well managed, in that parts of structures

or full structures are regenerated without changes in tissue function or uncontrolled cell growth. For example, urodeles are remarkably resistant to carcinogenesis in their regenerative territories, and there are examples where application of chemical carcinogens evokes super-numerary regenerates, rather than tumours (see for a review Brockes, 1998b).

It is possible that regeneration from differentiated cells provides a quicker, more localised and less costly form of regeneration than from stem cells, since a large number of cells near the injury can participate in regeneration. Additionally, no population of stem cells has to be maintained in the tissue and the risks of hyperplasia are avoided. In fact, most of the stem cells existent in the body, such as satellite cells in muscle, seem to be involved in the renewal of the differentiated cells but cannot cope with large injuries.

It is quite striking that in the liver there exist both mechanisms of regeneration (Michalopoulos and DeFrances, 1997; Thorgeirsson, 1996); the stem cells are activated when the differentiated cells cannot cope with the injury. These mechanisms may have evolved in the liver to protect animals in the wild from the catastrophic results of liver loss caused by food toxins.

How is the differentiated state controlled in the cells described in this chapter? Although in all the systems described there is still much to be learned on the activation of cellular plasticity, it seems that more than one signal may be needed to activate plasticity in vertebrates. A clear example is the one of the newt myotube/myofibers. At least two different signals seem to be necessary to induce cell cycle re-entry and dedifferentiation. In both situations there seems to be a positive, plasticity-triggering signal, released upon tissue damage *in vivo,* and existent in serum, *in vitro.* Another signal, such as fibre damage *in vivo* and release of contact inhibition *in vitro* may be important to prevent overgrowth, and restrict the response to the site of injury. This last signal seems to be dominant, as serum-stimulated contact inhibited myotubes do not re-enter the cell cycle (Tanaka et al.,

1997). A similar mechanism might operate in the mammalian liver where a collagenase injection is needed for growth factor stimulation of hepatocyte proliferation. A role for the extracellular matrix may be very general. For example, it has been suggested that extracellular matrix remodelling and proteolysis play a regulatory role in VSMC and newt PEC plasticity. The most striking example was the one described for the jellyfish *Podocoryne,* where upon matrix degradation, skeletal muscle transdifferentiates into smooth muscle and nerve cells. The extracellular matrix might exert a regulatory role on the cytoskeleton, which may be important for the destabilisation of the differentiated state. Destabilisation of actin filaments promotes plasticity in the jellyfish (Schmid, 1992; Schmid and Reber-Muller, 1995). Additionally, it has recently been reported that a purine-analogue that interferes with the tubulin cytoskeleton is able to trigger cellularization/dedifferentiation of mammalian myotubes (Rosania et al., 2000).

Although plasticity can be observed in mammals, it is clearly restricted to fewer cell types than in urodele amphibians. What underlies this difference? Although there may be differences in the early events after injury, like the closure of a wound, there is also a clear difference in the responsiveness of cells. Newt myotubes re-enter the cell cycle in response to mammalian sera. The same does not happen with their mammalian counterparts. Newt PECS are also more plastic then their mammalian and avian counterparts. The plasticity of the latter can be stimulated upon the particular combination of phenylthiourea and FGF-2, but these are extreme conditions.

An understanding of the molecular regulation of the differentiated state in the same cell type, in species with different regenerative abilities, should give us more insight into the regulation of the differentiated state and why that ability to regenerate is not present in higher vertebrates. The molecular basis of plasticity is the topic for the next section.

1.3 Molecular Mechanisms Involved in the Plasticity of the Differentiated State

1.3.1 Cell Cycle and Differentiation

Differentiation and the maintenance of the differentiated state are a product of the interaction of molecules that regulate the cell cycle and molecules involved in differentiation. I will therefore start by reviewing the molecular players involved in the cell cycle. I will discuss how the differentiation process is coupled to the exit from the cell cycle and how the maintenance of the post-mitotic state might be achieved. Our knowledge in this field has gained from the study of a few paradigmatic cell types that I will discuss. I will then discuss, at the molecular level, the regulation of cellular plasticity.

1.3.1.1 Overview of the Regulation of Cell Cycle Progression

1.3.1.1.1 Basic Mechanisms

1.3.1.1.1.1 Cell Cycle Compartments and Checkpoints

The cell division cycle consists of a consecutive series of co-ordinated processes, generally invariant in their order, that produce two daughter cells from a single parent. Two events in the cell cycle ensure that each daughter cell receives a full complement of the hereditary material: the replication of chromosomes and their segregation to each daughter cell. According to these events, the cell cycle can be divided into four phases: G1, S, G2, and M. During S phase, DNA is synthesised and the genome is duplicated. During mitosis or M-phase, chromosomes condense and segregate into the daughter cells. The interval following M and preceding S phase is called gap1 or G1, and the interval following S and preceding M is called gap 2 or G2. The G1 phase has been further subdivided into early G1, mid G1, in which cell growth takes place, and late G1, in which final preparations for DNA replication occur (Studzinski and Harrison,

1999). For the majority of cells, G2 is a phase where levels of mitotic inducers increase. This phase is important for monitoring of chromosome replication and preparation for mitotic spindle assembly (Baserga, 1999).

The correct traversal of the various compartments of the cell cycle is controlled by a series of regulatory steps referred to as checkpoints. If an event in the cell cycle is not completed or has been improperly completed, checkpoints delay progression through the cell cycle, to provide time for repair or completion of the event. The cell cycle block is not released until the defect has been corrected. These mechanisms not only assure normal cell division but are also required for growth arrest under conditions of environmental stress or after DNA damage (Clarke and Gimenez-Abian, 2000; Kristina, 1998; Nurse et al., 1998).

One of the most important checkpoints in the cell cycle occurs late in G1, when cells translate many intracellular and extracellular input signals to decide whether to commit themselves to division. After this point, termed the restriction point, cells do not require further mitogenic stimulation to progress through the cell cycle (Pardee, 1989).

There are also checkpoints during S, G2 and M phases, which monitor the accuracy of DNA replication and correct chromosomal alignment and segregation, ensuring that each daughter cell receives a full complement of genetic information identical to the parental cell (for review and other references on checkpoints see Clarke and Gimenez-Abian, 2000; Pines and Rieder, 2001; Wassmann and Benezra, 2001).

1.3.1.1.1.2 Mechanisms that Drive Cell Cycle Progression

The transitions between the different phases of the cell cycle are driven by the successive activation of a family of cyclin-dependent kinases (CDKs). These proteins are serine-threonine kinases, which exert their influence by phosphorylation of other cell cycle regulatory proteins. Although the levels of

CDKs remain relatively constant throughout the cell cycle, their activity changes due to several mechanisms of regulation:

1.CDK activation and interaction with the substrate requires binding to a partner protein from the cyclin family. In mammalian cells, there are at least 9 CDKs and 11 cyclins. Several specific combinations of cyclins and CDKs are implicated in major cell cycle transitions. The cyclin levels oscillate throughout the cycle, contributing to the substrate specificity of the CDKs (Puri et al., 1999).

2.Phosphorylation of CDKs is important for the activation and inhibition of cyclin-CDK activity (Lew and Kornbluth, 1996; Puri et al., 1999). For example, phosphorylation of CDKs within the conserved residues Thr-14 and Tyr-15 located in the catalytic cleft inhibits cyclin-CDK function, and dephosphorylation of these residues is required for activation of the complexes (Ekholm and Reed, 2000; Lew and Kornbluth, 1996). Dephosphorylation is accomplished by the CDC25 phosphatases (Ekholm and Reed, 2000).

3. There are inhibitory effectors of the cyclin-CDK complex. There are two families of CDK inhibitors (CDIs), distinguished on the basis of their sequence similarity and substrate specificity. The INK4 (for inhibitor of CDK4) family of proteins competes with D cyclins for binding to CDK4 and CDK6 and inhibits their kinase activities. This family includes $p16^{INK4a}$ (p16), $p15^{INK4b}$ (p15), $p18^{INK4c}$ (p18) and $p19^{INK4d}$ (p19) (Vidal and Koff, 2000). The INK4 family plays an important role in tumourigenesis. *p16INK4a* is a single mutagenic target in many human tumours (Ruas and Peters, 1998; Serrano, 2000). The other family of kinase inhibitors is the CIP/KIP family (for CDK interacting protein/kinase inhibitory protein), which includes $p21^{CIP}$ (p21), $p27^{KIP}$ (p27), and $p57^{KIP2}$ (p57).(Vidal and Koff, 2000). These proteins display a preference for fully assembled CDK/cyclin complexes. They inhibit cyclin-CDK2,4 and 6 complexes *in vitro* but preferentially act on CDK2 complexes *in vivo* (Vidal and Koff, 2000). Although generally associated with situations where cell growth is inhibited, the CDIs also regulate

cell cycle progression and CDK activity in proliferating cells (Vidal and Koff, 2000). Both p21 and p27 seem to play an important role as assembly factors for cyclinD-CDK4 or 6 (Cheng et al., 1999; LaBaer et al., 1997).

4. Another mechanism for regulation of the activity of cyclin-CDK complexes is subcellular compartmentalisation. These complexes shuttle between the cytoplasm and the nucleus, while many of their substrates are nuclear (Takizawa and Morgan, 2000; Yang et al., 1998).

1.3.1.1.1.3 Phase Transitions

1.3.1.1.1.3.1 Entry into S Phase-the Restriction Point and the Role of pRb

Considerable efforts in the cell cycle field have been put into explaining the transition through the restriction point in molecular terms. The retinoblastoma gene product (pRb) is a tumour suppressor protein thought to play a major role in that transition by inhibiting the passage through the restriction point in its active, non-phosphorylated form (Kaelin, 1999; Mulligan and Jacks, 1998; Weinberg, 1995). Mitogenic and growth-inhibitory signals control progression through the restriction point, by controlling pRb phosphorylation status (Mittnacht, 1998). Phosphorylation converts pRb into an inactive form, devoid of ligand-binding ability; the reverse reaction, catalysed by phosphatases, reconstitutes active pRb (Mittnacht, 1998). In mammalian cells, pRb is hyperphosphorylated from late G1 to the end of M phase, just before entry of the daughter cells into a subsequent G1 (Mittnacht, 1998; Weinberg, 1995).

The retinoblastoma protein contains several functional domains: the N-terminus, domain A, a spacer domain, domain B and the C-terminus. The A, B and spacer domains form a central "pocket", a domain critical for pRb tumour suppressor function (Adams, 2001; Harbour and Dean, 2000a; Qin et al., 1992). The main activity of pRb in the regulation of S phase entry is due to binding to the E2F family of transcription factors, through the pocket domain. The E2F family is composed of at least 5 proteins (E2F1-5) which are active when they form

heterodimeric complexes with one of the E2F-related transcription factors DP-1,2, or 3 (Harbour and Dean, 2000b; Studzinski and Harrison, 1999). E2F transactivates genes whose transcription is important for S-phase entry and DNA replication, such as c-myc, B-myb, cdc2, cyclin A, cyclin E, CDK2, thymidine synthetase, thymidine kinase, cdc6, and DNA polymerase α (Geng et al., 1996; Harbour and Dean, 2000b; Studzinski and Harrison, 1999). E2F overexpression induces quiescent cells to enter S-phase (Johnson et al., 1993; Qin et al., 1994). E2Fs have a preference for binding hypophosphorylated pRb, which blocks the ability of E2Fs to activate gene transcription and induce S phase entry (Flemington et al., 1993; Harbour and Dean, 2000b; Helin et al., 1993). Binding to pRb also results in active repression of transcription of E2F target genes (Weintraub et al., 1995). This transcriptional repression may be mediated through binding of the pRb-E2F complex to histone deacetylases, which remodel the chromatin, leading to a tight association between the nucleosomes and preventing the access of transcription factors to their cognate elements in gene promoters (Harbour and Dean, 2000b).

Phosphopeptide analysis suggests that there are more than sixteen distinct sites of phosphorylation on pRb. The aminoacid sequence of those sites is typical of sites modified by the CDKs (Kaelin, 1999; Knudsen and Wang, 1996). During the G1/S transition, pRb is a substrate of two G1 cyclin-CDK complexes: cylinD-CDK4/6 and cyclin E-CDK2 (Adams, 2001). Functional inactivation is sequential in that cyclin D-CDK4/6 activity is maximal during mid to late G1, whereas CDK2 activity is maximal at the end of G1 and during the G1/S phase transition (Adams, 2001; Lundberg and Weinberg, 1998; Mittnacht, 1998). The relative contribution of different kinases to the phosphorylation of different pRb aminoacid residues is not well characterised; nor is the relative contribution of each of those sites to inactivation of different pRb functions (Adams, 2001; Mittnacht, 1998).

Mitogenic signals control the activity of the G1-cyclin-CDK complexes through a variety of mechanisms such as regulation of the expression and localisation of cyclins, and degradation of CDIs (Jones and Kazlauskas, 2001; Marshall, 1999; Sahai et al., 2001). For example, growth factor-dependent activation of the Ras/Erk pathway increases cyclin D1 mRNA and the PI3K/Akt pathway stabilises these proteins (Jones and Kazlauskas, 2001; Peeper et al., 1997).

The established view of the regulation of the restriction point is that, upon mitotic stimulation, the complex formed by cyclin D-CDK4/6 will phosphorylate pRb and release E2F. Cyclin E and E2F are both transcriptional targets of E2F and are upregulated as a result of pRb inactivation; release of E2F establishes a positive feedback loop that drives cells into S phase (Fig. 1.3.1, black arrows; Adams, 2001; Harbour and Dean, 2000a; Harbour et al., 1999; Lundberg and Weinberg, 1998). According to the view presented above and the fact that the INK4 family proteins inhibit specifically CDK4/6 kinases, the pathway constituted by p16, CDK4/6-cyclinD and pRb behaves as a single target to escape growth control during tumourigenesis (Serrano, 2000; Sharpless and DePinho, 1999). That is in agreement with the fact that inactivation of *p16* and *Rb* is mutually exclusive in cancers and the fact that the presence of functional pRb was found to be required for growth suppression by p16 (Lukas et al., 1995; Medema et al., 1995; Serrano, 2000).

Other pathways possibly involved in the transition through the restriction point

There are two other members of the pocket protein family: p107 and p130 (Mulligan and Jacks, 1998). The pocket domain is the most conserved region among the three; sequences outside this domain are conserved between p107 and p130 but not pRb (Adams, 2001). These other members of the pocket protein family have only been described in mammalian cells and have been less studied than pRb (Bartek and Lukas, 2001). Recent findings have shown that p16-

mediated cell cycle arrest can also occur in mouse embryonic fibroblasts lacking the *Rb* gene (Bruce et al., 2000). In these cells, the p16-induced arrest is not mediated exclusively by pRb, but depends on the non-redundant functions of the two other pRb-family members (Bruce et al., 2000). Additional work in mouse embryonic fibroblasts lacking the three pocket proteins has shown that at least one of them is required for the p16-mediated cell cycle arrest (Dannenberg et al., 2000; Sage et al., 2000). According to these findings, the role of p107 and p130 in regulating the restriction point in mammalian cells should not be discounted (Fig. 1.3.1, blue and black).

Recent reports have questioned whether the pocket protein phosphorylation pathways are solely responsible for allowing progression past the G1 restriction point. Evidence is mounting to implicate the Myc proto-oncogene as a central element of a pathway (red pathway, Fig. 1.3.1) parallel to the pRb-E2F pathway (black pathway, Fig. 1.3.1). The c-myc proto-oncogene is a transcription factor of the helix-loop-helix/leucine zipper family, whose endogenous expression is induced by mitogens. Ectopic expression of c-myc induces DNA synthesis in quiescent cells, and it has been implicated in the regulation of cyclin E-CDK2 activity, E2F dependent transcription, and cell growth (Bartek and Lukas, 2001; Nasi et al., 2001). Overexpression of Myc can bypass the p16/pRb growth inhibitory pathway (Alevizopoulos et al., 1997; Santoni-Rugiu et al., 2000). A mutant (MadMyc) that actively represses c-myc target genes can cause G1 arrest independent of a functional pRb (Berns et al., 1997).

Cyclin E has different downstream effectors from pRb. Evidence for this comes from the fact that cyclin E-CDK2 can promote S phase in the presence of a non-phosphorylatable pRb mutant (Alevizopoulos et al., 1997; Lukas et al., 1997). Some of the targets of cyclin E-CDK2 are p27, cdc45 and p220NPAT, implicating an effect of that complex in the firing of the origins of replication and histone biosynthesis (Fig. 1.3.1, green; Bartek and Lukas, 2001). Cyclin E may be a

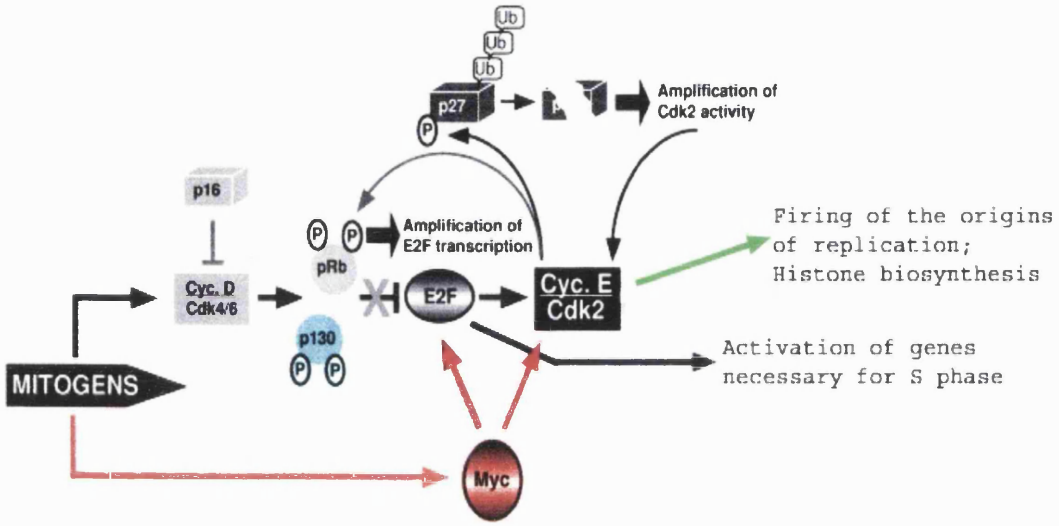

Figure 1.3.1 G1/S-promoting pathways. In black, p16-cycD/CDK4,6-pRb-E2F pathway. Mitogens lead to phosphorylation of pRb, which releases E2F. E2F promotes the transcription of genes necessary for replication, among which is CDK2. The activation of the cyclin E/CDK2 complex targets p27 for degradation and leads to further inactivation of pRb, creating a positive feedback loop that drives the cell into S phase. In blue, other members of the pocket protein family may also regulate the restriction point. In red, the myc proto-oncogene can also drive cells into S phase, by activating the transcription of E2Fs and regulating the activity of the cyclin E/CDK2 complex. In green, cyclin E/CDK2 has other downstream effects than just pRb phosphorylation. Adapted from Bartek and Lukas (2001).

convergence point of both the E2F/pRb and the Myc pathway, though different effectors of these pathways should not be ignored (Arata et al., 2000; Bartek and Lukas, 2001).

1.3.1.1.1.3.2 Progress Through S-phase, G2 and Entry into Mitosis

Progression through and exit from S phase is thought to involve the inhibition of E2F. CyclinA-CDK2 phosphorylates E2F-DP1 heterodimers on DP1, inhibiting their activity (Krek et al., 1994; Puri et al., 1999). Rereplication might be prevented due to cyclin A-CDK2 phosphorylation of cdc6, a component of the complexes at the origins of replication, targeting cdc6 for nuclear export and degradation by

ubiquitin-mediated proteolysis (Jallepalli et al., 1997; Leatherwood, 1998; Petersen et al., 1999b).

The first irreversible event in mitosis is nuclear envelope breakdown (NEB). NEB is triggered by the sudden accumulation in the nucleus of the active complex formed by Cyclin B-CDK1 (also known as Cdc2), initially called the maturation (mitosis) promoting factor (MPF; Masui and Markert, 1971; Pines and Rieder, 2001). Cyclin B levels rise during G2 and peak in mitosis (Takizawa and Morgan, 2000). The complex formed by cyclin B-CDK1 is held inactive by phosphorylation of CDK1 at Thr 14 and Tyr15 by the protein kinases Wee1 (Coleman and Dunphy, 1994; Heald et al., 1993) and Myt1 (Booher et al., 1997; Mueller et al., 1995). This complex is mainly found in the cytoplasm (Hagting et al., 1998; Takizawa and Morgan, 2000). During late prophase, most cyclin B-CDK1 complexes are translocated from the cytoplasm to the nucleus. At the same time, inhibition of Wee1 and Myt1, and activation of Cdc25C, a dual-specificity protein phosphatase that dephosphorylates CDK1, results in the activation of cyclin B-CDK1. Activation of Cdc25C is catalysed by at least two kinases: polo kinase and cyclin B-CDK1 (Abrieu et al., 1998; Karaiskou et al., 1999; Kumagai and Dunphy, 1996; Kumagai and Dunphy, 1997). This establishes a positive feedback loop that once activated drives cells irreversibly to mitosis (Karaiskou et al., 1999; Takizawa and Morgan, 2000).

1.3.1.1.1.3.3 Exit from Mitosis and Entry to G1

After NEB, the mitotic spindle, a bipolar structure composed of microtubules and associated proteins, is assembled. The chromosomes become fully condensed and align along the midline of the mitotic spindle. Specialised protein complexes, called kinetochores, organised on each centromere, attach sister chromatids resulting from DNA replication to spindle microtubules. There are several mechanisms that assure proper spindle formation and sister chromatid attachment to microtubules (reviewed in: Clarke and Gimenez-Abian, 2000;

Wassmann and Benezra, 2001). Once everything is in place, the anaphase promoting complex (APC), a large ubiquitin-ligase complex, targets proteins that hold sister chromatids together for proteolysis, and anaphase begins. The APC is activated in part by cyclin B1-CDK1 (Pines and Rieder, 2001). It also targets cyclin B for proteolysis, leading to inactivation of cyclin B-CDK1 as cells reach telophase. The rapid decrease in cyclinB-CDK1 activity results in nuclear envelope reformation around the separated chromosome masses, and cytokinesis. If non-degradable forms of cyclin B are expressed, the chromosomes complete anaphase normally, but do not decondense; the nuclear envelope does not reform, and cytokinesis does not occur (Clute and Pines, 1999; Pines and Rieder, 2001; Wheatley et al., 1997).

The separation of the two daughter cells is achieved by formation of a cleavage furrow, a structure resulting from the ingression of the membrane around the middle of the cell, perpendicular to the spindle axis. The cleavage furrow constricts down to an intercellular bridge (midbody) connecting the two daughter cells. The midbody is ultimately severed, resulting in full separation of the cells. Cytokinesis is a complex process since the timing, the localization, and the assembly of the cleavage furrow must be precisely regulated so that there is proper segregation of the genetic material and cytoplasm between both daughter cells. Preparation for cytokinesis starts during anaphase with the assembly of two important structures: the central spindle and a contractile ring at the cell equatorial cortex (Robinson and Spudich, 2000; Straight and Field, 2000). In late anaphase, after chromosome segregation, in each end of the cell, microtubules outside the spindle (astral microtubules) begin to grow out of the cell cortex (Robinson and Spudich, 2000). Some of these microtubules are reorganised to extend across the equatorial plane of the cell, forming arrays of bundled microtubules, called the central spindle or midzone (Robinson and Spudich, 2000; Straight and Field, 2000). The contractile ring is a structure formed by several

proteins, including actin and actin cross linking molecules, myosin II and other motor proteins, that drives furrow ingression. An interplay between the microtubules, the contraction of the contractile ring and recruitment of new membrane leads to ingression of the cleavage furrow at the correct time and position (Field et al., 1999; Hales et al., 1999; Robinson and Spudich, 2000; Straight and Field, 2000).

1.3.1.2 Cell Cycle Arrest and Differentiation: Molecular Players and Interactions

The most intensively studied model for differentiation is skeletal myogenesis and it has yielded a paradigm for thinking about the relationship of growth and differentiation. This was possible in part due to the availability of cultured myogenic cell lines that recapitulate *in vitro* the process of differentiation *in vivo*. Therefore, considerable attention will be given to myogenesis in this section. When possible, examples from other cell types will be given.

Myogenic cells, called myoblasts, can be propagated in culture in growth factor rich media. Although proliferating myoblasts express MyoD, a member of the myogenic transcription factor family, which act in cooperation with the MEF2 family of transcription factors to activate muscle-specific gene expression, they do not differentiate. Upon growth factor withdrawal, they exit from the cell cycle at G0/G1 phase, the transcriptional function of MyoD is activated and they begin to express muscle specific genes. They then fuse to form myotubes (Olson, 1992; Wei and Paterson, 2001; Zhu and Skoultchi, 2001).

Differentiation in this system is clearly associated with cell cycle arrest. For example, forced expression of CDK inhibitors p16 or p21 in proliferating myoblasts results in the activation of MyoD transcriptional function, even in the presence of serum (Skapek et al., 1995). Conversely, overexpression of positive cell cycle regulators that act in G1, such as c-jun, c-myc, c-fos, cyclin D1 and E2F1, inhibits myogenesis (Guo and Walsh, 1997; Skapek et al., 1996; Skapek et al., 1995; Wang et al., 1995; Wang et al., 1996). Failure of myogenesis is observed in p21 and

p57 double null mice (Zhang et al., 1999b). In other tissues, similar effects have been reported (Studzinski and Harrison, 1999). For example, increased levels of p21 are associated with differentiation in the hematopoietic system (Liu et al., 1996a; Liu et al., 1996b) and in the intestine epithelia (Gartel et al., 1996). Lens fibre differentiation is impaired in p27 and p57 double mutant mice (Zhang et al., 1998).

What is the connection between molecules involved in the cell cycle and molecules involved in differentiation? In muscle, cyclin D1 dependent kinases and CDK 2 can inhibit MyoD activity through phosphorylation (Rao et al., 1994; Skapek et al., 1996; Skapek et al., 1995), and independently of phosphorylation in the case of CDK4 (Zhang et al., 1999a). Inhibitors, such as p57, can stabilize myoD both by inhibiting its phosphorylation through CDKs and by direct interaction with the transcription factor (Reynaud et al., 2000; Reynaud et al., 1999).

The retinoblastoma protein also promotes the expression of late markers of muscle differentiation. *Rb-/-* mice die prior to E14, before detailed analysis of myogenesis is possible. When *Rb-/-* mice are rescued by the expression of a *Rb* transgene (mg*Rb*:*Rb-/-*) , which expresses low levels of pRb, they survive to birth (Zacksenhaus et al., 1996). At E17.5 the mutant foetuses express the transgenic pRb in the brain, but not in muscles. These foetuses have defects in skeletal muscle differentiation, such as shorter myotubes and reduction in the expression of late muscle-specific genes (Zacksenhaus et al., 1996). Additionally, pRb is necessary for MyoD activation of MEF2 function (Novitch et al., 1999). This requirement for pRb to promote muscle-gene expression can be partially bypassed by co-expressing MyoD with an artificially activated form of MEF2C (Novitch et al., 1999). This function of pRb is independent of its cell cycle arrest inducing activity. Expression of an E2F-Rb chimeric protein containing only the small pocket domain of pRb induces cell cycle arrest but restores only part of the expression of late differentiation markers obtained with the full-length pRb

(Novitch et al., 1999). Additionally, pRb mutants that are unable to associate stably with E2F and are defective in promoting cell cycle arrest are still able to stimulate cellular differentiation (Sellers et al., 1998). These results suggest that pRb is implicated in a second pathway for MEF2C activation that is independent of pRb effects on the cell cycle.

In other tissues pRb also promotes differentiation by virtue of its interaction with a variety of transcription factor families such as CCAAT/enhancer-binding proteins (C/EBPs), a family of transcription factors crucial for adipocyte differentiation (Chen et al., 1996b; Classon et al., 2000) and c-jun in keratinocytes (Nead et al., 1998). It has been suggested that some of these pRb functions in differentiation may be due to a role of pRb as a transcriptional activator. It may perform this function by recruiting BRG1/HBRM, which facilitate transcription by disruption of regular nucleosome arrays in conjunction with transcription factors that act to induce differentiation (Zhang et al., 2000; Zhang, 1999; Zhu and Skoultchi, 2001).

pRb may also have other roles in differentiation, notably inhibition of apoptosis. *Rb -/-* mice have increased cell death in tissues where *Rb* is normally expressed: nervous system, liver, lens, and skeletal muscle precursor cells (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Myotubes in which pRb is inactivated by the expression of viral oncoproteins, such as E1A, also undergo cell death (Latella et al., 2000). Deregulated expression of E2F1 causes apoptosis in differentiated cardiomyocytes and neurons differentiated from p19 embryonal carcinoma cells (Azuma-Hara et al., 1999; Kirshenbaum et al., 1996). However apoptosis in *Rb* mutant skeletal muscle does not seem to be E2F1 nor p53 dependent, since apoptosis persists in mg*Rb:Rb-/-;E2F1-/-* and mg*Rb:Rb-/-;p53 -/-* compound mutant muscles (Jiang et al., 2000).

In addition to a role in differentiation, MyoD can also induce cell cycle arrest. MyoD can inactivate CDK4 (Zhang et al., 1999a) both by direct binding, and by

activating p21 expression (Guo et al., 1995; Halevy et al., 1995; Wei and Paterson, 2001). The expression of p21 is the first irreversible step in cell cycle withdrawal during myogenesis. After p21 expression is induced, it is no longer down regulated by mitogen stimulation (Guo et al., 1995; Halevy et al., 1995; Wang and Walsh, 1996; Wei and Paterson, 2001). The induction of p21 prevents the formation of active cyclin E/CDK2, cyclin A/CDK2 complexes, involved in the initiation of DNA synthesis.

Genetic ablation of CDIs has implications in the differentiation and development of several cell types (reviewed in Zhu and Skoultchi, 2001). *p27-/-* mice have revealed a prominent role for this molecule in the decision to withdraw from the cell cycle, as it results in a gene-dosage-dependent increase in body size as a result of multiorgan hypercellularity. Although the cells withdraw from the cell cycle, they undergo extra divisions (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Oligodendrocyte differentiation provides a good example. The levels of p27 accumulate during the course of proliferation, correlating with the onset of differentiation. *In vitro*, p27-/- oligodendrocyte precursor cells undertake one or two extra divisions before initiating differentiation (Durand and Raff, 2000). Recently, it has been shown that CDIs might also have direct dual functions in cell cycle regulation and differentiation. p27Xic1, the *Xenopus laevis* p27 and p21 homologue has been shown to have two different domains with distinct functions. It promotes Muller glial cell differentiation in *Xenopus laevis* retina through a part of the molecule distinct from the one necessary to inhibit CDKs (Ohnuma et al., 1999). Inhibition of cell cycle progression through the latter domain does not induce differentiation (Ohnuma et al., 1999). This type of protein with dual function was also found in other developmental contexts (Ohnuma et al., 2001). For example, geminin inhibits DNA replication through the carboxyl terminus and can convert prospective epidermis into neural tissue in the gastrula

ectoderm, through its amino-terminus (Kroll et al., 1998; McGarry and Kirschner, 1998).

Although more attention was given to myogenesis in the present discussion, it may be concluded that differentiation in many tissues involves a complex interplay between cell cycle molecules and transcription factors. Molecules previously known to have a role in the cell cycle can have direct effects in differentiation, and molecules involved in differentiation can directly impinge on the cell cycle machinery. This may be a way of co-ordinating the number and the phenotype of cells in each tissue.

1.3.2 Maintenance of Stable Cell Cycle Arrest and Phenotype in Differentiated Cells

Are the same molecules involved in arresting proliferation during differentiation and in maintaining the post-mitotic arrest? Most of the studies, such as gene deletion, only address the question of whether those genes are important for terminal differentiation to occur. As such, the use of viral proteins that induce re-entry into the cell cycle has been an important tool for investigating the stability of the post-mitotic state. Two of these proteins are the SV40 large T antigen and the adenoviral protein E1A.

Large T may promote growth through a variety of interactions, such as binding to pocket-proteins, p53 and the transcriptional co-activator p300 (reviewed in Ali and DeCaprio, 2001). It induces DNA synthesis and mitosis in mouse myotubes (Endo and Nadal-Ginard, 1989; Endo and Nadal-Ginard, 1998; Iujvidin et al., 1990).

The adenoviral protein E1A induces DNA synthesis and mitosis in differentiated skeletal myotubes (Crescenzi et al., 1995), adipocytes (Crescenzi et al., 1995), and cortical neurons (Suda et al., 1994), and DNA synthesis without subsequent mitosis in cardiomyocytes (Kirshenbaum and Schneider, 1995; Liu and Kitsis, 1996). E1A also interacts with a variety of cell cycle regulatory

proteins, such as pocket proteins (Moran, 1993), p300 (Eckner et al., 1994), p27 (Mal et al., 1996) and p21 (Keblusek et al., 1999).

Different mutants of E1A have been used to investigate the interactions that are important for its function. An E1A mutant (E1A.RG2) that is defective for p300 binding is still efficient in inducing DNA synthesis in myotubes (Table 1.3.1.; (Mal et al., 2000). Thus p300 may not play an essential role in the maintenance of the post-mitotic state of fully differentiated multinucleated myotubes. In contrast, the ability to induce cell cycle re-entry is lost in an E1A mutant (E1A.928; Table 1.3.1.) which is unable to bind to pocket proteins, and in an E1A mutant unable to bind to p21 and p300 (E1A.dl2-36; Table 1.3.1 ; Mal et al., 2000).

Table 1.3.1 **Binding properties of E1A mutants and their ability to induce DNA synthesis in differentiated muscle cells.** +, positive for binding to a cellular protein; -, negative for binding to a cellular protein. From (Mal et al., 2000).

	Binding			BrdU-positive
Mutant	p300	pRb	p21	C2C12 myotubes
wt E1A	+	+	+	+
E1A.928	+	-	+	-
E1A.RG2	-	+	+	+
E1A.dl2-36	-	+	-	-

The role of pocket proteins in maintaining the post-mitotic arrest of multinucleated myotubes, particularly pRb, has been further substantiated with

other experiments. During myogenesis pRb expression is upregulated and remains high, whereas that of p107 is normally downregulated (Schneider et al., 1994). In *Rb-/-* myotubes, p107 expression remains high. The expression of p107 and of p130 in *Rb -/-* myotubes is not sufficient to maintain a stable cell cycle arrest. Upon serum stimulation, p107 is downregulated and these cells re-enter the cell cycle (Schneider et al., 1994). Cell cycle re-entry was also observed in *Rb-/-* embryonic fibroblasts driven to differentiate into myocytes by ectopic MyoD expression (Novitch et al., 1996). Myofibers of mg*Rb:Rb-/-* mice contain abundant large nuclei that have undergone several rounds of endoreplication without mitosis, indicating that there are defects in the post mitotic arrest (Zacksenhaus et al., 1996). The function of pRb in maintaining the post-mitotic arrest might be broader than just inhibiting the function of E2Fs. In fact endoreplication persists in the myofibres of mg*Rb:Rb-/- ; E2F-/-* mice (Jiang et al., 2000). Additionally, overexpression of E2F4 or E2F1 does not induce cell cycle re-entry *per se* in these cells (Pajalunga et al., 1999; Puri et al., 1998).

The CDI p21 is expressed at high levels in differentiating skeletal myotubes (Franklin and Xiong, 1996; Phelps et al., 1998). When an E1A mutant E1A.928 that binds p21 but not pRb is expressed in differentiated myotubes there is no DNA synthesis (Mal et al., 2000). In this mutant cyclin E-CDK2 is activated and pRb is phosphorylated (Mal et al., 2000). The fact that expression of this mutant does not lead to DNA synthesis might be due to a requirement for phosphorylation of other pRb sites. Other CDIs may also have a role in maintaining post mitotic arrest. The single knockout mice for p21, p18, p27, or p57 do not present muscle phenotypes (reviewed in Zhang, 1999), but the p21, p57 double mutant mice show endoreplication in the nuclei of multinucleated myotubes (Zhang et al., 1999b).

The molecular regulation of the post-mitotic state might thus depend on the function of several proteins. Another example is the case of neurons. 15% of cortical neurons from adult mice transfected with E1A are induced to enter S

phase *in vivo* (Suda et al., 1994). It is not clear whether E1A is acting through the E2F-pRb pathway to induce S phase. Although E2F1 overexpression can induce S phase re-entry in cortical neurons from adult mice *in vivo* and in cultured rat sensory neurons (Smith et al., 2000; Suda et al., 1994), a mutant version of E1A that is able to inactivate pocket proteins, but not p300, does not induce DNA synthesis in cultured cortical neurons (Slack et al., 1998). The interaction of this E1A mutant with CDIs was not described (Slack et al., 1998). Further studies are necessary to clarify the importance of pocket proteins in the maintenance of the differentiated state in neurons.

CDIs may play an important role in maintaining the post-mitotic arrest in neurons. Ectopic entry into the cell cycle was observed, both in lens fibre cells in the p27 and p57 double-mutant mice (Zhang et al., 1998) and in neurons from the central nervous system in the p19 and p27 double-mutant mice (Zindy et al., 1999).

Although much remains to be learned about the precise mechanisms that maintain cells in a post-mitotic state, there is evidence favouring redundancy in the set of molecules that are involved. It becomes clearer that the maintenance of the stability of the post-mitotic state cannot be ascribed to one single type of molecule. Thus, different players, such as pocket proteins and CDIs, and positive cell cycle regulators, such as E2F, may each play an important role.

1.3.3 Molecular Regulation of Plasticity

The molecular regulation of the plasticity of the differentiated state has only recently started to be addressed and the information available is still incomplete. Examples of such plasticity are hepatocyte proliferation during liver regeneration; VSMCs proliferation and newt myotube dedifferentiation and re-entry into the cell cycle.

There are two phases of hepatocyte proliferation associated with liver development in the rat. The first one is in the last 3 days before birth; the liver

triples in size due to a surge in hepatocyte proliferation. After birth, hepatocytes undergo a period of temporary arrest, after which they synchronously re-enter the cell cycle (Awad and Gruppuso, 2000). They then enter the quiescent state associated with the adult hepatocyte phenotype. Both of the declines in hepatocyte proliferation are associated with an accumulation of hepatocytes in the G1 phase of the cell cycle and a decrease in cyclin D/CDK activity (Awad and Gruppuso, 2000). There is an increase in p57 upon the growth arrest that occurs in foetuses, and an increase in p15 upon the transition to the adult quiescent hepatocyte phenotype (Awad et al., 2000). p21 and p27 are also present in the adult liver (Albrecht et al., 1998).

After PH, the activity of CDK2, CDK4 and cyclin D1-associated kinases are upregulated, and maximal activity of these enzyme complexes corresponds to peak DNA synthesis. Re-entry is also associated with the appearance of phosphorylated pRb (Albrecht et al., 1998). This is in agreement with the fact that overexpression of cyclin D1 in primary hepatocytes causes DNA replication even in the absence of growth factors (Albrecht and Hansen, 1999; Fausto, 2000). Paradoxically, p21 is induced after PH and is maximally expressed after peak DNA synthesis (Albrecht et al., 1998). Overexpression of cyclin D1 in hepatocytes leads to an increase in p21 (Albrecht and Hansen, 1999). It has been proposed that this dual activation of activators and repressors of cell cycle progression during liver regeneration might be important for precise control of liver growth (Fausto, 2000).

VSMCs provide a very interesting case of the molecular regulation of plasticity. Within normal vessels p27 seems to be the most abundant CDI (Tanner et al., 1998). VSMC proliferation after injury correlates with low-level expression of p21 and p27, and high CDK2 activity. When the cells stop proliferating, there is up-regulation of p21 and p27, which may contribute to the reestablishment of the quiescent phenotype (Tanner et al., 2000; Tanner et al., 1998; Wei et al., 1997; Yang

et al., 1996). p21 and p27 overexpression limits VSMC proliferation *in vivo* (Chang et al., 1995; Yang et al., 1996).

VSMCs undergo hypertrophic, but not hyperplastic growth, in response to Ang II, in serum-free media. Serum and PDGF-BB can induce hyperplastic growth. p27 has been implicated as a molecular switch between hyperplastic and hypertrophic growth (Braun-Dullaeus et al., 1999). While both serum and Ang II treated quiescent VSMCs upregulate positive cell cycle regulators, such as proliferating cell nuclear antigen (PCNA), cyclin D1, CDK2 and CDK1, the activity of CDK2 and CDK1 is only induced after serum treatment. This induction of CDK activity was correlated with down regulation of p27 protein levels after serum, but not Ang II treatment (Braun-Dullaeus et al., 1999). Similar results were obtained when serum was substituted by the mitogenic factor PDGF-BB (Servant et al., 2000). Phosphorylation of pRb was only observed in PDGF-BB and not Ang II treated cells (Rao, 1999).

Recently, it was proposed that components of the ECM may control specific cell cycle proteins in VSMCs (Assoian and Marcantonio, 1996). The inhibitory effect of polymerised collagen on VSMCs growth is mediated by alpha2 integrins, and is associated with up-regulation of p21 and p27 (Koyama et al., 1996). Additionally, it was proposed that TSP-1 promotes VSMC proliferation and migration, through a mechanism dependent on p21 regulation (Sanz-Gonzalez et al., 2000).

As described previously, newt myotubes in culture show a striking distinction from myotubes of other vertebrates in that they re-enter the cell cycle after serum stimulation (Tanaka et al., 1997). They then arrest stably at the end of S-phase without entering mitosis, or undergoing any pathological event such as apoptosis (Tanaka et al., 1997). The re-entry to S-phase parallels the behaviour of mouse myotubes that are null for both copies of the *Rb* gene, and of *Rb-/-* mouse

embryonic fibroblasts driven to differentiate into myocytes by ectopic MyoD expression (Schneider et al., 1994; Novitch, 1996).

The newt *Rb* gene was cloned (Fig. 1.3.2) and expressed, and affinity-purified antibodies were prepared to the protein. The newt myotubes are clearly not mutant for *Rb* since they express both *Rb* mRNA and *Rb* protein (Tanaka et al., 1997). Nonetheless, pRb does appear to be central to the regulation of the post-mitotic state in newt myotubes, since serum stimulation was associated with the appearance of the hyperphosphorylated form of pRb after immunoprecipitation. Furthermore, the phosphorylation of pRb is necessary as evidenced by the inhibitory effects of expressing pRb constructs (wild type and constitutively active) and the CDK4/6 inhibitor p16. Expression of human p16 in the A1 myotubes completely blocked S-phase entry after serum stimulation, implicating pRb as an endpoint to the serum-stimulation pathway (Tanaka et al., 1997). Interestingly, both newt myotubes and mouse *Rb-/-* myocytes appear to be blocked rather stably in G2 (Novitch et al., 1996; Tanaka et al., 1997).

Three recent reports have generated new approaches to address the plasticity of the skeletal muscle cell. The first was the generation of newt-mouse hybrid myotubes by fusion of mouse C2C12 and newt A1 myogenic cells (Velloso et al., 2001). The mouse nuclei re-enter the cell cycle upon serum stimulation of the hybrids, while mouse-homokaryon myotubes remain arrested (Velloso et al., 2001). Manipulation of this system may allow further study of the molecular regulation of the plasticity in the newt myotube. The other two reports concern another aspect of the plasticity of the urodele myotube and myofibre: the ability to dedifferentiate *in vivo* (Echeverri et al., 2001; Kumar et al., 2000; Lo et al., 1993; Velloso et al., 2000). It was reported that the molecule Msx1, a transcriptional inhibitor, may be involved in that transition (Odelberg et al., 2000). *Msx1* and *Msx2* were originally identified based on their homology to the *Drosophila muscle segment homeobox* (*Msh*) gene (Hill et al., 1989). Newt *Msx1* gene is expressed in

normal limb and tail, as well as their respective blastemas (Crews et al., 1995; Simon et al., 1995). The *Msx2* gene is upregulated after amputation and expressed in the cells of the wound epithelium before the wound is closed (Carlson et al., 1998; Koshiba et al., 1998). Later, it is also found in the mesenchyme. Expression of *Msx1* in mammalian myoblasts inhibits their fusion (Song et al., 1992). Expression of *Msx1* in myotubes, through an inducible retroviral vector, leads to the generation of mononucleate cells in about 5% of myotubes and loss of p21 and muscle regulatory factors expression (Odelberg et al., 2000). The second report on the reversal of the mononucleate to multinucleate transition was achieved by treatment of C2C12 mouse myotubes with myoseverin (Rosania et al., 2000). Myoseverin is a substituted purine derivative isolated from a combinatorial library, which induces scission of multinucleate myotubes into proliferating mononucleate cells. This transition is associated with changes in the microtubule cytoskeleton and also regulation of a number of genes associated with wound healing and extracellular matrix remodelling, among which is the FGF4 receptor and the 3/10 metalloprotease inhibitor (Rosania et al., 2000). The authors suggest that myoseverin may activate a programme that is related to the one observed in newt myotubes during regeneration. As such, myoseverin may be a useful tool to start to dissect out the molecules involved in the regulation of the differentiated state in urodele myotubes.

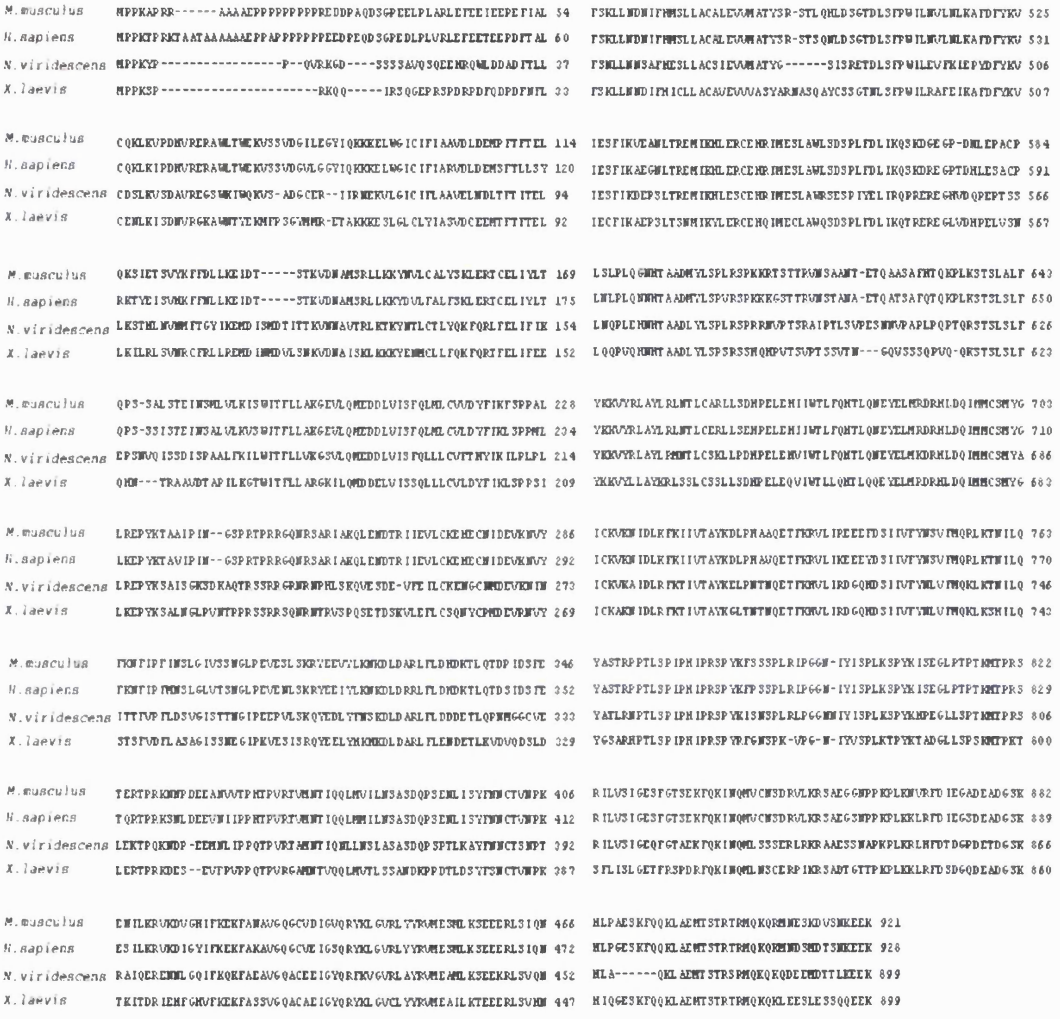

Figure 1.3.2 Sequence of newt Rb. The amino acid sequence of the newt (*N. viridescens*) Rb protein, predicted from the cDNA sequence, as compared with that of mouse, human and *Xenopus laevis*. (-) Positions at which gaps have been introduced to optimise the alignment. As with *Xenopus laevis* pRb, newt pRb lacks the distinct NH2 –terminal proline-rich sequence found in the mouse and human sequence. The predicted size of the encoded protein is 103 kD.

1.3.4 Discussion

In this Chapter, I have described some examples of the regulation of the differentiated state. It seems clear that the same molecules that play an important role in regulating cell cycle progression in proliferating cells are associated with differentiation and with the maintenance or plasticity of the differentiated state. That is the case with pocket proteins, cyclin/CDK complexes and CDIs.

The ability to regulate the expression and activity of cyclin/CDK complexes and CDI's seems to be essential for the plasticity of the differentiated state. In hepatocytes, the proliferative response to PH seems to be associated with the up-regulation of cyclinD/CDK complexes (Albrecht et al., 1998), while in VSMCs, proliferation is associated with down-regulation of p27 (Servant et al., 2000). Downstream of these positive and negative regulators may be pRb phosphorylation. Phosphorylation of pRb occurs during cell cycle re-entry in differentiated cells, such as hepatocytes (Albrecht et al., 1998), smooth muscle cells (Rao, 1999) and newt myotubes (Tanaka et al., 1997). Nevertheless, in none of these cases it was demonstrated that pRb phosphorylation is sufficient to trigger S phase entry. Other effectors of cyclin/CDK complexes and CDIs may be involved in the stability of the differentiated state. Accordingly, inactivation of pocket proteins is not enough to trigger cell cycle re-entry in mammalian skeletal myotubes; inactivation of p21 is also necessary (Mal et al., 2000).

What could be upstream of pRb phosphorylation in newt myotubes? The attempts to clone newt CDIs, such as p21 and p16, have not been successful (unpublished results from different people in this laboratory). It would be tempting to speculate that urodeles do not have CDIs, in order to explain cell cycle re-entry and pRb phosphorylation by newt myotubes. On the other hand it may be that plasticity is achieved in urodele-differentiated cells similarly to the mechanisms above described for hepatocytes or VSMCs, with regulation of cell cycle effectors by mitogenic signals.

1.4 Regulation of The Differentiated State in The Cardiac Muscle Cell

1.4.1 Heart structure and Differentiation of Cardiomyocytes

1.4.1.1 Structure and Development of the Heart

The structure of the heart has changed considerably throughout evolution (Chen and Fishman, 2000; Sedmera et al., 2000). The fruit fly *Drosophila melanogaster* has an open circulation system. The contractile dorsal vessel has similar functions to the vertebrate heart, pumping hemolymph through the interstices of tissues (Chen and Fishman, 2000). In vertebrates, the blood is driven throughout the tissues in a circulatory fashion, within a distinctive vascular system that passes through an oxygenating interface (gills or lungs). New elements constitute the circulatory system in vertebrates: the ventricle, the valves, an endothelium lining of vessels and heart, and a pacemaking system. The ventricle, which is a thick-walled high-pressure-generating chamber, guarantees that the blood goes at sufficiently high pressure to perfuse the tissues. The valves are important to prevent backflow of blood. The endothelium, which is a single-cell lining of all vessels and of the heart, provides scaffolding for vessel formation during embryonic growth, and is an important source of vaso-regulatory factors. Finally, an elaborate pacemaking–conducting system coordinates the heartbeat, so that all parts of a single chamber contract nearly simultaneously, and guarantees the coordination between different chambers (Chen and Fishman, 2000; Fishman and Olson, 1997; Srivastava and Olson, 2000).

Within vertebrates, the heart structure has changed considerably. Vertebrates such as fish have a two-chambered heart, with a ventricle and an atrium. Blood-circulation is a one-way through system. The heart pumps deoxygenated blood returning from the body, forwarding it to the gills where it is oxygenated and then supplied to the body (Chen and Fishman, 2000). The advent of air breathing during evolution brought a need for a separate pulmonary circulation and

separation of oxygenated from deoxygenated blood. Amphibians have an atrial septum but rely upon streaming of blood within the ventricle to keep oxygenated blood separated from deoxygenated blood (Duellman and Trueb, 1986; Sedmera et al., 2000). The separation of the blood within the amphibian ventricle seems to be due to a combined effect of a spiral valve in the *conus arteriosus*, a large tube from which all the arterial arches emerge, and the spongy subdivisions of the ventricle (Fig. 1.4.1). The spiral valve separates the streams of blood to the lungs and skin from those going to the rest of the body. The spongy subdivisions of the ventricle are the result of the corrugation of the inner wall of the ventricle during development (Sedmera et al., 2000). These myocardial protrusions are called the trabeculae. In adult amphibians, almost the entire thickness of the ventricular wall is made of this trabecular layer (Fig. 1.4.1).

Reptiles, birds and mammals have both atrial and ventricular septae to keep oxygenated blood separate from deoxygenated blood (Chen and Fishman, 2000; Sedmera et al., 2000). The ventricles of birds and mammals have coronary vascularization, and are thickened as compared to those of lower vertebrates (Sedmera et al., 2000).

The heart is the first organ to form and function during vertebrate embryogenesis (Stainier, 2001). Formation of the heart involves a precisely orchestrated series of molecular and morphogenetic events. Subtle perturbations in this process can have severe consequences, such as congenital heart disease (reviewed in Srivastava, 2001). Cardiomyocytes originate soon after gastrulation from bilateral symmetric populations of cells in the anterior lateral mesoderm (Stainier, 2001). These cells are thought to commit to a cardiogenic fate in response to an inducing signal that emanates from the adjacent endoderm (Nascone and Mercola, 1995; Schultheiss et al., 1995). Continued folding of the mesoderm and endoderm brings both lateral cardiogenic layers towards the ventral midline. There they fuse in an anterior-to-posterior progression to form

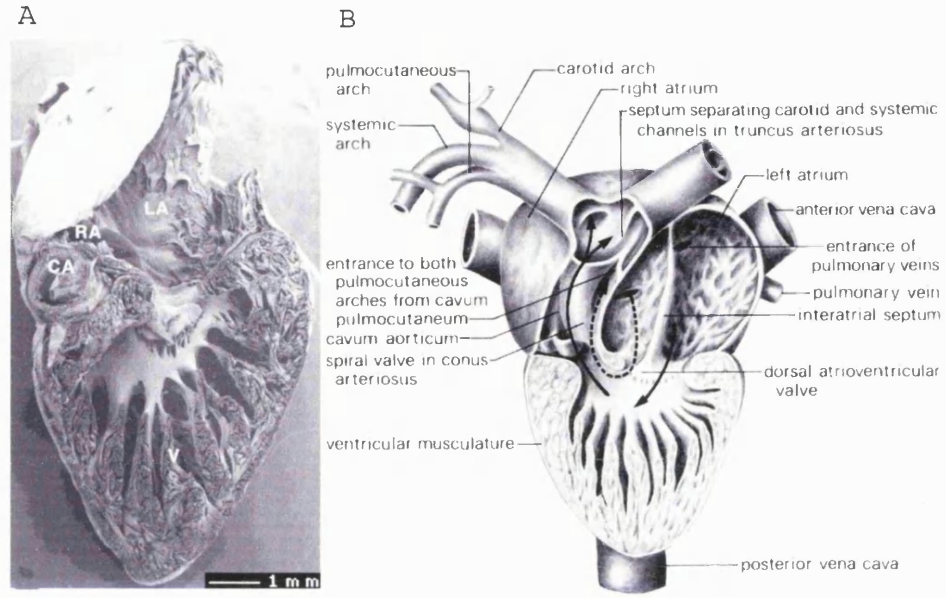

Figure 1.4.1 The structure of the amphibian heart. (A) Dorsal half of frog heart (Rana sp.). Shows clear separation between the left (LA) and right (RA) atrium. Note the thin compact outer myocardium and the trabecular sheets in the ventricle (V). The conus arteriosus (CA) possesses a spiral valve which in conjunction with the ventricular trabecular sheets assures fairly efficient blood compartmentalization. From Sedmera et al. (2000). (B) Scheme of the heart of the anuran *Rana catesbiana* partly sectioned to show internal structure. The circulatory pathway of oxygenated blood is indicated by solid arrows and that of deoxygenated blood by broken arrows. From Duellman and Trueb (1986).

the primitive heart tube. This consists of the outer myocardium and an inner endocardial tube, separated by a thick extracellular-matrix layer called the cardiac jelly (Stainier, 2001; Tam and Schoenwolf, 1999).

The expression of muscle-specific contractile-protein genes is first detected just as the most-anterior regions of the two heart cardiogenic layers begin to fuse. Differentiation and assembly of the contractile apparatus, the myofibrils, proceed in an anterior-to-posterior progression and, by the time of primitive heart tube formation, cardiomyocytes have begun to contract rhythmically (For a review on myofibrillogenesis see Gregorio and Antin, 2000; Gregorio et al., 1999). This tube then undergoes morphogenetic movements that lead to the formation of a looped, multi-chambered organ (Srivastava and Olson, 2000; Stainier, 2001).

1.4.1.2 The Cardiac Muscle Cell

1.4.1.2.1 Morphology of the Cardiomyocyte

The typical ventricle cardiomyocyte is an elongated cell, 100 to 150 μm in length and 20 to 35 μm in width (Severs, 2000). The contractile unit of the cardiomyocyte and other striated muscles is the sarcomere (Fig. 1.4.2; Barral and Epstein, 1999; Gregorio and Antin, 2000). There are three systems of filaments in the sarcomere: thin filaments, thick filaments, and elastic filaments constituted, among other proteins, by actin, myosin and titin, respectively. Muscle contraction is brought about by the interaction between myosin heads and adjacent actin filaments. Upon ATP hydrolysis, the myosin head moves along the actin filament toward the Z lines (see Fig. 1.4.2), causing actin to slide against the myosin filament, resulting in shortening of the sarcomere and muscle contraction. Thin filaments are anchored in the Z lines by the crosslinking protein α-actinin, and capped by CapZ. Actin filament capping inhibits the elongation and depolymerization of the filaments. Also associated with the actin molecules are tropomyosin molecules, which stabilise the thin filaments. Each tropomyosin molecule binds one troponin complex (composed of troponins T, I and C), and together they mediate the Ca^{2+} regulation of myosin ATPase, necessary for muscle contraction. Several proteins bind to myosin, including myosin-binding-protein C (MyBP-C), MyBP-H, M protein and myomesin. Titin constitutes the elastic filaments. Single molecules of titin reach from the Z line to the centre (M line) of the sarcomere (Fig. 1.4.2). This molecule centres thick filaments in the sarcomere and acts as an elastic spring element during muscle contraction (Barral and Epstein, 1999; Gregorio and Antin, 2000).

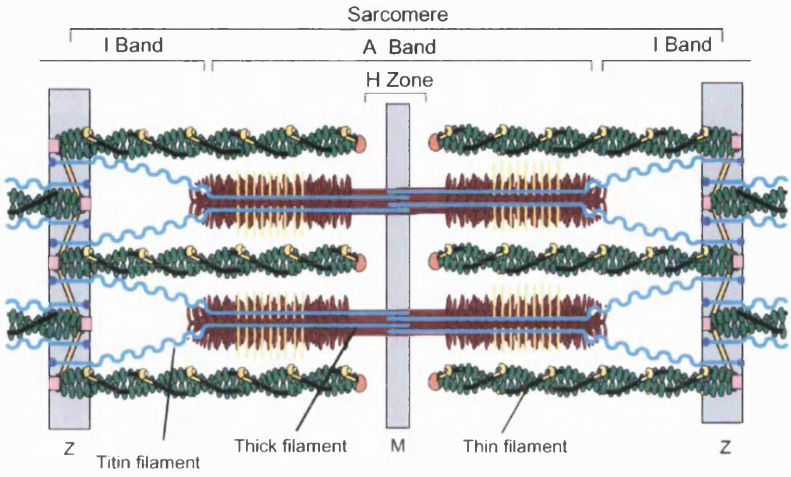

Figure 1.4.2 Major components of a cardiac muscle sarcomere (a single contractile unit). Actin (green); myosin (dark red); Rod-like tropomyosin molecules (black lines); troponins (yellow); α-actinin (gold); CapZ (pink squares); tropomodulin (bright red); titin (turquoise); myosin-binding-protein C (MyBP-C; yellow transverse lines); T-cap (telethonin; dark blue); M- M lines; Z- Z lines. From Gregorio and Antin, 2000.

Within the cell, each myofibril is surrounded by a network of interconnecting membranes: the transverse tubules, or T tubules, which extend inward from the plasma membrane, and the sarcoplasmic reticulum, an adjacent sheath of flattened vesicles (Sommer, 1982). Upon depolarisation, influx of calcium, through specific channels in the plasma membrane, triggers the opening of calcium release channels in the sarcomeric reticulum membrane, resulting in major release of calcium into the cytoplasm and subsequent myofibril contraction. The increase in Ca^{2+} concentration in the cytosol is transient because the Ca^{2+} is rapidly pumped back into the sarcoplasmic reticulum by an abundant Ca^{2+}-ATPase in its membrane (Severs, 2000).

Neighbouring cardiomyocytes are joined by intercalated disks. These structures couple the cells electrically and mechanically, via gap junction, the fascia adherens, and the desmosome (Severs, 2000; Sommer, 1982).

Atrial cardiomyocytes are different from ventricular cardiomyocytes. They are long and slender, have few or no transverse tubules, and more abundant

caveolae, and produce atrial natriuretic peptide, functioning as secretory cells. Myocytes from the conduction system, responsible for the generation of the electric impulse generation, also have a different morphology (Severs, 2000).

1.4.1.2.2 Differentiation of the Cardiomyocyte

Although the structure of the heart differs considerably throughout phylogeny, vertebrate and invertebrate hearts are constituted, among other cell types, by a spontaneously contractile myogenic cell type, the cardiomyocyte. As such, insight into cardiomyocyte differentiation has come from studies in vertebrate and invertebrate model organisms (Chen and Fishman, 2000; Fishman and Olson, 1997; Srivastava and Olson, 2000).

Cardiac muscle cells share phenotypic characteristics with skeletal muscle cells, such many proteins and the structure of the sarcomere, but express distinct sets of regulatory genes. For example, the myogenic determination factor genes *MyoD*, *Myogenin*, *Myf5* and *MRF4*, which are critical in skeletal muscle differentiation, are not expressed in cardiac muscle cells (Stainier, 2001).

Several genes seem to be important for heart development and cardiac muscle cell specification, such as *Mef2*, *Tbx5*, *FGF8*, *Hrt*, *Nkx2.5*, bone morphogenic proteins *(BMPs)*, and members of the *Gata* family (for a review see: Chen and Fishman, 2000; Srivastava and Olson, 2000; Stainier, 2001). *Nkx2.5* is a homeobox transcription factor from the Nk2 family and is the vertebrate homologue of *tinman* (in reference to the heartless tin character in the Wizard of Oz), a mutation that blocks dorsal vessel formation in *D. melanogaster* (Bodmer, 1993; Bodmer et al., 1990). Mutations in *Nk2.5* have been related to severe defects in heart formation in mouse and human (Srivastava, 2001; Stainier, 2001). Dominant-negative versions of Nkx2.5 block cardiogenesis in *Xenopus laevis* and zebrafish embryos (Fu et al., 1998; Grow and Krieg, 1998), however *Nkx2.5* is not essential for the specification of the cardiac lineage in mice (Lyons et al., 1995).

This may be due to functional redundancy among the 5 members of the Nk2 family (Chen and Fishman, 2000).

Nkx2.5 cooperates with zinc-finger transcription factors of the Gata family to activate cardiac gene expression (Durocher et al., 1997). *Gata5* is necessary for the initiation of *nkx2.5* expression in zebrafish (Reiter et al., 1999). The *Gata5* gene is expressed in the myocardial lineage of zebrafish prior to the onset of gastrulation until the initiation of a heart beat, and overexpression can lead to ectopic foci of beating myocardial tissue (Reiter et al., 1999). A recent study in *Xenopus laevis* has suggested that the Gata 6 protein maintains heart cells in the precursor state and that reduction in the expression of Gata 6 is a prerequisite for the progenitors to proceed in their differentiation (Gove et al., 1997). Studies in the mouse also point to a critical role for *Gata* genes in myocardial differentiation, although substantial redundancy between mouse *Gata4*, *Gata5* and *Gata6* seems to mask much of their role in this process. Mouse embryos mutant for *Gata4* have bilateral heart tubes and a diminished number of cardiomyocytes (Kuo et al., 1997; Molkentin et al., 1997), but *Gata4* -/- embryonic stem cells can differentiate into beating myocytes (Narita et al., 1997). *Gata5* -/- mouse embryos are viable and *Gata6* -/- mouse embryos die soon after implantation (Koutsourakis et al., 1999; Morrisey et al., 1998; Stainier, 2001).

Later on, T_3 exerts a variety of effects on the transcription of several muscle genes in cardiac myocytes (Gupta et al., 1999). For example, in the murine ventricle, α-MyHCs expression is dependent on T_3, whereas the expression of β-MyHC is repressed by it (Morkin, 1993; Morkin, 2000). This results in a change in the expression of cardiomyocyte-specific genes, although the genes that are expressed at each developmental stage may vary with phylogeny and muscle activity (Baldwin and Haddad, 2001; E. Ehler, personal communication).

1.4.2 Proliferation of Cardiomyocytes and Regulation of the Differentiated State

1.4.2.1 Proliferation During Development and After Birth

Numerous mitogens, anti-mitogens, and their receptors have been implicated in heart development (MacLellan and Schneider, 2000). Examples are FGFs, TGFβ, insulin-like growth factor 1 (IGF-1), neuregulin, AngII and cytokines signalling trough gp130 (see for review MacLellan and Schneider, 2000, McLellan and Schneider, 1999). However, the genetic ablation of many of these genes has resulted in lethality or in the lack of detectable phenotype, possibly due to functional redundancy, making it difficult to identify the precise role of these molecules. Moreover, results from genetic ablation and overexpression studies have to be interpreted carefully, since indirect effects, such as the death/survival of cells and changes in the heart work-load due to modifications within the foetal circulation and extracardiac tissues, might also affect cell numbers in the heart (MacLellan and Schneider, 2000).

FGF receptors are expressed during development and down-regulated as cells stop proliferating (Jin et al., 1994; Pasumarthi et al., 1995). FGF-1 and FGF-2 are expressed in the developing rat myocardium (Burton et al., 1999a; Spirito et al., 1991), their abundance increasing between E12 and post natal day (P) 10 (Burton et al., 1999a). In avians, FGF-1, 2 and 4 can trigger the proliferation of precardiac mesoderm and are expressed in the developing heart (Zhu et al., 1996). A dominant negative FGFR1, which is kinase-defective preventing autophosphorylation in trans, decreases proliferation of cardiomyocytes during the first week of chick embryonic development (Mima et al., 1995). FGFR1-deficient embryos can generate mesoderm but display severe early growth defects, not allowing an analysis of the effect on cardiomyocyte proliferation (Deng et al., 1994; Yamaguchi et al., 1994).

Transgenic mice that overexpress IGF-1 (Reiss et al., 1997) present cardiomyocyte hyperplasia. IGF-1 mutant mice die perinatally without a specific

cardiac phenotype (Baker et al., 1993; Liu et al., 1993). When these mice are rescued by mild expression of IGF-1 they survive to adulthood, and no heart phenotype is observed (Lembo et al., 1996). Therefore, while IGF-1 is capable of inducing cardiac myocyte proliferation, it is not clear that normal cardiac development requires this signal.

Cardiac muscle cell proliferation occurs in two distinct phases in murine development. While the first phase occurs during embryogenesis, the second phase occurs after birth, peaking at P 4-6 (Fig. 1.4.3.; Soonpaa et al., 1996). In the first phase, contractile cardiomyocytes are diploid, and mitosis and cytokinesis match DNA synthesis. Mitosis involves a complex process of disassembling and reassembling the contractile apparatus (Kasten, 1972; Rumyantsev, 1991; Borisov and Rumyantsev, 1991). The disassembly of the myofibrils seems to proceed after the entry into mitosis and may be associated with the activity of cyclin B/CDK1 complexes, which are active at that time. The second phase of proliferation occurs after birth. At this point, karyokinesis may occur in the absence of cytokinesis, and polyploid binucleate cells arise (Brodsky, 1991; Brodsky et al., 1980; Brodsky and Uryvaeva, 1977; Soonpaa et al., 1996). As described for hepatocytes, the pattern of polyploidization varies from species to species (Fig. 1.4.4 ; Brodsky, 1991). The proliferation of the mammalian cardiomyocyte during development resembles that described for the hepatocyte: there is proliferation at the same time as differentiation, and post-natal proliferation is associated with polyploidization. Brodsky suggested that the generation of polyploid cells in the heart might be different from the one described in the liver, since in the heart mononucleate diploid cardiomyocytes may give rise directly to polyploid mononucleate cells (Brodsky and Uryvaeva, 1977; Brodsky and Uryvaeva, 1985).

It is unclear why hepatocytes and cardiomyocytes become polyploid. It remains to be determined whether polyploidization presents any advantage for

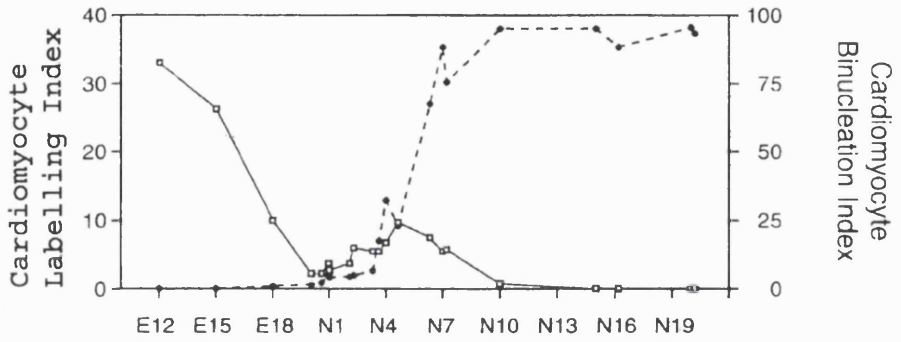

Figure 1.4.3 Cardiomyocyte DNA synthesis and binucleation during murine development. Experimental mice were given a single injection of tritiated thymidine. Four animals were analysed at each time point. A labelling index was obtained via autoradiography of isolated cell preparations, to allow proper identification of cells. Cardiomyocyte labelling index (left y-axis, open squares); cardiomyocyte binucleation index (right y-axis, dark circles). From Soonpaa et al. (1996).

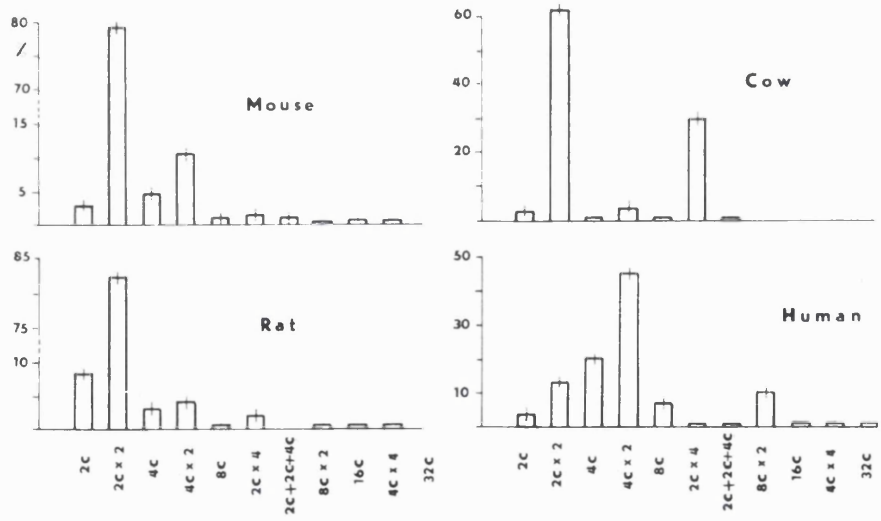

Figure 1.4.4 Heterogeneity of polyploid cardiomyocyte populations in the adult mammalian ventricle. The figure shows the percentage of cells in each category for each animal. 2C (C represents haploid DNA content), mononucleate cell with diploid nucleus; 2Cx 2 (the number after x represents the number of nuclei in the cell), binucleate cell with diploid nuclei; 2C+2C+4C, one cell with two diploid and one tetraploid nuclei. From Brodsky (1991).

these cells. Polyploidy may occur to amplify genes. Supporting this hypothesis is the observation that polyploid (4C) cardiomyocytes might have the 4 sets of genes active (Brodsky and Uryvaeva, 1985), thus being able to produce more contractile proteins. Polyploidization may thus allow the myocardium to increase its mass

more rapidly, with less disruption of the cytoarchitecture and requiring less intercellular junctions (Brodsky, 1991; Brodsky and Uryvaeva, 1985). Alternatively, polyploidization could be a side effect of proliferation in the post-natal environment. Some authors have proposed that binucleation may arise from the inability of the differentiated cardiomyocytes to properly disassemble their myofibrils during mitosis, precluding cytokinesis (Borisov and Rumyantsev, 1991). This may result from down-regulation of molecules from the cell cycle machinery, which are involved in the disassembly of the contractile apparatus. Mononucleate polyploid cells may result from down-regulation of mitotic cyclins and CDKs (McLellan and Schneider, 1999). Further studies are required to clarify these issues.

After the post-natal phase of proliferation and polyploidization, the muscle mass in the heart increases by hypertrophic growth of the cardiomyocytes (Fig. 1.4.5; MacLellan and Schneider, 2000; McLellan and Schneider, 1999).

1.4.2.2 Cardiomyocyte Proliferation in the Adult Mammal

In adult mammals, there is very little proliferation of the ventricular cardiomyocyte. For the normal adult mouse ventricle, estimates of DNA synthesis have ranged from 0.04% to 0.005% (MacLellan and Schneider, 2000; Soonpaa and Field, 1997; Soonpaa and Field, 1998). In several species, such as human, some limited capacity may be retained for cell cycle re-entry in response to mitogenic or hemodynamic stimuli, although this seems to be confined to a minute fraction of adult ventricular myocytes (Anversa and Kajstura, 1998; Beltrami et al., 2001; Kajstura et al., 1998). Further studies are necessary to quantify this response.

As consequence of the limited capacity of the mammalian ventricular cardiomyocyte to proliferate after injury, there is formation of a scar and induction of hypertrophy in the remaining cardiomyocytes, which can lead to pathology. The hypertrophic growth of cardiomyocytes can be initiated by growth factors, cardiac agonists, or passive mechanical stress, which promote the

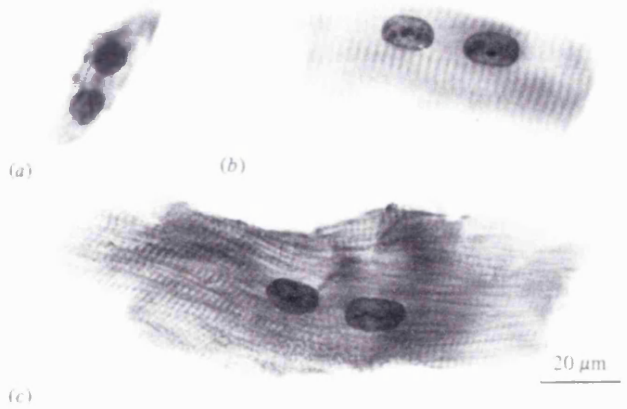

Figure 1.4.5 Hypertrophic growth of the mammalian ventricular cardiomyocyte. Binucleate cardiomyocytes from the heart ventricles of (A) a 1-day-old, (B) a 1-month-old, (C) a 1 year-old mouse, each photograph at the same magnification. From Brodsky and Uryvaeva (1985).

induction of immediate-early transcription factors such as Fos, Myc, and Jun (MacLellan and Schneider, 2000; Molkentin and Dorn, 2001). The expression of these genes is followed by characteristic changes in cardiac-specific gene expression, involving the reactivation of several genes not normally expressed in the adult ventricle but seen in the embryonic and neonatal heart, such as atrial natriuretic factor (ANF) and specific isoforms of contractile proteins (MacLellan and Schneider, 2000; Molkentin and Dorn, 2001).

In contrast to the ventricular cardiomyocytes, atrial cells can re-enter the cell cycle more easily after ventricular infarction or functional overloading (Borisov and Rumyantsev, 1991). This reactivation may be related to the fact that atrial cells are less differentiated, being smaller and with fewer myofibrils. Nevertheless, adult atrial cells do not seem to be able to undergo cytokinesis, and extensive polyploidization occurs after reactivation of these cells (Borisov and Rumyantsev, 1991).

Interestingly, adult ventricular cells may be more easily reactivated to undergo DNA synthesis when in culture (Claycomb, 1991; Claycomb and Bradshaw, 1983; Claycomb and Moses, 1988; Nag and Cheng, 1986), suggesting that there may be a

regulation of the differentiated state at the level of the extracellular matrix or the cytoskeleton, since the shape of the cells changes considerably when put in culture. The ability to undergo cytokinesis has not been described for these cells and further studies are necessary to quantify their ability to undergo karyokinesis.

Since the proliferation of ventricular cardiomyocytes *in situ* is so reduced, research has focused on trying to understand the regulation of the differentiated state in these cells. Here, I will review the regulation of the cell cycle machinery of mammalian cardiac muscle cells.

1.4.2.3 Regulation of the Cell Cycle and the Differentiated State in Cardiac Myocytes

It has been suggested that a cell intrinsic timer may control when cardiac myocytes withdraw from the cell cycle (Brodsky, 1991; Burton et al., 1999a). Studies in culture have shown that, in certain conditions, embryonic cardiomyocytes or stem-cell derived cardiomyocytes might withdraw from the cell cycle in a similar schedule to the one described *in vivo* (Burton et al., 1999a; Klug et al., 1995; Ueno et al., 1988). Additionally, sibling embryonic cardiomyocytes in culture tend to divide a similar number of times before they stop, whereas cells from different clones divide a variable number of times before they stop (Burton et al., 1999a). It has been proposed that this cell intrinsic timer may count time and not the number of cell divisions, because cardiomyocytes cultured at 33°C divide more slowly but stop dividing at around the same time as when cultured at 37°C (Burton et al., 1999a). *In vivo*, a similar result has been obtained. Newborn mice separated into groups of four or sixteen animals per single mother grew faster and slower, respectively. The liver and heart weights of these mice differed by a factor of 3-4 on the day of weaning. The animals were then fed equality. The difference in liver weight was eliminated, due to a temporal adjustment of the proliferation of hepatocytes. The definitive number of hepatocytes was established by 3 weeks of age in the fast-growing mice and by 3 months in the slow-growing ones. In contrast, proliferation of cardiomyocytes

ceased at 3-4 days after birth and polyploidization stopped at 3 weeks both in slow and fast growing mice, as if defined by an intrinsic temporal program. The fast growing mice had on average 20% more cardiomyocytes then their slow growing siblings (Brodsky and Delone, 1990; Brodsky, 1991). In another experiment embryonic cardiomyocytes placed in the kidney capsules of an adult mouse also proliferated and withdrew from the cell cycle in a similar schedule to the one observed *in situ* (Brodsky, 1991; Brodsky and Uryvaeva, 1985).

Extensive work has been done in order to characterise which molecules are involved in the timing mechanism and cell cycle exit in cardiomyocytes. Since there are no cell lines that recapitulate this process *in vitro*, most of these studies have been performed by looking at the expression, targeted disruption, or overexpression of several molecules involved in the cell cycle and differentiation. I summarise here the most informative experiments done in this field, which have been performed in mammals.

Cyclin D1 and D3 (Kang and Koh, 1997; Soonpaa et al., 1997), Cyclin E (Flink et al., 1998) and Cyclin B (Kang and Koh, 1997) are detected in embryonic myocardium and are down-regulated during the neonatal period, which correlates with cardiac myocyte cell cycle exit. Very low levels are detected in the adult (Flink et al., 1998; Kang and Koh, 1997; Soonpaa et al., 1997). Transgenic mice that over-express cyclin D1 in the ventricle, using the α-MyHC promoter, display a 40% increase in heart weight at 14 days after birth, ascribed to a twofold increase in cardiac myocyte number (Soonpaa et al., 1997). Adult ventricular cardiomyocytes of these animals are able to some extent to undergo DNA synthesis (170 times more cells undergoing DNA synthesis, as compared to non-transgenic control). The absolute magnitude is nevertheless small (0.05%; Soonpaa et al., 1997). Enhanced DNA synthesis in the adult cardiac myocytes of this animal was associated with multinucleation and the appearance of large, aberrant nuclei (Soonpaa et al., 1997). Although a detailed time course was not performed in this

study, multinucleation seemed to occur in the postnatal period: abnormal multinucleation profiles similar to those seen in the adult were also observed in flow cytometry analyses of 14d old transgenic animals (Soonpaa et al., 1997). As such, DNA synthesis in the adult may be dissociated from karyokinesis and cytokinesis, although further investigation of this question is necessary to support this hypothesis. Although this experiment points to an important role of cyclin D/CDK in the regulation of the cardiomyocyte cell cycle, it was not addressed whether the complex cyclin D1/CDK4 was active; the DNA synthesis observed could be the result of titration of the cell cycle inhibitors, p27 and p21 by cyclin D1.

CDK4, CDK2 and CDK1 are also down-regulated after birth (Flink et al., 1998; Kang and Koh, 1997). Overexpression of CDK2 in the mouse leads to a labelling index of 0.06% in adult ventricular cardiomyocytes, 100 times higher than that of age-matched wild type mice. Additionally, the cardiomyocytes of this transgenic mouse are less mature; 45% are mononuclear in comparison to 8% in the wild type and they express foetal markers, such as B-MyHC and ANF (Liao et al., 2001).

p107 is preferentially expressed during embryonic development in the ventricle; it is down-regulated after birth (Kim et al., 1994). p130 is expressed at low levels in the embryonic myocardium and continues to be expressed after birth in the adult (Chen et al., 1996a; Flink et al., 1998). pRb is expressed in low levels in embryos, just after birth, and in adults (Kim et al., 1994). pRb is phosphorylated in embryos, shifting to a less phosphorylated form after birth (Flink et al., 1998). Some authors have claimed that pRb is the most abundant pocket-protein in the adult myocardium (MacLellan and Schneider, 2000; McLellan and Schneider, 1999), though this aspect is still unclear. A cardiac-restricted deletion of the *Rb* gene has been implemented, using the Cre/lox system (MacLellan et al., 1998).

This approach resulted in small cardiac enlargement (8% at 8 weeks) (MacLellan et al., 1998), but further detail has still not been published.

p18, p57, p21 and p27 have been shown to be expressed in the mammalian heart (Burton et al., 1999a; Burton et al., 1999b; Poolman et al., 1998). p18 is detectable in the rat heart from E18 onwards, and after birth, but no longer in the adult heart (Burton et al., 1999a). p18 may play a role in cell cycle exit, since p18-deficient mice develop gigantism and widespread organ enlargement (Franklin et al., 1998). Additionally, p18 is induced by T_3 in an embryonic cardiomyocyte culture, where T_3 has been shown to be important for permanent cardiomyocyte cell cycle withdrawal (Burton et al., 1999a). It has been suggested that p18 might be part of the machinery that leads cardiomyocytes to cell cycle withdrawal, but not for the maintenance of the stability of the differentiated state, since it is not expressed in the adult heart (Burton et al., 1999a).

p27 is expressed throughout development and in the adult heart (Burton et al., 1999b; Flink et al., 1998; Koh et al., 1998; Poolman et al., 1998). Interestingly, its cellular localisation changes from cytoplasmic in the embryo, to nuclear in the adult, which may be related to a role in maintaining the stability of the differentiated state (Koh et al., 1998). Equal change in localisation has been observed in the cell cycle withdrawal of cardiomyocytes in culture (Burton et al., 1999a). Mice lacking p27, as described for the p18-/- mouse, show gigantism including a bigger heart (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996).

p57 is highly expressed during rat heart development, but its expression decreases after birth, and is not detected in the adult (Burton et al., 1999b; Koh et al., 1998). p21 is expressed during heart development and its expression increases after birth and is maintained in the adult (Burton et al., 1999b; Horky et al., 1997; Poolman et al., 1998). Mice that lack p21 are phenotypically normal (Deng et al., 1995).

The lack of a dramatic phenotype in the mutant mice described above is presumably secondary to redundancies in the action of molecules involved in the cell cycle. Accordingly, double mutant mice for p18 and p27 show a greater enlargement of the heart, as compared to the single mutants (Franklin et al., 1998). Further knowledge should come from studies of double-mutant mice, the development of conditional mutant mice and the establishment of cell culture systems that may reflect the withdrawal of cardiomyocytes from the cell cycle.

Which molecules could be involved in preventing cardiomyocytes from proliferating after cell cycle withdrawal? As for other cell types described in the previous section (1.3.2), the most informative experiments have come from the use of viral proteins. The first of these tumour suppressor-binding proteins to be studied in cardiac muscle was SV40 large T antigen. Expression of T antigen in cultured neonatal rat cardiac myocytes resulted in proliferation (Sen et al., 1988).

Similar studies have been performed using E1A. E1A reactivates DNA synthesis and down-regulates cardiac-restricted promoters in embryonic and neonatal cardiomyocytes (Hasegawa et al., 1997; Kirshenbaum and Schneider, 1995; Liu and Kitsis, 1996). In consequence, cells are blocked in G2 and undergo apoptosis. The use of mutant forms of the E1A protein has allowed the dissection of the molecular pathways involved in maintaining the post-mitotic arrest in the cardiomyocytes (Fig. 1.4.6). Mutations that disrupt E1A-pocket protein binding, but not E1A-p300 interaction, remained effective for induction of DNA synthesis in embryonic cardiomyocytes (Liu and Kitsis, 1996). Analysis of p300 expression during embryogenesis shows ubiquitous expression, including in the heart (Yao et al., 1998). The molecular function of p300 might be complex, since p300-deficient mice die *in utero*, with a hypoplastic ventricle with poor trabeculation and reduced rates of DNA synthesis (Yao et al., 1998). In neonatal rat cardiomyocytes inactivation of one of the pathways, the pocket protein or the p300, is sufficient to trigger cell cycle entry (Fig. 1.4.6 in black and blue; Akli et al.,

1999). It may be that p300 is more important in the regulation of the cell cycle in embryonic cells, and that both p300 and pocket proteins play a role in maintaining the post-mitotic arrest in the cardiomyocytes after birth.

The role of pocket proteins in maintaining the post-mitotic arrest in cardiomyocytes has been further demonstrated by overexpression of E2F. As E1A, E2F induces DNA synthesis accompanied by apoptosis in neonatal cardiomyocytes *in vitro* (Kirshenbaum et al., 1996) and of adult cardiomyocytes *in vitro* and *in vivo* (Agah et al., 1997a), with arrest in G2. E2F may have targets other than cyclin E, since overexpression of cyclin E is not sufficient to induce cell cycle re-entry in neonatal rat cardiomyocytes (Fig. 1.4.6 in green; Akli et al., 1999). Interestingly, E1A binding to pocket proteins has effects beyond those produced by E2F-1 alone, which could be due to the potential involvement of other endogenous pRb-binding proteins or of alternative E1A targets (Fig. 1.4.6 in red; Akli et al., 1999). In fact, in contrast to E2F-1, an E1A mutant that binds pocket proteins can drive S-phase entry in the presence of p21, and independent of an increase in CDK2 function (Akli et al., 1999).

Induction of hypertrophy by pressure overload leads to down-regulation of p27 and p21 (Burton et al., 1999b; Li and Brooks, 1997) and up-regulation of cyclin D2, D3 and CDK4,6 (Brooks et al., 1998; Li et al., 1998; MacLellan and Schneider, 2000; Tamamori et al., 1998). *In vitro*, hypertrophic agents also induce the activity of cyclin D-CDK complexes (Tamamori et al., 1998). As such, cardiac hypertrophy may re-use the same pathways associated with the G1 phase of the cell cycle in order to promote cellular growth. This is in agreement with the fact that signalling pathways that regulate G1 players during proliferation, such as ras signalling, are involved in hypertrophy (Sugden and Clerk, 2000). It is still not clear what are the effectors downstream of the cyclin D-CDK complexes that are important for hypertrophy and whether pRb may be involved in the hypertrophic response.

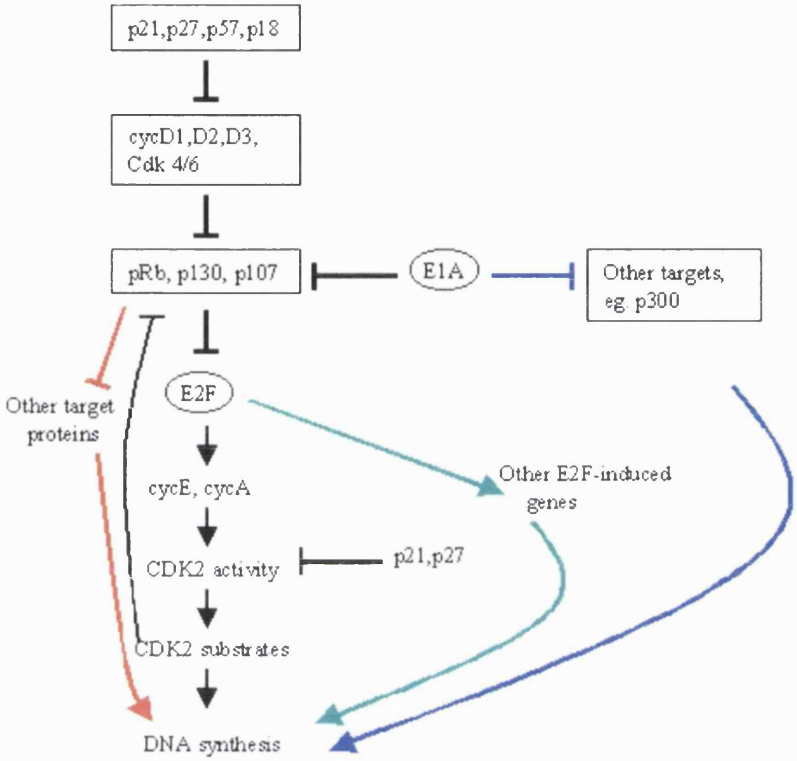

Figure 1.4.6 Regulation of the post-mitotic state in mammalian cardiomyocytes; pathways affected by the E1A protein. Adapted from MacLellan and Schneider (2000) and Akli et al. (1999).

In VSMCs, where both types of growth, hyperplastic and hypertrophic, co-exist, down-regulation of the inhibitors is the crucial point for cell cycle re-entry to occur (section 1.3.3). Despite down-regulation of the same inhibitors, little DNA synthesis seems to occur during the hypertrophic response in cardiac myocytes (Brooks et al., 1998; Soonpaa and Field, 1994; Soonpaa and Field, 1997). It may be that other changes are necessary for induction of DNA synthesis. Further study of conditional mutant mice for molecules involved in the cell cycle and differentiation, and further dissection of the cellular interactions of E1A and large T-antigen proteins, should help to clarify the complex regulation of the differentiated state in mammalian cardiomyocytes.

1.4.3 Heart Regeneration and Cardiomyocyte Proliferation in Lower Vertebrates

Cardiomyocytes from lower adult vertebrates, such as those from frogs and newts, have been shown to proliferate after injury to the ventricle (Oberpriller and Oberpriller, 1991; Rumyantsev, 1991), while control uninjured ventricles do not show a proliferative response (Oberpriller and Oberpriller, 1991; Oberpriller et al., 1989; Oberpriller et al., 1995; Rumyantsev, 1973; Rumyantsev, 1991). The proliferative response of the ventricular cardiomyocyte in the frog was only addressed *in vivo*, and there are some limitations to these studies, as I will discuss below. There is an extensive body of work characterising the proliferative response of the newt cardiomyocyte *in vivo*, and some experiments were also done to pursue this problem *in vitro*.

The amphibian ventricle has little connective tissue and no blood vessels, having a trabeculated structure instead (Bader and Oberpriller, 1979; Oberpriller et al., 1995). Comparison of the amphibian ventricular myocytes to their mammalian counterparts shows that amphibian cardiomyocytes have fewer myofibrils, have smaller and less complex gap junction complexes, and probably no T-tubule system (Millhouse et al., 1971; Sommer and Johnson, 1969). Moreover, adult newt cardiomyocytes are mononucleated with diploid nuclei (Oberpriller et al., 1995).

1.4.3.1 Regeneration in the Frog's Heart

Ventricles of frogs from the species *Rana temporaria* were crushed with thin forceps so that practically all muscle fibres between the mid and apical parts of the ventricles were damaged (Rumyantsev, 1973). Cardiomyocytes near the wound underwent DNA synthesis and mitosis. Increase in the size of nuclei and nucleoli was seen in cardiomyocytes around the wound 3 days after injury. This was proposed to be related to reactivation of the chromatin (Rumyantsev, 1973). This has also been shown for myofibre nuclei during regeneration in axolotls (Hay, 1959). Cardiomyocytes around the wound showed dedifferentiation, as

some of the myofibrils were loose, forming free myofilament bundles. Dedifferentiation was maximal in the second and third week after injury (Rumyantsev, 1973).

The time course of the cardiomyocyte proliferative response was analysed. Single or multiple injections of ^{3}H-thymidine were given to the animals at different days after injury, and the percentage of labelled cardiomyocytes was assessed in the vicinity of the damaged zone (Fig. 1.4.7, Rumyantsev, 1973). The identification of cardiomyocytes in this study was based only in their structure under the light microscope. Additionally, there was no quantitative definition of "vicinity of the damaged zone". This study has shown that the proliferative response starts at around 5 days after the injury and is maximal during the second and third week (Fig. 1.4.7). It was also stated that cardiomyocytes located far from the site of injury did not resume DNA synthesis (Rumyantsev, 1973).

Interestingly, granulation and scar tissues formed and proliferated within the wound (Rumyantsev, 1973). Rumyantsev suggested that this scar tissue, together with the reduced ability of cardiomyocytes to move into the wound, might be responsible for the incomplete myocardium restoration observed in these animals after injury (Rumyantsev, 1973).

In a similar experimental set up, the ventricle of *Agama caucasica*, a lizard, was crushed with thin forceps near the *apex cordis*, causing extensive damage to the muscle fibres. Upon cumulative injections of ^{3}H-thymidine into the animal, it was shown that 73% of the cardiomyocytes near the injured zone would incorporate the label. Again, there was no quantitative definition of the area occupied by this zone. Cardiomyocytes located far from the site of injury did not resume DNA synthesis (Rumyantsev, 1991).

1.4.3.2 Proliferation of the Newt Cardiomyocyte

The early events of heart regeneration in the newt have been described by Becker and colleagues (1974). These authors observed that, upon removal of 30 to

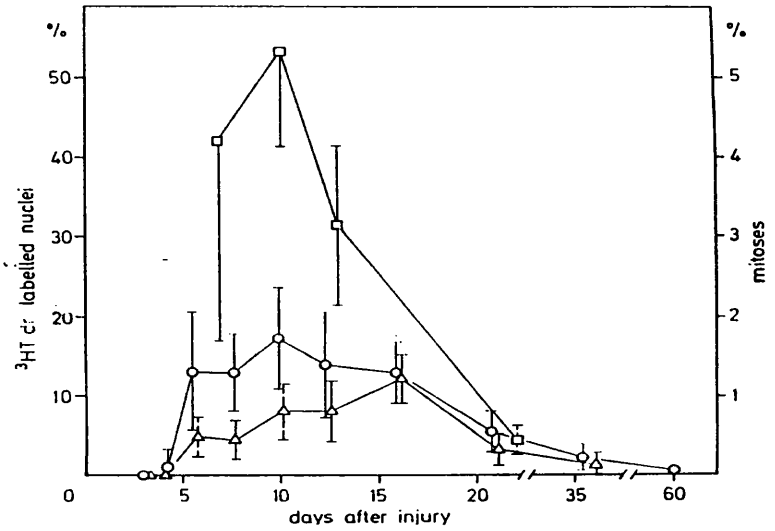

Figure 1.4.7 Percentages of cardiomyocyte mitosis (triangles) and of nuclei labelled after a single or three successive ^{3}H-thymidine injections (circles and squares, respectively) in the injured frog ventricle. Only myocytes in the vicinity of the damaged zone of the frog ventricle were counted. Continuous vertical bars are 95% confidence limits, discontinuous being standard errors of the mean. From Rumyantsev (1973).

50% of the adult newt ventricular myocardium, there was pronounced contraction of the myocardium at the amputated site, resulting in a decrease of the diameter of the wound. Additionally, the bleeding was initially very vigorous, decreasing rapidly, and ceasing with clot formation at approximately 60 sec (Becker et al., 1974). The authors followed the circulation of blood after amputation, by observing the movement of blood cells in skin capillaries in the tail fin. They observed that there was complete circulatory stasis within 5 min of the amputation, and that slow directional movement began generally after 90 to 120 min. Circulation became normal 3-5 hrs after amputation (Becker et al., 1974).

Becker and colleagues (1974) also described that a clot would initially fill the gap left by amputation, but that gradually, different types of cells would appear, such as cardiomyocytes. They claimed that "by 24 hours, sections appeared completely normal and there was no evidence of injury other, than, occasionally, a small mass of degenerating erythrocytes exterior to the myocardium proper " (Becker et al., 1974). It must be said that no figure was shown in this paper to

support this statement. As I will describe here, subsequent observations of the regeneration of the newt ventricle showed that this process takes much longer.

The majority of the work done in the field of newt heart regeneration was performed in the laboratory of Oberpriller and colleagues. These authors have shown that upon amputation of the apex of the newt ventricle, there was induction of blood clot formation, coagulation necrosis, macrophage activity and the formation of a connective tissue scar with some proliferating myocytes in the place of the injured muscle (Oberpriller and Oberpriller, 1971; Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995).

In an initial experiment, these authors cut 1/8th of the newt ventricle at its apex, closed the body wall incision, and killed the animals at different days, from 6 to 30 days after amputation. Animals were injected with ^{3}H-thymidine 3 hours prior to killing (Oberpriller and Oberpriller, 1974). At 6 days, a blood clot spanned the gap created in the ventricular wall, and there were many degenerating red blood cells. There was also an increase in the cytoplasmic density of the cardiac muscle adjacent to the clot. This was named coagulation necrosis, as it was thought to be necrosis induced by the clot. At 16 days, there were macrophages and leukocytes in the clot, there was no more coagulation necrosis of the cardiomyocytes, and there were mitotic cardiomyocytes in the trabeculae around the wound, as identified by ultrastructural analysis. There was a great increase in the number of connective tissue fibres in the wound area at 20 days. At this time point, ultrastructural analysis indicated that several myofibril-containing cells were labelled with ^{3}H-thymidine. The authors claimed that the most probable origin of these cells is from cardiomyocytes in the trabeculae adjacent to the wound area, since no reserve cells were observed in the newt heart. At 30 days, the wound area was mainly composed of loose connective tissue extending into and within the wound. Scattered cardiomyocytes were also present, as identified

by ultrastructural analysis, which had fewer and smaller myofibrils than control animals (Oberpriller and Oberpriller, 1974).

After the localised response of the newt cardiomyocyte was described, the authors thought there would be an increase in the proliferative response if more cardiomyocytes were exposed to the wound. This was achieved by mincing the amputated apical region of the ventricle and replacing it onto the amputation gap. There was an increase by two fold in the proliferation of newt cardiomyocytes in this experimental set up (Bader and Oberpriller, 1979; Oberpriller and Oberpriller, 1991). At 70 days, these grafts did not appear as a series of individual graft pieces held back together at the edge of each piece, but they appeared instead as a continuous muscular structure covered by epicardium and lined with endocardium, forming partial new wall, which seemed to be functional in some cases (Fig. 1.4.8 A; Bader and Oberpriller, 1979; Oberpriller and Oberpriller, 1991). This minced system has allowed the detailed examination and quantitation of the proliferative events.

A time course of the proliferative events was performed. The tip of the ventricle was cut, minced and grafted back into the ventricle. Animals were sacrificed one hour after being injected intraperitoneally with ^{3}H-thymidine. At 15 days, 80% of the cells found in mitosis in the minced muscle were cardiomyocytes, as assessed by electron microscopy (Bader and Oberpriller, 1979). The percentage of labelled cells in the minced graft (morphological criterion were used to distinguish cardiomyocytes from other cells) was determined (Fig. 1.4.8 B, Bader and Oberpriller, 1979). The results found are very similar to the ones described earlier by the same authors (Bader and Oberpriller, 1978), showing that there is a peak of DNA synthesis at around 16 days after mincing the muscle. The response of the newt cardiomyocytes seems similar to the one of the frog, with the peak of proliferation occurring in the second and third week after injury.

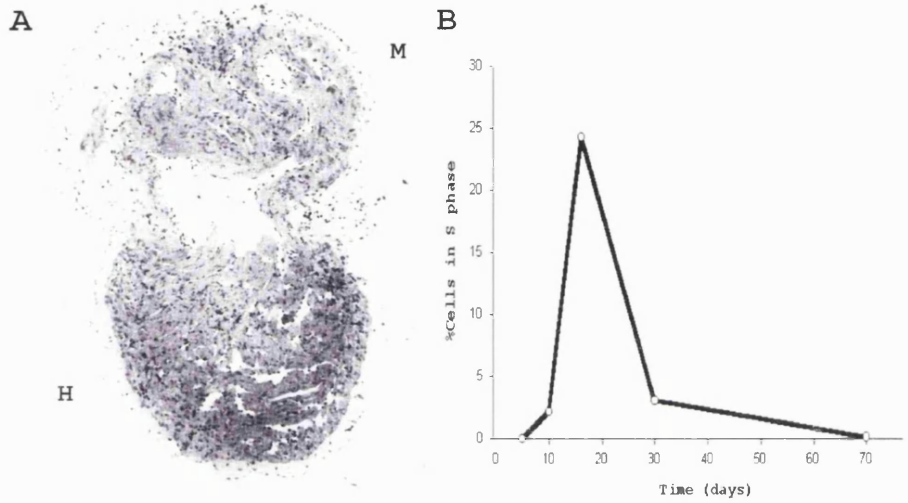

Figure 1.4.8 Minced ventricle experiment. (A) Graft of minced ventricle 45 days after mincing. The minced area (M) can be seen where there has been partial reorganisation of the minced myocardium containing areas of excessive connective tissue growth, especially in the epicardium. H-host area. From Oberpriller and Oberpriller (1991). (B) Proliferative response of the ventricular cardiomyocyte in the minced graft experiment. Animals were sacrificed one hour after being injected intraperitoneally with tritiated thymidine. The percentage of labelled cells in the minced graft (morphological criterion used to distinguish cardiomyocytes from other cells) was determined. The results found are very similar to the ones described earlier by the same authors Bader and Oberpriller (1978). Data from Bader and Oberpriller (1979).

Dedifferentiation of the newt cardiomyocyte upon injury, as discussed above for the frog cardiomyocyte, was described *in vivo* and in organ culture. Bader and Oberpriller (1979) described how cardiomyocytes from the minced cardiac muscle grafts appeared to undergo partial dedifferentiation, involving the extensive loss of myofibrillar structure. At 70 days after mincing and grafting, when proliferation had ceased, cardiomyocytes had regained the differentiated appearance typical of adult newt cardiomyocytes of non-injured newt hearts (Bader and Oberpriller, 1979). Other authors cut the newt ventricle in small pieces of approximately 0.5-1 mm and placed them in culture in media containing 10% foetal bovine serum (FBS; Nag et al., 1980; Nag et al., 1979a; Nag et al., 1979b). Concomitant with DNA synthesis and mitosis, they also observed the disruption

of the myofibrillar organisation, resulting in the distribution of scattered patches of myofibrils and free myofilaments (Nag et al., 1980; Nag et al., 1979a; Nag et al., 1979b). Although the proliferative response had stopped after two months in culture, the authors did not observe a reorganisation of the myofibrils as observed *in vivo* (Nag et al., 1980). This could be due to the constant presence of signals promoting dedifferentiation in the culture media.

There was some indication that polyploidy and multinucleation can occur in newt cardiomyocytes since, 45 days after amputation and mincing of the newt heart, 7% had a polyploid nucleus and 4% of the cardiomyocytes were binucleate. In control animals, 1% of the cardiomyocytes had a polyploid nucleus and less than 1% were binucleate (Oberpriller et al., 1989). This issue was not addressed in the studies performed in the frog ventricle.

After amputation and mincing of the tip of the newt ventricle, DNA synthesis of cardiomyocytes was significantly higher in the minced muscle and limited to a radius of 500μm of the wound (Oberpriller et al., 1989). These data suggest that the stimulatory signals for cardiomyocytes may arise at the site of injury. When the newt atrium was directly injured, there was also formation of a connective tissue scar with proliferating myocytes localised within 500 μm of the wound (McDonnell and Oberpriller, 1984). In contrast, upon ventricle injury, there was spatially diffused induction of DNA synthesis and mitosis in myocytes in the atria (McDonnell and Oberpriller, 1983), indicating that different types of stimuli may operate on the cardiomyocytes from the atria.

More data has arisen from tissue culture studies, demonstrating that dissociated and cultured ventricular cardiomyocytes from adult newts are able to synthesise DNA and undergo mitosis and cytokinesis while maintaining some contractibility (Nag et al., 1979b; Tate et al., 1987; Tate and Oberpriller, 1989; Tate et al., 1989). After I had started my PhD, it was shown that multinucleation of

newt cardiomyocytes due to acytokinetic mitosis may also happen in culture (Matz et al., 1998).

It has been shown that DNA synthesis in cardiomyocytes cultures might be stimulated by growth factors such as PDGF, FGF-1 and FGF-2 while others, such as TGF β, might repress it (Oberpriller et al., 1995; Soonpaa et al., 1992; Soonpaa et al., 1994). Unfortunately, the data from these studies may have little significance, since those experiments were done in the presence of media containing 10% FBS, which contains a variety of growth factors and might itself induce proliferation. Additionally, the effect reported by the authors was rather small (20% for PDGF for example; Oberpriller et al., 1995; Soonpaa et al., 1992).

1.5 Focus for Experiments in this Thesis

The amphibian adult ventricular cardiomyocyte is one of the few well documented systems where an adult cardiomyocyte has been shown to divide. No other adult system has been shown to be as responsive to injury and as proliferative when placed in culture. It is not clear whether there is a particular group of less differentiated cells in the amphibian heart that is responsible for the response to injury. Additionally, it is not known whether these newt cells are able to respond to injury signals because they have different receptors, different intracellular regulation or both. Urodeles possess general high regenerative capacity and plasticity of the differentiated state, and because of the work described above, provide a potential model system to study heart regeneration and regulation of its differentiated state. There are similarities in the proliferative response of the cardiomyocyte and other differentiated newt cells, such as the localisation near the injury site. This may indicate that a similar signal may be responsible for their proliferation. Clarification of the cellular and molecular mechanisms involved in the newt cardiomyocyte response to injury may bring additional insight to urodele regeneration, and to the regulatory mechanisms that do not allow cardiomyocytes to proliferate in the adult mammalian heart.

The main purpose of this PhD was to investigate the cellular and molecular mechanisms involved in the plasticity of the differentiated state of newt ventricular cardiomyocytes. I was interested in two aspects of the plasticity: the ability to go back into the cell cycle and the regulation of the phenotype of these cells, namely whether they would be able to transdifferentiate into other cell types. Since it had been shown that newt cardiomyocytes can re-enter the cell cycle in culture, I decided to establish a culture system in order to address the first question. The first experimental chapter of this thesis (Chapter 3) describes the establishment of this culture and its characterisation regarding the differentiated phenotype, the timing of the proliferative response and the stimuli that trigger

that response. To address the second question, I decided to implant fluorescently labelled, purified newt cardiomyocytes into the regenerating tail blastema of the *Amblystoma maculatum* larvae. As discussed in this introduction, the environment of a regenerating blastema plays an important role in evoking plasticity of the differentiated state, providing cells with the signals to re-enter the cell cycle and to re-differentiate into different cell types. Unfortunately, difficulties in correctly identifying the implanted cells, due to the existence of endogenous fluorescent cells in the majority of the animals, led me to abandon this project and focus solely on the regulation of the proliferative response of these cells.

Since it appeared that in the culture system the cells were proliferating in a similar schedule to the one described in vivo, I decided to address the cellular regulation of the proliferative response of the newt cardiomyocyte in this culture (Chapter 4). This has allowed the use of time-lapse microscopy to study the response at the single cell level. The results obtained showed that the majority of the newt cardiomyocytes can re-enter the cell cycle, and that their individual proliferative potential may be heterogeneous. This heterogeneity can explain at the cellular level results previously obtained after injury to the newt heart *in vivo*.

Since I had shown that the majority of the newt cardiomyocytes could re-enter the cell cycle, I decided to focus on the molecular regulation of this response. The study with myoseverin has proposed the hypothesis that there may be a molecular program of regeneration that is triggered upon injury (Rosania et al., 2000). It is tempting to think that re-entry into the cell cycle may be part of that program. Two players in a possible pathway for plasticity have been described in the newt myotube culture. Phosphorylation of pRb, a major regulator of the post-mitotic state (see section 1.3), upon serum stimulation, plays a pivotal role in cell cycle re-entry (Tanaka et al., 1997). Additionally, the factor (Fa) that induces DNA synthesis is a product of thrombin proteolysis of serum (Tanaka et al., 1999). I decided to test the hypothesis of whether this pathway is conserved in the newt

cardiomyocyte. The fact that the response of the newt cardiomyocyte to injury is localised to the area surrounding the wound, and that I had shown (Chapter 3) that mammalian serum stimulates re-entry by these cells, made that hypothesis more attractive. The experiments studying the regulation of pRb and Fa in the newt cardiomyocyte are described in the fifth chapter.

-Chapter 2-

MATERIALS AND METHODS

2.1 CELL CULTURE

All the cells and solutions were hand held inside a tissue culture hood using aseptic techniques.

2.1.1 General

2.1.1.1 Amphibian Media and Incubator

All the solutions used for newt cell cultures were diluted with distilled water (dH_2O) for urodele osmolarity, as previously described by Ferretti and Brockes (1988). Two different media were used: amphibian modified Eagles minimum essential media with Earle's salts (AEMEM) and amphibian modified Leibovitz's L15 (AL15). AEMEM is composed of 65% EMEM with Earle's salts (Gibco); 50 i.u/ml penicillin; 50 μg/ml streptomycin; 0.28 i.u./ml insulin; 0.29 mg/ml glutamine) and 10% heat-inactivated (56°C, 30 minutes) foetal bovine serum (FBS, First Link). AL15 medium is composed of 65% L15 (Gibco); 50 i.u/ml penicillin (Gibco); 50 μg/ml streptomycin (Gibco); 0.28 i.u./ml insulin from bovine pancreas (SIGMA); 0.29 mg/ml glutamine (Gibco) and 10% heat-inactivated foetal bovine serum (FBS, First Link). L15 is an aminoacid buffered medium, supporting cell growth in non-CO_2 equilibrated environment. AL15 was used for long-term maintenance of the cells outside the incubator, such as microinjection and time-lapse microscopy. Phosphate buffered saline was also diluted to amphibian osmolarity (APBS) (75% PBS, 25% dH_2O).

Cells were always kept in a humidified atmosphere in an incubator at 25°C, 2% CO_2 in air (to equilibrate the pH of AEMEM at 7.4-7.6 as described by Ferreti and Brockes (1988)). Whenever AL15 was used inside the incubator, flasks were sealed with parafilm to prevent gas exchange.

2.1.1.2 Counting Cells

After resuspension in 1-5 ml of medium, cells were counted on a haemocytomer with 4 chambers of 10^{-4} ml. The number of cells in the four

chambers was averaged, and the final cell number was calculated from the number of cells per 10^{-4} ml and the total volume in which the cells were resuspended.

2.1.2 Primary Culture

2.1.2.1 Animal Maintenance

Adult *Notophthalmus viridescens* were obtained from Charles Sullivan and Co. (Nashville Tennessee, USA). Newts were kept at 12-14°C in tanks containing 30-100 animals and fed twice a week on bloodworms.

2.1.2.2 Harvesting Ventricles

Newts were anaesthetised by immersion for 10-15 min in 0.1% w/v Tricaine (3-aminobenzoic acid ethyl ester; SIGMA). Newts were sterilised by dipping in 1.2% sodium hypochlorite for 2 min, after which they were rinsed in sterile water. Under the stereomicroscope, the upper chest was opened and the heart was taken out with forceps. The ventricle (white as compared to the atria) was cut with scissors and put inside a flask with AL-15 medium (with no serum or insulin) and 50μg/ml gentamycin[1] (SIGMA). This media was changed once, to take blood and debris out and the flask was sealed with parafilm and stored overnight in the incubator.

2.1.2.2 Dissociation of the Ventricles

After an overnight incubation, ventricles were placed in a round/flat bottomed vial with 2mls of a sterile enzyme solution containing: 0.5% BactoTrypsin (Difco), 380U/ml Collagenase (E.C. 3.4.24.3- SIGMA), 0.15% Bovine albumin (Fraction V- SIGMA), 0.30% D-glucose (BDH) and 50 μg/ml gentamycin (SIGMA) diluted in APBS. The tissues were dissociated in a shaking waterbath

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[1] Gentamycin was added in all the steps until the cells were plated as it was found that it would avoid the frequent contamination of newt cardiomyocytes by *Pseudomonas aeruginosas.*

(GRANT) at 27°C with 90 rotations/min for 2 hrs. Thereafter the speed was reduced to 60 rotations/min for another 6 hours. The enzyme solution was changed every two hours. At 4 and 6 hrs the contents of the flask were gently triturated, the solution was removed and fresh enzyme solution was added. The solution removed at 4 hrs was discarded since I had observed that most of the cells in that fraction were not cardiomyocytes (by immunofluorescent staining for sarcomeric myosin heavy chain). Solutions removed at 6 and 8 hrs were neutralised by the addition of 4 mls AMEM-10% FBS, and pooled together. Solutions were passed through a 100μm microsieve to remove clumps of cells (Cell microsieve; Biodesign, Inc. NY). The microsieves were made by placing squares of a 100μm mesh (2cm x 2cm) over 20 ml universal vials and depressing the mesh in the centre to form a funnel shape. Meshes were attached to the vials at three points by heating with the tip of an incandescent scalpel. The solution that passed through the mesh was centrifuged on a table top centrifuge at 400 RPM (27*g*) for 10 min. The pellet was collected and cells were resuspended in AMEM-10% FBS and preplated in 10 cm non-coated culture dishes for app. 3 days. At the end of this period most of the non-myocytes had attached to the culture dish. This procedure resulted in cultures in which greater than 90% of the cells were cardiomyocytes.

2.1.2.3 Culturing Newt Cardiomyocytes

Tissue culture dishes were pre-coated, first with poly-L-lysine then with laminin. Poly-L-lysine (20μg/ml in distilled water) was added to tissue culture dishes (Nunc). Fourty five minutes afterwards the solution was aspirated. After the dish was dry, laminin (SIGMA; from basement membrane of Engelbreth-Holm-Swarm Mouse sarcoma; 15 μg/ml in serum free media) was added to the dish. After 60 min the solution was aspirated and the cells were added.

Media containing the preplated newt cardiomyocytes was centrifuged on a table top centrifuge at 400 RPM (27*g*) for 10 min. Cells were counted, resuspended

in AMEM (usually AEMEM-10%FBS unless otherwise stated) and plated at a density of approximately 4000-5000 cells/cm^2.

For long-term observation of cells, cells were plated in tissue culture dishes where a grid (app. 0.2-0.5 mm^2) was scored with a scalpel and was oriented (A-Z; 1-30). Orientation of the grid was performed with a sterile needle (25G; Microlance) by using a Narishige MMO 202 micromanipulator mounted on a Zeiss Axiovert 135 microscope.

2.1.3 Newt Myogenic Cells

Newt A1 cells, originally isolated from muscle explants (Ferretti and Brockes, 1988), were grown as monolayers in AEMEM on gelatine-coated plastic culture flasks (0.75% w/v porcine skin gelatine in H_2O, SIGMA).

2.1.3.1 Passaging

Cells were washed in 2-5 ml APBS, and digested with 1-3 ml trypsin/EDTA 0.05% (Gibco) diluted from a 10X stock to 1X using APBS. The enzyme was neutralised by the addition of 2-5 ml AEMEM-10%FBS. A cell pellet was collected by centrifugation on a table top centrifuge at 1000 RPM (179 *g*) for 3 min, and the cells were resuspended in 1-5 ml AEMEM-10%FBS. A1 cells were passaged once a week. After trypsinization, cell pellets were resuspended in AEMEM-10% FBS and counted. Typically $2x10^5$ A1 cells were plated on a 162 cm^2 tissue culture flask.

2.1.3.2 Myotube Formation

A1 cells were plated on gelatine-coated 100 mm or 60 mm tissue culture dishes scored with a sterile scalpel to form a fine mesh pattern in order to limit the size of the myotubes. The cells were grown to high density, so that almost no cytoplasm was visible between the cell nuclei. Fusion was induced by lowering the serum concentration in the medium to 0.5% for 5 days.

2.1.3.3 Myotube Purification

The myotubes were sieved for purification from mononucleate cells, as described previously (Lo et al., 1993; Tanaka et al., 1999). The myotubes were digested with trypsin, and resuspended in 5 ml AEMEM-10%FBS The suspension was sieved through a 100 μm pore size nylon mesh (Biodesign, Inc., New York). Cells clumps retained by the mesh were discarded and the flow through fraction was sieved through a 35μm mesh (does not retain mononucleate cells). The myotubes retained by the 35 μm mesh were rinsed off the mesh by inverting it onto a 60 mm tissue culture dish with 2 ml of AEMEM (0% or 10 % serum), and rinsing the mesh with another 2 ml of the same medium. The cells were plated onto 35 or 60 mm dishes coated with gelatine or fibronectin (20 μg/ml, 1hr, room temperature).

2.1.4 Labelling of Cardiomyocytes with PKH-Cell Tracker Dye

The PKH-26 fluorescent cell tracker dye (SIGMA) is stably incorporated into cell membranes (Horan and Slezak, 1989). After preplating, cardiomyocytes were centrifuged and the pellet was resuspended in AEMEM-0%FBS (lipids in the serum bind to the dye). The cells were centrifuged again and resuspended in the diluent supplied with the PKH-labelling kit. The cell suspension was added to the dye solution ($3x10^{-6}$ M) and incubated at room temperature for 5 min with mixing at 1 min intervals. Staining was arrested by adding an equal volume of serum and incubating for 1 min. This was followed by dilution in AEMEM 10%FBS and two washes by centrifugation and resuspension in AEMEM 10%FBS.

For lineage tracing experiments the PKH-labelled cells were then mixed with unlabelled cells (1:24). A total of $4x10^4$ cells were seeded on a 35 mm tissue culture dish scored with a grid, thereby preventing the movement of the cells in between squares. Under the inverted microscope, I ensured that only one mononucleate labelled cell was present per square by removing others, using a microinjection pipette attached to a micromanipulator (Narishige MMO 202) mounted on a Zeiss Axiovert 135 microscope.

2.1.5 BrdU, ^{3}H-and ^{14}C-Thymidine Incorporation

Cells were labelled by adding 3-bromo-2-deoxyuridine (BrdU; SIGMA) 10 μg/ml. For experiments with radioactive-labelled thymidine an initial concentration of 1 μCi/ml of ^{3}H-thymidine was used. As no cells under observation underwent mitosis, conditions were optimised, and a detectable signal with no inhibitory effect on cell growth was obtained with ^{14}C-thymidine at specific activity of 0.54 mCi/mmol, 0.01μCi/ml (see next section). This was particularly important for experiments presented in Chapter 4. In cases where the labelling was performed for more than 3 days, fresh media and label were added every 3 days.

2.1.5.1 Titration of the Concentration of Labelled Thymidine

In parallel experiments, with or without ^{3}H-thymidine, cell division was observed only in the latter. There had been previous reports of negative effects of radiolabelling with ^{3}H-thymidine on cell cycle progression, such as cell cycle arrest, accumulation of p53 and DNA fragmentation (Solary et al., 1992; Yeargin and Haas, 1995). A separate experiment was carried out to assess the toxic effect of ^{3}H-thymidine on the cultured cardiomyocytes.

Cells were cultured in the presence of the same concentration of thymidine at different specific activities. The solution was added to the cells and 3 days afterwards the cells were fixed and stained for phosphorylated histone H3, a marker for mitosis (P-Histone H3; Hendzel et al., 1997; Velloso et al., 2000; Wei et al., 1999). The effect of ^{14}H-thymidine was also tested, since it had been reported to have less adverse effects than ^{3}H-thymidine in muscle precursor cells (Beauchamp et al., 1999). Initial experiments were performed in A1 cells. It was observed that the isotope was inhibiting proliferation, since the arrest in proliferation was dependent on the concentration of isotope but not thymidine (which was constant; see table 4.1 for a typical experiment). This inhibitory effect was common to ^{14}C and ^{13}C, although it seemed greater for ^{3}H-thymidine (Table 2.1). No arrest was seen with ^{14}C-thymidine at a specific activity of 0.54

mCi/mmol, 0.01μCi/ml (after dilution in the media), hundred times smaller than what was previously used in this laboratory (Table 2.1). I tested whether that concentration of thymidine would be innocuous in long-term experiments, after incubation of the cells with the radioactive label for 12 days. I observed that that concentration of ^{14}C-thymidine was innocuous in cultures of cardiomyocytes and A1 cells (Table 2.2), and detectable after one-week incubation with photographic emulsion.

Table 2.1. P-Histone H3 labelling of A1 cells cultured in the presence of ^{3}H-thymidine and ^{14}C-thymidine for 3 days, expressed as the quotient between the experimental and the control value.

SPECIFIC ACTIVITY OF THYMIDINE SOLUTION (SHORT ASSAY) + (Ci/mmol)	A1 CELLS	
	^{3}H-THYM	^{14}C-THYM
0	1± 0.1	
0.27	0.94±0.08	1.03 ± 0.22
0.54		0.98±0.06
2.7	0.5±0.05	0.85±0.09
13.5		0.5±0
54	0±0	0.28±0.01

The results here presented are the average of the counts of two dishes, plus or minus the difference between each experimental value and the mean. Approximately 3000 cells were counted per dish. The same effect was observed in two other experiments and with cardiomyocytes. + The total concentration of thymidine was kept constant. Solutions were diluted 1000x when added to the media.

Table 2.2. P-histone H3 labelling of A1 cells and cardiomyocytes cultured in the presence of ^{3}H-thymidine and ^{14}C-thymidine for 12 days, expressed as the quotient between the experimental and the control value.

SPECIFIC ACTIVITY OF ^{14}C -THYMIDINE SOLUTION (LONG ASSAY) + (Ci/mmol)	A1 CELLS	CARDIOMYOCYTES
0	1 ± 0.13	1 ± 0.05
0.054		1.08 ± 0.13
0.27	1.09± 0.03	
0.54	1.12±0.04	1.12±0.17
54	0 ±0	0 ±0

The results here presented are the average of the counts of two dishes, plus or minus the difference between each experimental value and the mean. Approximately 3000 cells were counted per dish. A similar result was obtained in another independent experiment. + The total concentration of thymidine was kept constant. Solutions were diluted 1000x when added to the media.

2.2 TRANSFECTION OF CELLS

2.2.1 Adenoviral Infection

The adenovirus used was kindly given by Dr. Michael Themis (Imperial College, London). This was a recombinant-adenovirus, belonging to the serotype 5 (Ad5) in a non-replicating form due to a deletion of the E1 early gene (E1A + E1B), necessary for replication. The virus contained the Green Fluorescent Protein (GFP) gene under the control of the eukaryotic CMV promoter. Virus stock was 10^{9} particles/ml. Four days after plating the cells were infected by incubation

with the adenovirus for 24 hours at a multiplicity of infection (MOI) ranging from 10 to 10000. Following incubation with the virus the cells were washed twice with APBS and given fresh media.

Prior to addition the adenovirus was incubated in AL15 (no serum) with polycations or cationic lipids for 20 minutes (DEAE dextran- 5 μg/ml; DOGS (lipopolyamine dioctadecylamidoglycylspermine)-5μg/ml; lipofectamine-5μg/ml; Protamine Sulfate - 5μg/ml and polyamine polyethyenimine (PEI) at 3μg/ml). This solution was then added to the cells. A volume of 150μl/cm^2 was added to each dish.

2.2.2 Microinjection of Plasmid DNA

2.2.2.1 Preparation of DNA for Microinjection

Plasmid DNA was used to transform competent B1H1 cells (Stratagene) according to the protocol of (Sambrook et al., 1989). The bacteria were spread on 1.5% bacto-Agar (Difco)-NZCYM media (Gibco) containing 100 μg/ml ampicillin and incubated overnight at 37°C. A single colony was used to innoculate 5 mL of NZCYM media (Gibco) containing 100 μg/ml ampicillin, and the culture was incubated with shaking at 37°C for 10 hours, and inoculated into 250 ml NZCYM 100 μg/ml ampicillin with shaking overnight. The following day another 250 ml of medium with fresh ampicillin was added and incubated for another 5 hours. Preparation and purification of plasmid DNA were by alkali lysis and equilibrium centrifugation on a CsCl gradient (Sambrook et al., 1989). The solution containing plasmid DNA and CsCl 1.55g/ml was introduced into Beckman pollyallomer centrifuge tubes, heat sealed, and equilibrated overnight at 80,000 RPM in a Beckman TLN100 rotor (room temperature). The lower band containing supercoiled plasmid DNA was collected, and a second equilibration was performed at 100,000 RPM for 4 hours at room temperature. The ethidium bromide was removed by water-saturated butanol extraction and the DNA was precipitated and resuspended in Tris-EDTA pH 8.0 (TE pH 8.0; 10mM Tris.Cl;

1mM EDTA (pH 8.0)) An aliquot was run on a 2% agarose gel to check for purity, and the concentration was determined spectroscopically by measuring the A_{260}/A_{280} ratio (Sambrook et al., 1989).

2.2.2.2 Microinjection

Dextran and plasmid DNA microinjection were performed using a Narishige MMO 202 micromanipulator connected to a pneumatic picopump PV820 (World Precision Instruments) mounted on a Zeiss Axiovert 135 microscope. Micropipettes were prepared with a P-97 micropipette puller (Sutter, USA) from glass capillaries (Clark Electromedical Instruments) with an inner filament and diameters of 1.2 mm (external) and 0.69 mm (internal). An hour before microinjection, cells were placed in serum free AL-15 media with 2,3-butanedione monoxime (BDM; 4mM) to inhibit myofibril contraction (Bartoli and Claycomb, 1997). The plasmid concentration for microinjection was 0.05-0.2 mg/ml, and the plasmids were diluted in TE. Plasmid DNA was injected into the nucleus. Dextrans (Texas red-conjugated or fluorescein-conjugated dextran 70 kD 15mg/ml in H_2O, Molecular Probes) were microinjected cytoplasmically. After microinjection cells were placed in AEMEM-10%FBS.

2.3 Processing of Cultured Cells

2.3.1 Fixation

Cultured cells were fixed, after rinsing with PBS, with 100% methanol at -20 °C for 5 min. For cells labelled with dextrans and GFP, fixation was in 4% paraformaldehyde in PBS containing 0.2% Triton-X100 pH 7.4, for 10 minutes. Cells containing alkaline phosphatase were fixed with acid alcohol (5% glacial acetic acid in ethanol) at -20 °C for 5 minutes. Cells for pRb staining were fixed in 4% PFA 5 min followed by permeabilisation with 100% methanol at -20 °C for 5 minutes.

2.3.2 Immunocytochemistry

The fixed cells were rinsed in PBS 3 times (10 min each) to remove the fixative. For BrdU staining (not when the cells were expressing alkaline phosphatase) a 10 min hydrolysis in 2N HCl was performed and followed by rinsing 3 times in PBS. After a 30 min blocking step in PBS containing 10% goat serum (PBS-GS), primary antibodies were added and incubated for 1 hour at room temperature or overnight at 4°C. This was followed by 3 washes in PBS-GS and secondary antibodies were added for 1 hour at RT followed by two washes in PBS-GS. All antibodies were diluted in PBS-GS.

For sarcomeric myosin heavy chain (sarcomeric MyHC) staining the primary antibody was directly coupled to Fluorescein-NHS (Molecular Probes).

In double and triple labelling experiments antibodies of different subclasses were used. For BrdU, p16 and MyHC staining, the cells were processed first for BrdU (nuclear), followed by p16 (cytoplasmic and nuclear) and then MyHC (cytoplasmic; directly coupled to fluorescein). For BrdU, MHC and alkaline phosphatase, the cells were first processed for BrdU, then MHC, followed by alkaline phosphatase (described in the next section). In this case the solution containing the antibody against BrdU had nuclease activity (Amersham RPN 202) [2]. Controls were stained with secondary antibodies only. The controls for the antibodies used in this thesis were all negative.

Staining of the nuclei with Hoechst 33258 was at a concentration of 1 μg/ml in PBS for 5 minutes. Cells were mounted in 90 % glycerol in 200 mM Tris-HCl buffer pH 8.2 or in Fluorescent Mounting Media (DAKO).

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[2] This solution containing antibody and nuclease was used to prevent the use of HCl. The nuclease exposes BrdU by enzymatic degradation of DNA . HCl would destroy the alkaline phosphatase activity.

2.3.3 Alkaline Phosphatase Cytochemistry

For alkaline phosphatase cytochemistry, samples were incubated in PBS at 65°C for 15 minutes to destroy the endogenous alkaline phosphatase activity. The cells were washed in 1mM $MgCl_2$/PBS and developed using ELF-97 (Molecular Probes).

2.3.4 Autoradiography

Isotopically labelled cells were processed for autoradiography after antibody staining. Cells were air dried and coated with Ilford K5 emulsion diluted 1:1 in H_2O. Development was after 2-7 days in Phenisol (Ilford), diluted 1:6 in H_2O for 6 minutes followed by fixation was in Hypam (Ilford) diluted 1:6 in H_2O, for 6 minutes.

2.4 Testing Thrombin Activity

For generation of activity in serum, thrombin (Enzyme Research Laboratories) was added to the cells at different concentrations (0.07–0.56 μM) in the presence of 1%-FBS containing media. Proteolytic activity of thrombin was measured using the chromogenic substrate tosyl-glycl-prolyl-arginine-4-nitranilide acetate (ChromozymTH, Boehringer). Samples were monitored at 405 nm every 20 s for at least 3 min to determine activity.

Crude thrombin was purchased from Calbiochem and added to the cells at a concentration of 100 μg/ml, as described before (Tanaka et al., 1999).

2.5 Microscopy

2.5.1 Imaging Live Cells and Time-Lapse Microscopy

Routinely the live cardiomyocytes were viewed using a phase contrast objective (10x-40x) on a Zeiss Axiovert 135 microscope. The images of live cells were obtained using a SONY CCD Black and White camera and Image Pro Plus software (Media Cybernetics, USA). The same set-up was used for long-term observations and time-lapse recording. For continuous time-lapse recording cells

were maintained in AL15-10%FBS medium at room temperature. A few drops of light mineral oil (SIGMA) were layered on top of the medium to prevent vapour condensation.

For long term observation of cells (pictures taken once or twice a day), cells were regularly kept in AEMEM-10%FBS in the incubator. For observation, the dishes were taken out of the incubator after sealing with parafilm. The fields under observation could be recognised due to orientation of the grid (A-Z and 1-30).

2.5.2 Imaging Fixed Samples

Cells processed for imunofluorescence were observed under bright field illumination using phase contrast optics, or under epi-illumination using standard fluorescein (excitation 450-490 nm, emission between 515-565 nm), rhodamine set (excitation 538-558 nm and emission above 590 nm), and UV (excitation 390- 420 nm and emission above 425 nm) filter sets on a Zeiss Axiophot 2 microscope. Images were collected on a CV-12 cooled monochrome digital camera (Photonic Sciences, UK) as 12-bit images with Image Pro Plus software (Media Cybernetics, USA). The individual grey scale images from each channel were converted to 8 bit images, and merged to obtain 24 bit colour images.

Alternatively, cells processed for imunofluorescence were observed using a Leica TCS SP confocal system. Images were acquired as Z-series stacks and full colour extended projection of the image was generated through Leica TCS imaging software. Each confocal optical section was averaged two to four times.

All images were further processed in Adobe Photoshop 4.0 (Adobe Systems, USA) for colour correction and contrast enhancement.

2.6 Immunoprecipitation and Western Blotting

Immunoprecipitation was performed essentially as described previously (Hu et al., 1991). Recipes for the solutions used can be found in (Sambrook et al., 1989) One 10 cm dish was used per experiment. Cardiomyocytes were plated in

AEMEM-10%FCS. After 10 days, the cells were rinsed with PBS and lysed for 30 min on ice with 700 µl of lysis buffer (250mM NaCl; 0.1%NP40; 50mM HEPES pH 7.5; 5mM EDTA; 10mM NaF; 0.1 mM orthovanadate; 50 µg/ml PMSF; 1µg/ml Leupeptin; 1 µg/ml aprotinin and 1mM DTT). Cellular debris was removed by centrifugation for 1 min at 10,000 *g*. For immunoprecipitation, samples were incubated with 4 µl SK70 for 1 h, at 4°C, then with 7 µl XZ56 for 1 h, at 4°C, with rocking, and further with 40 µl protein A-Sepharose for 1 h. The sample was then washed three times with wash buffer (155 mM NaCl, 20 mM Tris, pH 8, 5 mM EDTA, 0.05% NP-40, 0.02% NaN3). The sample was then split in two aliquots, one aliquot was incubated with 600 units in a total of 70 µl of lambda phosphatase (NEB) at 30°C for 30 minutes. The sample was then washed once with wash buffer. Both samples were then solubilised in 40 µl 2x SDS loading buffer (100mM Tris.Cl (pH 6.8); 200 mM dithiothreitol; 4% SDS; 0.2% bromophenol blue; 20% glycerol) for 10 min at 80°C. Samples were resolved on 7.5% SDS polyacrylamide gels (NOVEX), Western blotted onto nitrocellulose (Sambrook et al., 1989), blocked in 1% dried milk in Tris Buffer Saline /0.1% Triton X-100 for 30 min, and then probed with SK70 at a 1:200 dilution followed by HRP-conjugated donkey anti-rabbit secondary antibody (Amersham Corp.) and enhanced chemiluminescence (ECL; Amersham Corp.).

2.7 Antibodies and Plasmids

2.7.1 Antibodies

Mouse monoclonal antibodies were used against BrdU (IGg1, BU-20, Amersham), muscle specific myosin heavy chain (IgG2a, A4.1025 Dr. Simon Hughes, Randall Institue, King's College, London), p16 (IgG1, DCS-50.2, Dr. Gordon Peters, Imperial Cancer Research Fund, London), troponin (CT3, Dr. Elisabeth Ehler, ETH, Zurich), titin (9D10, Dr. Elisabeth Ehler, ETH, Zurich), pRb (51β, Dr. Sybille Mittnacht, ICR, London; XZ56, (Tanaka et al., 1997)). Other antibodies were polyclonal rabbit anti mouse: anti-phospho histone H3 (Upstate

Biotechnology, New York, USA); pRb (SK70, (Tanaka et al., 1997); 23, Dr. Sybille Mittnacht, ICR, London; 652, Dr. Sybille Mittnacht, ICR, London). Primary antibodies were used at a dilution of 1:100 to 1:500.

Secondary antibodies were FITC or TRITC conjugated goat anti-mouse, rabbit anti-mouse, or swine anti-rabbit (DAKO) or subclass specific FITC, TRITC, or biotin conjugated goat anti mouse (Jackson), or Alexa blue conjugated goat anti-mouse (Molecular Probes). Streptavidin conjugated to cascade blue was from Molecular Probes.

2.7.2 Plasmids

PTL1-p16 was constructed in the lab by Dr. Cristiana Velloso (Velloso et al., 2000), by inserting an EcoRI-BamHI fragment of pcDNA3WTp16 (a gift of Drs. David Parry and Gordon Peters, Imperial Cancer Research Fund, London, UK) into the BamHI-NotI site of PTL1 (Ragsdale et al., 1989) which contains an SV40-promoter. PCAP (PTL1-Alk. Phos.) is PTL1 containing human placental alkaline phosphatase under the control of the SV40 promoter. It was made by Mr Phillip Gates in the laboratory (Schilthuis et al., 1993).

-Chapter 3-

Establishment and Characterisation of a Primary Cell Culture of Adult Newt Ventricular Cardiomyocytes

3.1 INTRODUCTION

Mammalian ventricular cardiomyocytes cease proliferation soon after birth (Soonpaa et al., 1996), and post-natal growth of cardiac muscle is largely achieved by an increase in cell size (hypertrophy; MacLellan and Schneider, 2000; Soonpaa and Field, 1994; Soonpaa and Field, 1997). The repair of cardiac lesions, such as those caused by infarction, is limited to compensatory hypertrophy and fibrosis. In contrast, adult amphibian ventricular cardiomyocytes, such as those from the frog and the newt, can re-enter the cell cycle and undergo mitosis after injury to the ventricle (Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995; Rumyantsev, 1973; Rumyantsev, 1991). Although the plasticity of the adult newt cardiomyocyte has been recognised and investigated for some time, the underlying regulatory mechanisms are not yet understood.

In vivo studies on the plasticity of the adult newt cardiomyocyte are limited in the sense that it is difficult to identify and manipulate the cells *in vivo*. It would be advantageous to investigate this problem with a cell culture model where observation and manipulation of single cells is feasible, in conjunction with their unequivocal identification using cell-type specific antibodies. Explants, dissociated cultures and cell lines obtained from newt tissues have proven very useful in studies of the plasticity of other newt tissues such as skeletal muscle [for a review see (Brockes, 1997; Brockes, 1998a). To date, there is no report on the isolation of any newt cell line that differentiates into cardiomyocytes. However, explants and dissociated cultures of newt hearts have been described (Ferretti and Brockes, 1988; Nag et al., 1979b; Tate et al., 1987; Tate and Oberpriller, 1989). The plasticity of the cardiomyocytes seems to be preserved in these cultures, as DNA synthesis and mitosis were reported (Nag et al., 1980; Nag et al., 1979a; Oberpriller et al., 1995; Tate et al., 1989). Consequently, these dissociated primary cultures may be useful to investigate the cellular and molecular regulation of the plasticity of the adult newt cardiomyocyte.

The signals that stimulate cell cycle re-entry and division of the newt cardiomyocyte in dissociated culture have yet not been identified. It could be a molecular signal present in serum, since the studies reported to date have been performed in medium containing 10% FBS (Nag et al., 1980; Nag et al., 1979a; Oberpriller et al., 1995; Tate et al., 1989). Alternatively, the processes of mincing the heart for explant culture and dissociating the cells could liberate some stimulating signals from the tissue or free the cells from negative interactions such as contact inhibition. It has been shown that FBS can induce cell cycle re-entry in newt skeletal myotubes, and that this re-entry is sensitive to contact inhibition (Tanaka et al., 1997). It would be important to establish whether FBS is involved in regulating cell cycle re-entry in the newt cardiomyocyte, and whether inhibitory factors also exist.

This chapter describes the establishment and characterisation of a primary culture model to study the plasticity of the adult newt ventricular cardiomyocyte. In addition, I investigated whether the overall dynamics of *in vivo* cardiomyocyte proliferation are maintained in culture, and whether FBS is promoting DNA synthesis of newt cardiomyocytes.

3.2 RESULTS

3.2.1 Dissociation, Purification and Culture of Newt Cardiomyocytes

Dissociation of the ventricles was achieved by a modification of the protocol established by Tate and collaborators; see Materials and Methods). Approximately 170 different preparations of cells were performed according to this protocol for the work presented in this thesis. In brief, ventricles were dissociated in a mixture of collagenase type V (380U/ml) and trypsin (0.5%) in amphibian PBS at 27°C, in a shaking waterbath. The dissociation period extended for 8 hours on average. Cardiomyocytes were collected between 6 and 8 hours after the start of the dissociation process. Routinely, 40-60% of the cells obtained were cardiomyocytes, as evidenced by the distinct elongated shape and the presence of striations (see Fig 3.1B and D). An average of $4X10^4$ cardiomyocytes were obtained from one ventricle.

The purification of the cardiomyocytes was achieved by differential adhesion with pre-plating in an uncoated culture dish (Fig. 3.1). Blood cells, connective tissue cells and some pigmented cells attached to the plastic (Fig. 3.1), whereas the cardiomyocytes remained in solution, as they require specific substrata for adhesion. The cardiomyocyte-enriched suspension was then plated on laminin-coated dishes (see Materials and Methods). After 3 days, approximately half of the cardiomyocytes had attached to the dish. Most cells were contracting spontaneously by day 10^3, and continued to beat at least 2 months after plating. Routinely, the enriched cultures were mainly composed (90-97%) of cardiomyocytes as assessed by immunofluorescence microscopy using a fluorescein-conjugated anti-myosin heavy chain antibody (Fig 3.1F).

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[3] Time was defined as the number of days after plating the cells on laminin.

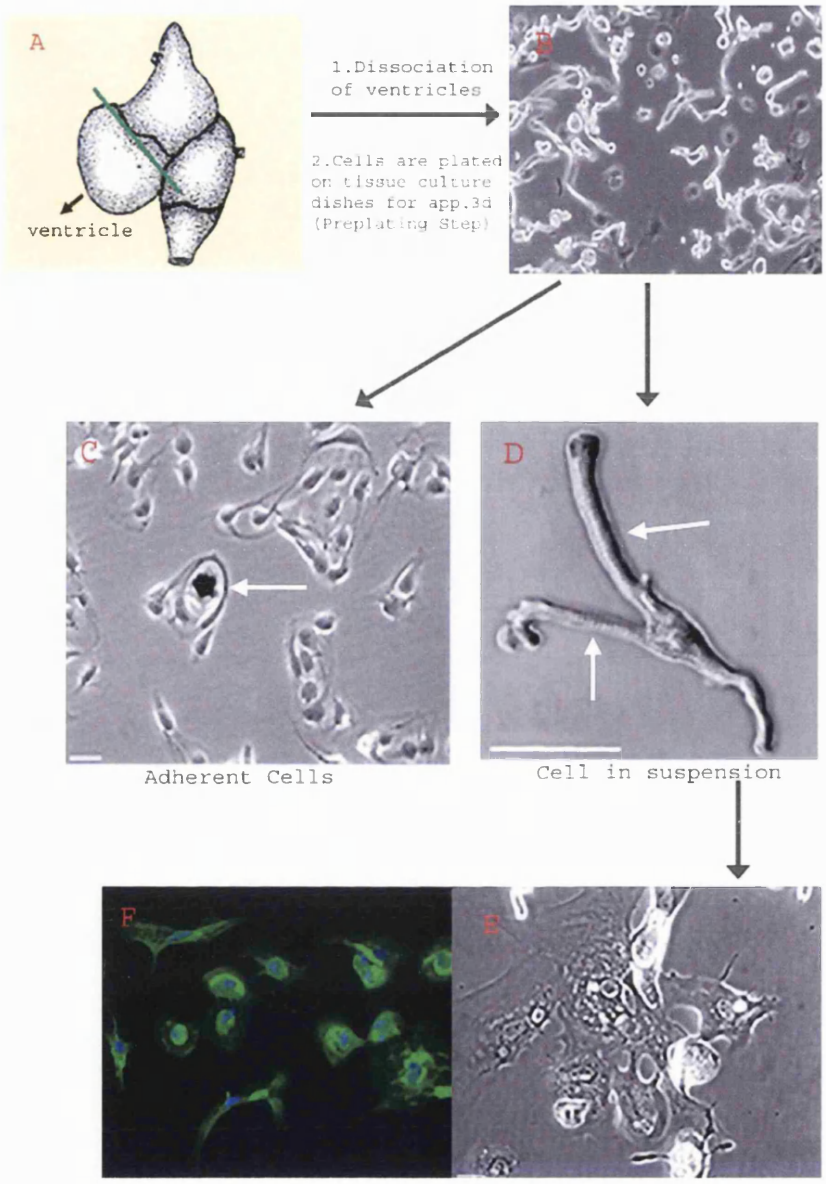


Figure 3.1 Dissociation, purification and culture of newt ventricular cardiomyocytes. After dissociation of the ventricles, cells were plated in a non-coated tissue culture dish for 3 days. Whilst non-muscle cells attached to the plastic, cardiomyocytes remained in suspension. Cardiomyocytes were then plated on laminin. (A) Schematic diagram of the newt heart. (B-E) Photomicrographs of live cells. (B) Preplating step: while some cells have attached, the cardiomyocytes remain in suspension (note that they are elongated and branched). (C) Cells that adhered during the preplating step. Observe that most of them resemble fibroblasts and pigmented cells (arrow). (D) Isolated cardiomyocyte in suspension; branches and striations are visible at this magnification (see arrows). (E) Group of cardiomyocytes 8 days after plating onto laminin. Note that newt cardiomyocytes have adhered to the substrate, spread and established connections with one another. At this time, cells have also started to contract spontaneously. (F) Fluorescent micrograph of fixed cardiomyocytes stained with an FITC conjugated antibody to sarcomeric-myosin heavy chain and in blue for nuclei (Hoechst). Observe that the majority of the cells are cardiomyocytes. Scale bars for all images-50μm.

3.2.2 Characterization of the Culture

3.2.2.1 Cell Morphology and Markers

Upon isolation, newt cardiomyocytes had a morphology that was distinct from their mammalian rod-shaped counterparts (Severs, 2000) as they often presented several (up to six) branches (Fig. 3.1B and D). Myofibrils were well organised, showing regular striations as seen in Fig. 3.1D.

After adhesion to the substrate, cell spreading was variable, both in timing and in the shapes taken up by the cells. While some cells spread without major changes in shape, the majority lost the branched pattern, acquired a rounded shape, and were birefringent when examined under phase contrast optics before spreading (Fig. 3.2). Several cells acquired vacuoles during this morphological change (Fig. 3.2). This process has been termed "metamorphosis" for the mammalian cardiomyocyte (Claycomb and Palazzo, 1980; Nag et al., 1996), seems to involve a complex reorganisation of the myofibrils, from orientation parallel to the long axis of the cell, to both partial disassembly and reorientation in several directions (Fig. 3.3 A,B,C and D). Additionally, the rate of spreading was variable. At 8 days after plating, cells in very different stages of spreading were seen in the same dish (Fig. 3.3 A-D). The majority of the cardiomyocytes were spread at 10 days (Fig. 3.3 F), although some cells spread later.

The spreading of cardiomyocytes was found to be dependent on the concentration of serum at six days (Fig. 3.3 F). Subsequently, the percentage of cells that had initiated spreading was the same, regardless of the concentration of serum in the medium (Fig. 3.3 F). The presence of serum had an effect on the size of the cells, since most of the cardiomyocytes were larger when maintained in medium containing 10% FBS than in serum-free media (not shown).

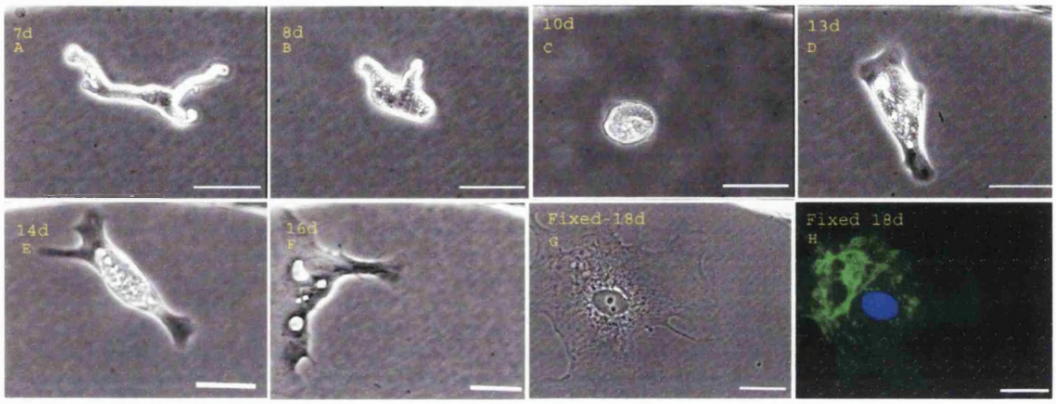


Figure 3.2 Morphological changes of a newt cardiomyocyte in culture. Micrographs of the same field on different days after plating on laminin (day number is indicated in the top left corner of the picture). (A-F) Live cell. (G-H) Fixed cell. (H) Cell stained with antibody for sarcomeric-MyHC (green) and DNA (blue). Note that the extensions of the cardiomyocyte retract towards the centre of the cell (From A to C), and that the cell spreads (C to G). Observe the vacuoles that appeared after the change in cell shape (F). Scale bar-50μm.

In order to analyse the expression of sarcomeric components of the newt cardiomyocyte, several experimental approaches were used. Ventricles were extracted and analysed by Western blot analysis by Dr. Elisabeth Ehler (ETH-Switzerland). Reactivity to several proteins representative of the different sarcomeric structures was found: α-cardiac actin, troponin I and T and tropomyosin (thin filaments), α-actinin (Z-disks), titin (elastic filaments), sarcomeric MyHC and β-MyHC (thick filaments) and myomesin (M-band) (E. Ehler, personal communication; Fig. 3.4 A). I have performed immunofluorescent staining of cells in culture for sarcomeric MyHC, titin and troponin, with the same antibodies used for the Western blots, and these reacted strongly (Fig. 3.4 B, C).

It has recently been described that embryonic and neonatal avian and mammalian heart express two myomesin isoforms, one of which is down-regulated after birth, so that only one isoform is expressed in the adult heart (Agarkova et al., 2000). In consequence, the embryonic myomesin isoform (EH-myomesin) has been described as the first true marker for embryonic cardiomyocytes. Only one antibody, of several that recognize different isoforms and domains of myomesin, was found to cross-react with the newt myomesin in

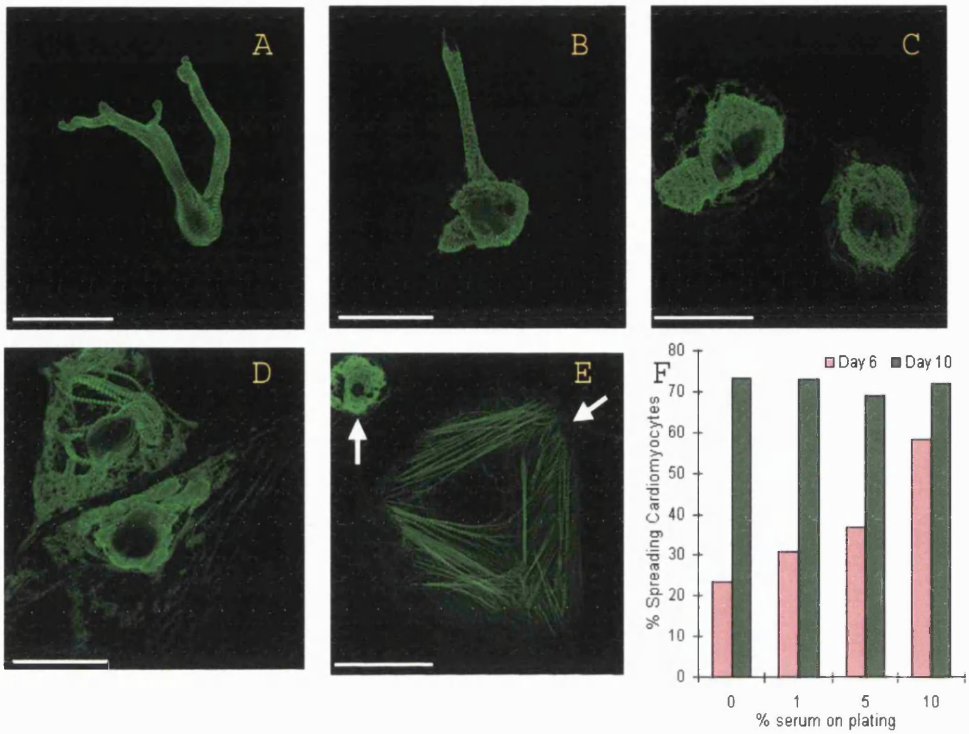


Figure 3.3 Heterogeneity and serum influence on cell spreading. (A-E) Confocal micrographs (Z-axis projections) of cells stained for sarcomeric-MyHC (green). Scale bar-50μm. (A-D) Cells were cultured in serum free medium for 8 days. Note the heterogeneity of cell spreading and the organisation of the myofibrils. (A) Cell not spread, myofibrils parallel to long axis of the cell. (B) One of the extensions of the cardiomyocyte has not retracted towards the centre of the cell. In the centre of the cell myofibrils surround the nucleus. (C) Two cardiomyocytes -myofibrils running around the centre (nucleus) of the cells. (D) Two cardiomyocytes that have spread more than in (C). (E) Cells cultured in 10% FBS for 8 days. Note the two cells in different stages of spreading (see arrows). (F) Cells were plated in different concentrations of serum. Cells were stained for sarcomeric-MyHC at 6 and 10 days and the percentage of spreading cells was scored, according to a criterion, where cardiomyocytes that had already started to extend from the round form were considered spread (such as the cells in figure D, and the right but not the left cardiomyocyte in Figure E). A minimum of 150 cardiomyocytes was counted per dish.

Dr. Elizabeth Ehler's experiments. This antibody (clone 673) recognizes the 5th fibronectin-like domain in the myomesin, common to both embryonic and adult isoforms. Using this antibody, only one myomesin band of the same molecular weight as the human and mouse adult myomesin isoform was found, both in the newt ventricle sample and in an extract of cardiomyocytes cultured for 10 days in media containing 10% FBS.

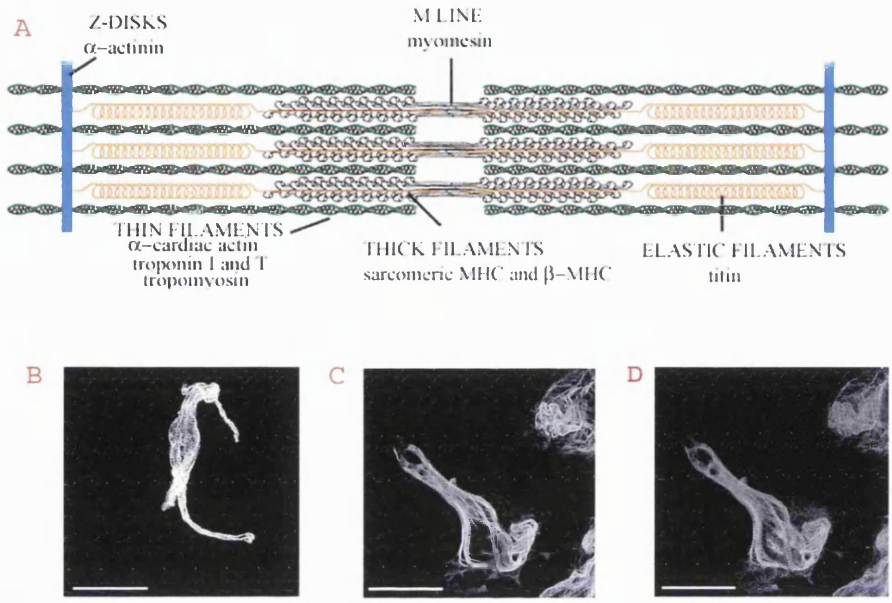


Figure 3.4 Expression of sarcomeric markers in newt cardiomyocytes. (A) Scheme of sarcomeric proteins found by western blot in newt ventricles (experiments were performed by Dr. Elizabeth Ehler to whom I sent frozen ventricles). Note that elements of the different sarcomeric structures were found to be present in newt ventricles. Adapted from Alberts et al. (1994). (B-D) Confocal micrographs of ventricular cardiomyocytes fixed and stained in culture for (B) troponin, (C) sarcomeric-MyHC and (D) titin. Note the cross striations in the staining of each protein. Scale bars-50μm.

3.2.2.2 DNA Synthesis & Mitosis

3.2.2.2.1 Newt Cardiomyocytes Undergo DNA Synthesis in Culture

Newt cardiomyocytes were plated in medium containing 10% FBS and were observed to enter S phase, as detected by incorporation of BrdU (Fig. 3.5 A, B). Cardiomyocytes were pulsed with BrdU at various times after plating, and it was observed that they entered S phase somewhat asynchronously, with a peak at around 10 days (25.8%; Fig. 3.5 C). The number of cardiomyocytes synthesising DNA during the pulse decreased to 5.1% at 22 days (Fig. 3.5 C). The asynchronous cell cycle re-entry might be a consequence of the asynchronous spreading, as incorporation of BrdU was rarely observed in cardiomyocytes that had not spread.

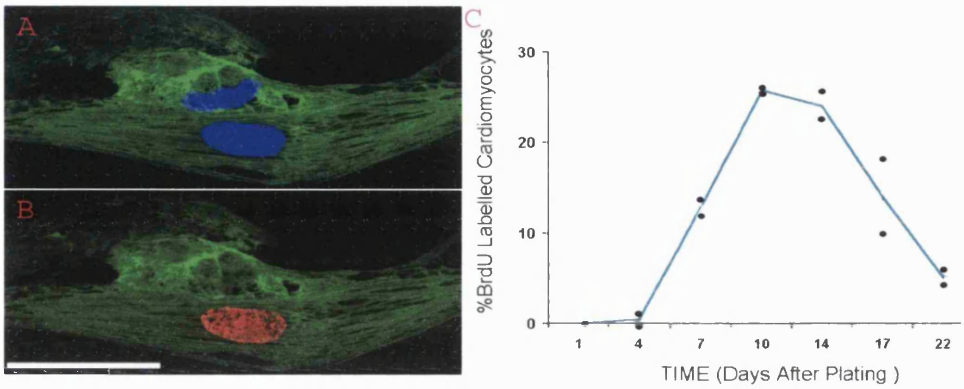


Figure 3.5 Newt cardiomyocytes undergo DNA synthesis. (A and B) Two cardiomyocytes stained for myosin heavy chain (green), DNA (blue) (A) and BrdU (red) (B). Note that the lower cardiomyocyte shows a BrdU-positive nucleus. (C) Cardiomyocyte DNA synthesis at different days after plating. Cells were pulsed with BrdU for 9 hours at various times after plating. Cells were fixed and processed for BrdU and sarcomeric-MyHC staining as described in Materials and Methods. In this and subsequent figures I have preferred to give the results of individual experiments expressed as the average of duplicate dishes, and the data points given for each condition. A minimum of 300 cardiomyocytes were scored per dish. Note the peak of DNA synthesis at around 10 days after plating. Scale bar-50μm.

3.2.2.2.2 Newt Cardiomyocytes Undergo Mitosis in Culture

Cardiomyocytes were observed to undergo mitosis between 8 and 14 days after plating (Fig. 3.6A). Newt cardiomyocytes in mitosis often remained flat and attached to their neighbours and were readily identified due to their large condensed chromosomes (Fig. 3.6A). Most of the myofibrils were only present in the periphery of the cell during mitosis (Fig. 3.6A1,C), but occasionally they extended into the cleavage furrow at cytokinesis. Ten to fourteen days after plating there was a peak of cells undergoing mitosis as evidenced by staining the cells with an antibody that recognises phosphorylated histone H3 (P-histone-H3; Fig. 3.6B, C; Hendzel et al., 1997; Velloso et al., 2000; Wei et al., 1999). At 10 days 1.3% of the cardiomyocytes labelled for P-histone-H3.

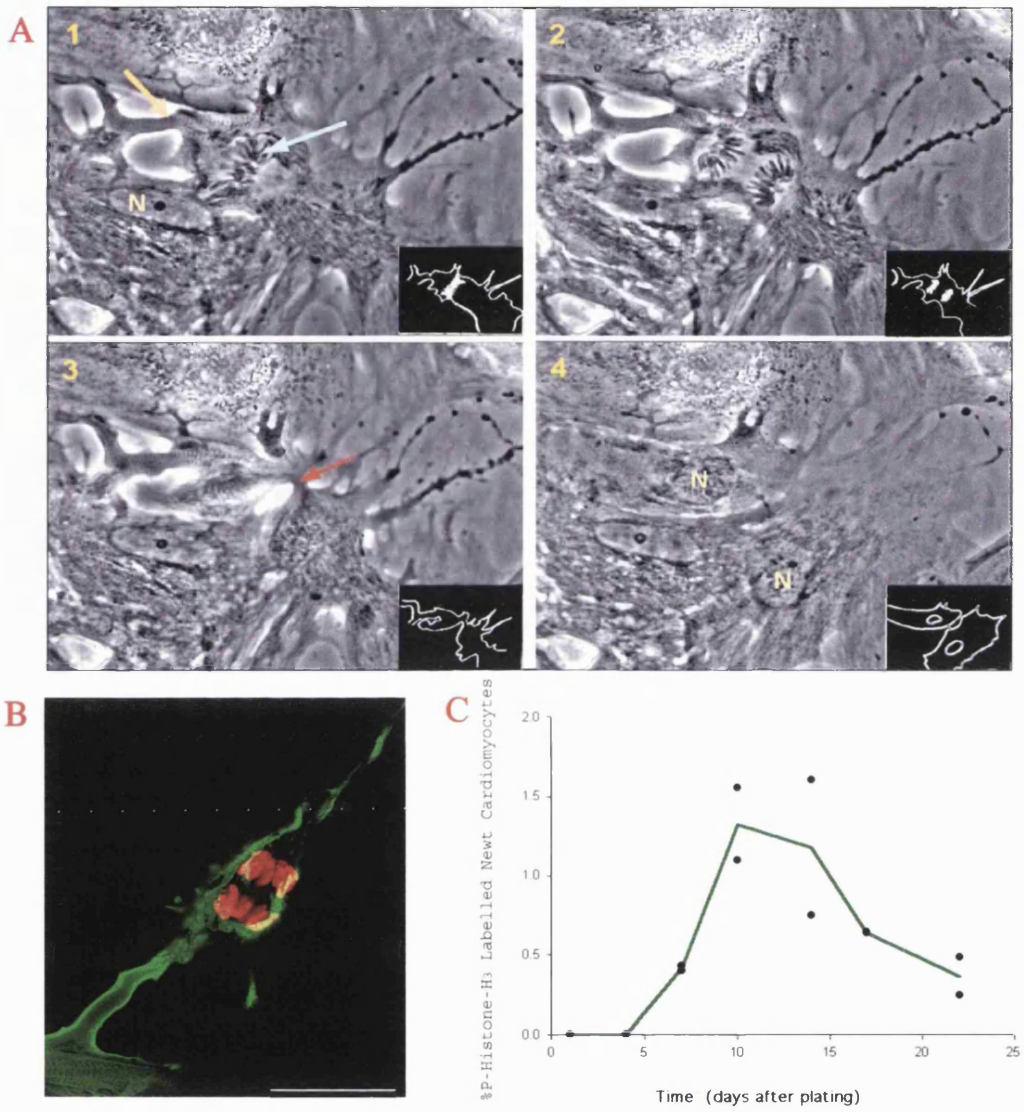


Figure 3.6 Newt cardiomyocytes undergo mitosis in culture. (A) An outline of the cell is seen in the right, lower side of each picture. 1 - Newt cardiomyocyte in metaphase. Note the myofibrils (yellow arrow) and chromosomes (blue arrow). N- nuclei of other cells in the culture. 2 - Late anaphase, 20 minutes after 1. 3 -Cytokinesis, 30 minutes after 2. Red arrow points to the cleavage furrow. 4 -The two daughter cells. Both cells were contracting. (B and C) Cardiomyocyte mitosis at different days after plating. (B) Cells were fixed and stained for sarcomeric-MHC (green) and P-histone-H_3 (red). Scale bar-50μm. (C) Quantification of the percentage of cardiomyocytes stained for P-histone-H_3 at different days after plating. A minimum of 400 cells was scored per dish.

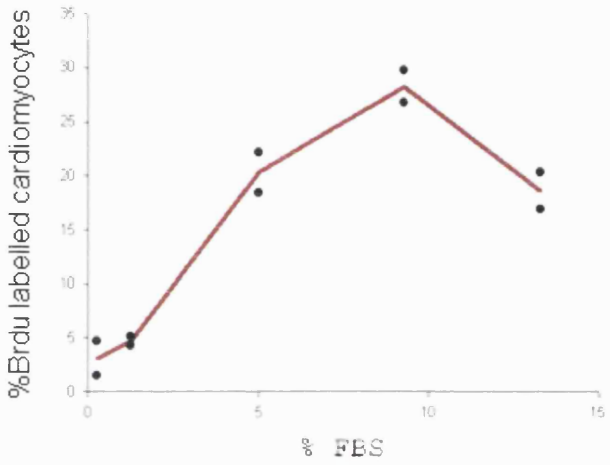


Figure 3.7 Serum enhances DNA synthesis in cardiomyocytes. Cells were plated in 10% FBS containing media. 2 days after plating the cells were washed and fresh medium containing 0.5% FBS was added. Four days afterwards the cells were stimulated with different concentrations of serum for four days. Cells were then labelled with BrdU for 8 hours, fixed and processed for BrdU and sarcomeric-MyHC as described. A peak of DNA synthesis at approximately 10% FBS was also observed in three other experiments using the same protocol.

3.2.2.2.3 Serum Induces Entry into S Phase by Newt Cardiomyocytes

Entry into S phase by adult newt cardiomyocytes in culture has only been reported in the presence of FBS (Nag et al., 1980; Oberpriller et al., 1995; Soonpaa et al., 1992). To ascertain whether serum was enhancing DNA synthesis, I performed a dose response experiment (see Fig. 3.7). It is clear that serum enhanced BrdU uptake by the cardiomyocytes and that the cardiomyocyte response was maximal in medium containing 10% FBS.

3.3 Discussion

The results presented in this chapter are essentially in agreement with those reported by others (Oberpriller et al., 1995; Tate et al., 1989), in that the plasticity of the adult newt cardiomyocyte, defined here as the ability to enter in S phase and divide, is maintained in a dissociated culture. Although these are highly differentiated cells, with well-defined sarcomeres (Fig. 3.4), newt cardiomyocytes enter the cell cycle extensively and divide when stimulated by mammalian serum (Fig. 3.5 and 3.6).

Since embryonic, but not adult, mammalian cardiomyocytes, can divide in response to injury, it would be important to compare their differentiated phenotype with that of the newt cardiomyocyte. As discussed previously, it is difficult to define phenotypic characteristics that unequivocally and universally classify cardiomyocytes as embryonic, neonatal or adult. Nevertheless, the characterisation of the newt cardiomyocyte indicates that it may possess an adult phenotype. For example, the spreading behaviour of the cells indicated that the majority of the cardiomyocytes went through an initial period, characterised by rounding of the cell and change in the organization of the myofibrils (Fig. 3.2 and 3.3), after which the cells spread and resumed spontaneous contractions. This behaviour has been described for adult mammalian cardiomyocytes, and is distinct from that of embryonic and neonatal cardiomyocytes, which do not go through such complex reorganization of the cytoskeleton upon adaptation to culture (Bugaisky and Zak, 1989; Nag and Lee, 1997; Nag et al., 1996; Rothen-Rutishauser et al., 1998). Additionally, the immunocytochemistry results show that newt cardiomyocytes have defined and organised sarcomeres, as expected for differentiated cardiomyocytes (Fig. 3.4). The preliminary results obtained by Dr. Elizabeth Ehler with a newt ventricle sample and an extract of cultured cardiomyocytes, suggest that newt cardiomyocytes express only one isoform of myomesin, of the same size as the mammalian adult isoform. Since the presence of the embryonic myomesin isoform is a probable marker for embryonic

cardiomyocytes (Agarkova et al., 2000), the observation that newt adult cardiomyocytes might only express the adult myomesin isoform is an indication of their differentiated phenotype. It would be important, however, to study the expression of myomesins in newt embryos as a positive control for the detection of the embryonic newt myomesin isoform.

The time course of cardiomyocyte DNA synthesis, in conjunction with the results previously obtained by Tate and collaborators (Tate et al., 1989), strongly supports the claim that the events observed in the context of dissociated culture might reflect the plasticity observed *in vivo*, both in the timing and the extent of the proliferative response (see Fig. 3.8). Different experimental protocols gave similar results with a peak of proliferative response between the 2nd to 3rd weeks in culture and 25 to 30% of the cardiomyocytes undergoing DNA synthesis. After this peak, the response decreased in all the experiments. These similarities suggest that the factors which stimulate and inhibit cell proliferation are the same *in vivo* and in dissociated cultures, thus making the culture system a valuable tool to investigate the cellular and molecular mechanisms that control the plasticity of the adult newt cardiomyocyte.

What are the signals that stimulate the cardiomyocytes to divide in culture? The cardiomyocyte response does not seem to be dependent on the continued presence of newt-specific factors, as the cells can re-enter the cell cycle and divide *in vitro* (Fig. 3.8). The results presented here show that entry into S phase is enhanced by mammalian serum (Fig. 3.7), though the contribution of other factors, such as cell damage or release of contact inhibition, cannot be eliminated. Hence, there may be a factor common to mammalian and newt serum that stimulates this proliferative response (Fig. 3.9A and B).

Cells stop proliferating in culture according to a similar schedule as observed *in vivo*, even in the presence of FBS (Fig. 3.8), indicating that there may exist cell-intrinsic factors that limit their response (Fig. 3.9B). These results are different from those observed for mammalian embryonic cardiomyocytes *in vitro*,

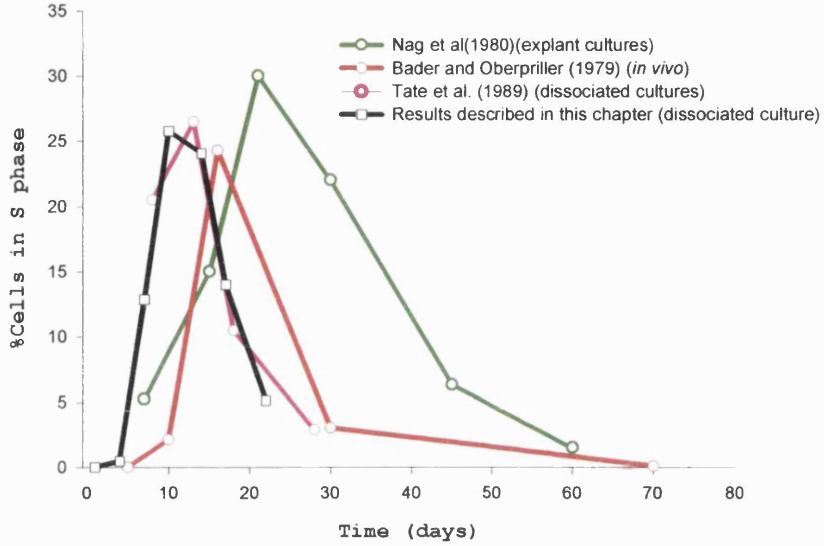
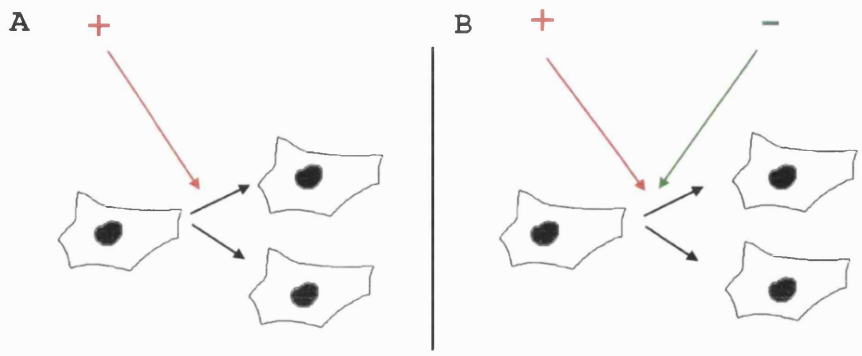


Figure 3.8 Comparison of the timing of cell cycle re-entry of adult newt cardiomyocytes in different experimental systems. In each experiment, different labelling methods were used. In Bader and Oberpriller's experiment (Bader and Oberpriller, 1979), the tip of the ventricles was cut, minced and grafted back into the ventricle in order to increase the number of myocytes near the wound surface. Animals were sacrificed one hour after being injected intraperitoneally with tritiated thymidine. The percentage of labelled cells in the minced graft (morphological criterion used to distinguish cardiomyocytes from other cells) was determined. The results found are very similar to the ones described earlier by the same authors (Bader and Oberpriller, 1978). In Nag's experiment (Nag et al., 1980) the newt ventricle was excised and cut into small pieces (0.5-1.0mm) that were cultured. The explants were then labelled with tritiated thymidine for 24 hrs before fixation. The percentage of labelled cells containing myofibrils was scored (by electron microscopy). In the experiment performed by Tate and collaborators (Tate et al., 1989), cells were dissociated, kept in culture and given pulses of tritiated thymidine for 24 hrs prior to fixation. Cardiomyocytes were recognized by the periodic acid-Schiff (PAS) reaction product (glycogen gives a positive PAS reaction) or by the presence of myofibrils. The experiment that I performed was described in Fig. 3.5. All the experiments *in vitro* were performed in medium containing 10% FBS.



\+ Signals that promote cardiomyocyte proliferation and are present both *in vivo* and *in vitro* in FBS.

– Signals that inhibit cardiomyocyte proliferation and are present both *in vivo* and *in vitro* (could be intrinsic to the cells).

Figure 3.9 Control of the proliferative response of newt cardiomyocytes. Model (A) Cells are exposed to a transient stimulatory signal that stimulates their proliferation. The down regulation of this signal would stop the division of the cells. Model (B) Cells are exposed to stimulatory signals and inhibitory signals that regulate their proliferation. According to the results presented in this chapter, the stimulatory signal is not specific to newt as it is present in FBS. In consequence, those results and other experiments in culture (Fig. 3.8) are a test for these models, favouring model (B). In fact, cells stop proliferating even when constantly exposed to FBS (Fig. 3.8), showing that there are factors that prevent cells from proliferating further.

where it is has been shown that FBS can override the intrinsic timer that would force these cells to stop dividing according to the *in vivo* schedule (Burton et al., 1999a).

Although the results presented in this chapter indicate that a minimum of 26% of the cardiomyocytes can enter into S phase (Fig. 3.5C), many questions remain to be answered. First, it is unclear whether those cells are ultimately undergoing mitosis and cytokinesis. Secondly, it is not known whether the broad curve of cells synthesising DNA shown in figure 3.5C is due to asynchrony of the entry into the cell cycle, or to cells going through more than one round of DNA synthesis. The clarification of these aspects is the focus of the next chapter.

-Chapter 4-

Analysis of the Proliferative Potential of Adult Newt Ventricle Cardiomyocytes

4.1 INTRODUCTION

Although the plasticity of adult newt cardiomyocytes has been recognised and investigated for a considerable time, the cellular mechanisms underlying the proliferation of these cells are not known. Several questions remain unanswered, such as the fraction of cardiomyocytes that can re-enter the cell cycle and undergo DNA synthesis, whether they are able to progress through the different phases of the cell cycle, and to what extent they proliferate. In the following paragraphs, I will briefly review previous studies which have addressed these questions commenting on their limitations, and explaining why I decided to use different experimental approaches.

What is the percentage of cardiomyocytes that contribute to the repair observed after injury to a newt heart? The most informative *in vivo* studies used an experimental protocol ingeniously designed to increase the surface of muscle exposed to the wound, where the apex of the newt ventricle was amputated, minced and replaced onto the amputation gap (Bader and Oberpriller, 1979; Bader and Oberpriller, 1978; Oberpriller et al., 1989). After 16 days, 24% of the cells[4] (excluding blood and endothelial cells) in the minced tissue became incorporated ^{3}H-thymidine, which had been injected into the animal one hour prior to fixation. (Bader and Oberpriller, 1979). These data are in broad agreement with the *in vitro* data discussed in the previous chapter, where 26% of the cardiomyocytes became labelled after a single exposure to BrdU or ^{3}H-thymidine (Fig. 3.5 C; Tate et al., 1989). Therefore, it is reasonable to estimate that a minimum of 26% of the cardiomyocytes can undergo DNA synthesis, but the total

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[4] The majority of the proliferating cells in the injured newt ventricle seemed to be cardiomyocytes, since 31 out of 37 mitotic cells in the minced tissue were myocytes, as assessed by electron microscopy (Bader and Oberpriller, 1979).

fraction of cells that enter the cell cycle following injury cannot be determined from these results.

Another approach to the problem was the use of cumulative labelling. Following the minced tissue protocol, ^{3}H-thymidine was injected daily from the 15th to the 20th day after amputation and the tissue was fixed 20 days after the last injection (Oberpriller et al., 1989). The tissue was dissociated after fixation, allowing proper identification of cell types. It was observed that 47% of the cardiomyocytes in the minced area became labelled (Oberpriller et al., 1989). In culture, continuous exposure to tritiated thymidine from 3 to 28 days after plating resulted in labelling of 34% of the cardiomyocytes (Tate et al., 1989). It must be taken into account that subsequent to the continuous labelling period the cells that incorporated ^{3}H-thymidine might have proliferated, giving rise to labelled progeny. As a consequence, the percentage of labelled cells observed is not an accurate estimate of the total fraction of cells that can enter S phase. In conclusion, these studies clearly indicate that following injury a significant number of cardiomyocytes re-enters the cell cycle and synthesizes DNA, but do not establish what is the overall percentage of cardiomyocytes that behaves in this way.

What is the proliferative ability of the cells that re-enter the cell cycle? It has been suggested that cardiomyocytes might divide once or twice after injury, based on the fact that the total number of cells increases 2-3 fold in the minced tissue graft (Bader and Oberpriller, 1979). Interpretation of these results requires a precise estimate of the percentage of cardiomyocytes that are able to enter and progress through the cell cycle.

Finally, are proliferating newt cardiomyocytes able to progress through all phases of the cell division cycle? Mammalian cardiomyocytes become polyploid and/or binucleate after birth due to G2 arrest and incomplete cytokinesis (Brodsky, 1991). Newt skeletal myotubes arrest in G2 after DNA synthesis

(Tanaka et al., 1997). There is some indication that polyploidy and multinucleation may also occur in newt cardiomyocytes since at 45 days after amputation and mincing of the newt heart, 7% had a polyploid nucleus and 4% of the cardiomyocytes were binucleate. In control animals, 1% of the cardiomyocytes had a polyploid nucleus and less than 1% were binucleate (Oberpriller et al., 1989). It is important to know how these percentages relate to the total fraction of cardiomyocytes that re-enters the cell cycle.

Successful investigation of the proliferative potential of different cell types has been achieved, for example, by approaches that allow the observation of single cells, using time-lapse microscopy (Burton et al., 1999a; Temple and Raff, 1986). In the previous chapter I have shown that adult newt cardiomyocytes are able to re-enter the cell cycle and divide in culture on a similar schedule to that observed *in vivo*. This chapter addresses the questions discussed above by following individual cells in culture by both lineage tracing and time lapse microscopy.

4.2 Results

4.2.1 Analysis of the Proliferative Potential by Lineage Tracing

In order to investigate the proliferative potential of the cardiomyocytes, a lineage tracing experiment was performed: $4x10^4$ cells, of which 4% were labelled with the fluorescent red membrane dye PKH-26 (SIGMA), were seeded on a 35mm tissue culture dish scored with a grid, thereby preventing the movement of the cells in between squares (see Fig. 4.1A). Under the inverted microscope, I ensured that only one mononucleate labelled cell was present per square by removing others (see Materials and Methods). The cells were fixed at 24 days after plating. A total of 238 cells were followed in three independent experiments. It was observed that 38% became multinucleate (i.e. contained two or more nuclei) and that 35% of the cardiomyocytes divided at least once (Fig. 4.1B, C and D). Approximately one third of the latter gave rise to 3 or more cells, suggesting that these cells underwent more than one mitosis (Fig. 4.1C).

Multinucleation is unlikely to be due to PKH-labelling since the percentage of multinucleate cardiomyocytes increased in all the cardiomyocyte preparations (see Fig. 4.2). One problem with the analysis of these experiments is that the size of cells increases as cultures age, and it becomes more difficult to identify cell boundaries. It has been suggested that cell fusion might occur in cultures of newt cardiomyocytes (Tate et al., 1989), embryonic chick cardiomyocytes (Przybylski and Chlebowski, 1972) and atrial myocytes from the squirrel monkey (Claycomb and Moses, 1985). In these studies, multinucleate cells with ^{3}H-thymidine labelled and non-labelled nuclei were observed. Therefore I decided to confirm the lineage tracing results using time-lapse microscopy, an approach that allows the distinction between multinucleation and overlapping or fusion of cells.

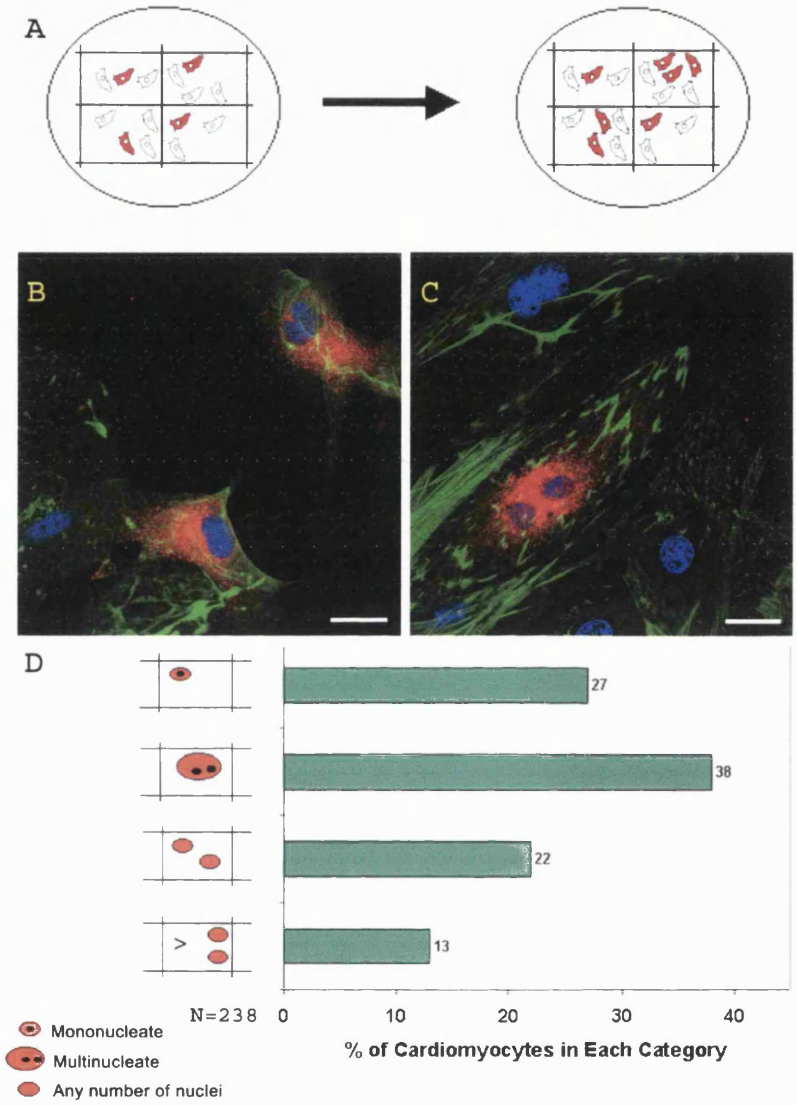

Figure 4.1 Lineage tracing analysis of the proliferative potential of adult newt cardiomyocytes. (A) Schematic representation of the experiment. Cells were labelled with the fluorescent red membrane dye PKH-26 (SIGMA) and seeded onto a tissue culture dish scored with a grid such that only one labelled mononucleate cell per square was present (see Materials and Methods). Cells were fixed after 24 days and stained for sarcomeric-MyHC and DNA. The number of red myocytes per square was counted. (B-C) Confocal micrographs of squares where two daughter cells (B) or a binucleate cell (C) were observed (PKH-red, sarcomeric MyHC-green, and Hoechst-blue). Scale bar-50μm. (D) Quantitation of the progeny of 238 cardiomyocytes from three independent experiments: (from the top) one mononucleate cell in the square (representing a cell that did not divide); one cell with more than one nucleus (cell that became multinucleate), two cells in the square (the mother cell divided once, giving rise to cells mononucleate or multinucleate daughter cells); more than two cells in the square (the mother cell divided more than once, giving rise to mononucleate or multinucleate daughter cells).

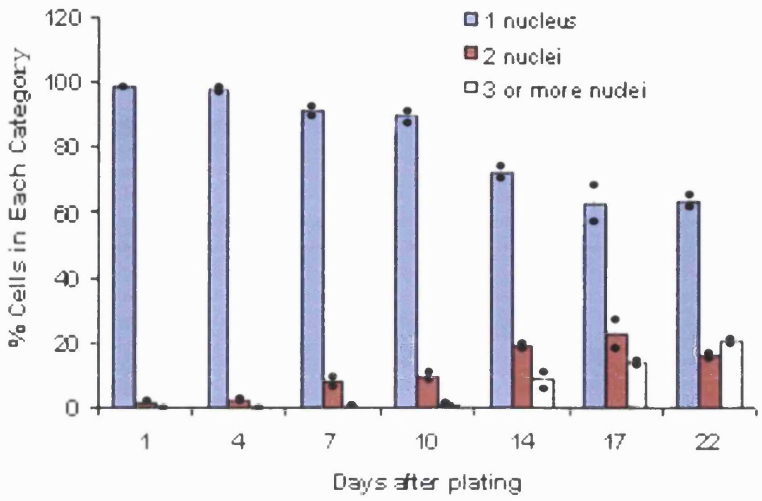

Figure 4.2 The number of nuclei in newt cardiomyocytes increases with time in culture. Cells were fixed at different days and stained for sarcomeric-MyHC and DNA (Hoechst). The number of nuclei per myocyte was scored. Note that the number of multinucleate cardiomyocytes increases from 7 to 17 days in culture, concomitant with cell proliferation (see Fig. 3.5 C). 150 cells were counted per well.

4.2.2 Analysis of the Proliferative Potential by Time Lapse Microscopy

4.2.2.1 Analysis of the Proliferative Potential: Mitosis and Cytokinesis

Cells were plated onto a culture dish scored with a numbered grid. Pictures of the grid squares were taken once or twice every day (see Materials and Methods). The progeny of 195 cardiomyocytes from five different experiments were followed.

A small number of non-sibling cardiomyocytes seemed to fuse[5] (less than 7%; Fig. 4.3), although further experimental evidence would be necessary to prove it. Most multinucleate cells resulted from incomplete mitosis (Fig. 4.4 A, B and C). This could be due to a problem in resolving the cleavage furrow, as mitosis often

[5] I shall call this process " fusion-like" in the rest of the text, since more experimental evidence would be needed to prove that there is fusion of membranes vs. overlapping of cells.

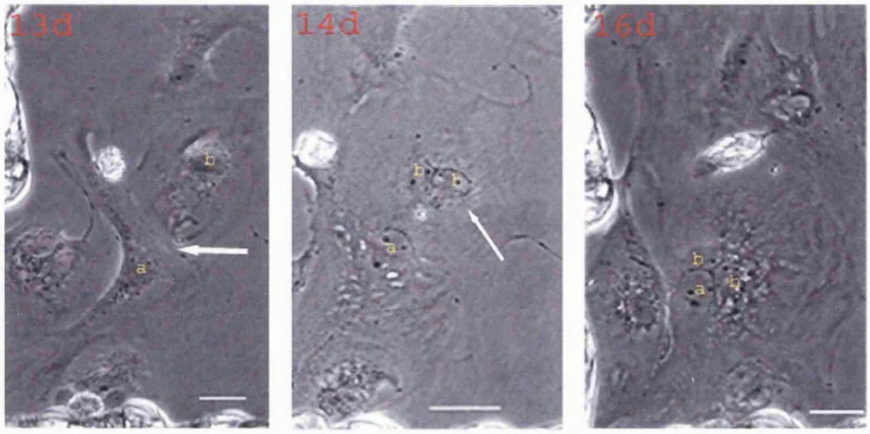

Figure 4.3 Cardiomyocytes may fuse. Cells were plated onto a dish scored with a grid, and pictures of the same square in the grid were taken once or twice a day. At 18 days, the plate was fixed and stained for sarcomeric MyHC and Hoechst (not shown). The number on the top left corner of each picture shows the number of days elapsed since the cells were plated. Two cells a and b (letters indicate the position of the nuclei) at 13d are clearly separated (arrow). The arrow points to the separation between the two cells, where there is juxtaposition of their cytoplasmatic membranes. At 14 days b becomes binucleate (arrow); the boundary between both cells is not clear. At 16 days the three nuclei are very closely juxtaposed and it is not possible to distinguish whether this is one or two cells. Scale bar – 50 μm.

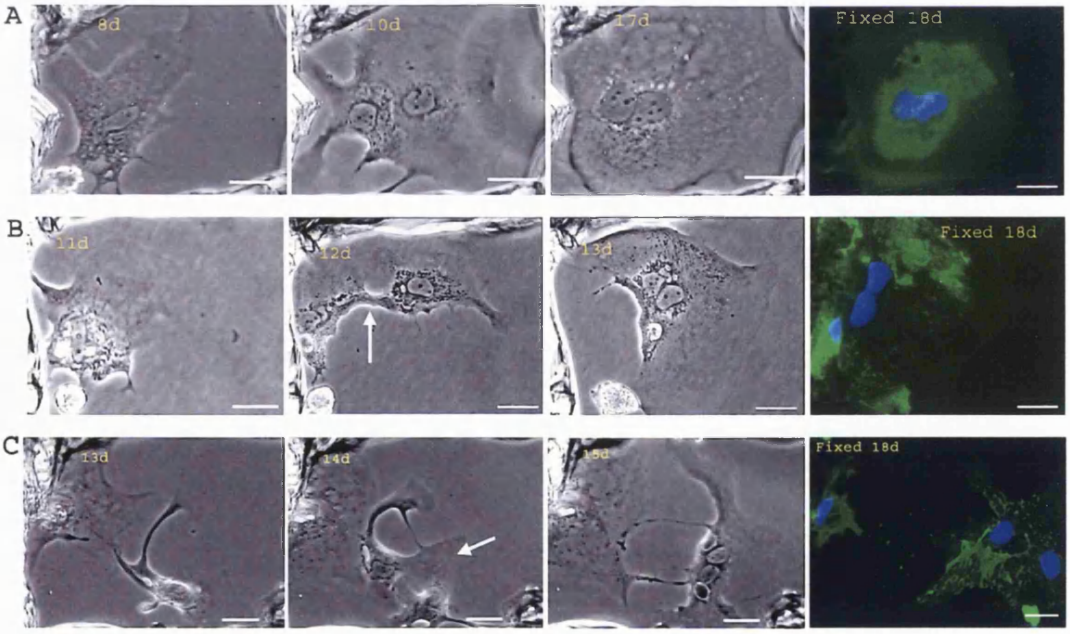

Figure 4.4 Cardiomyocytes enter mitosis giving rise to multinucleate cardiomyocytes. Description of experiment as in legend to Fig. 4.3. At 18 days, the plate was fixed and stained for sarcomeric MyHC (green) and Hoechst (blue). The number on the top left corner of each picture shows the number of days elapsed since the cells were plated. (A-C) Different examples of mononucleate cardiomyocytes that gave rise to binucleate cells. Note that in (B) the cardiomyocyte divides at 12d, but both daughter cells remain connected (see arrow). The nuclei of the cells become closer one day after. In (C) the cardiomyocyte on the right (13d) divides at 14d but, as in B, the daughter cells do not come completely apart (see arrow) and the nuclei are close together at 15d. Scale bar – 50 μm.

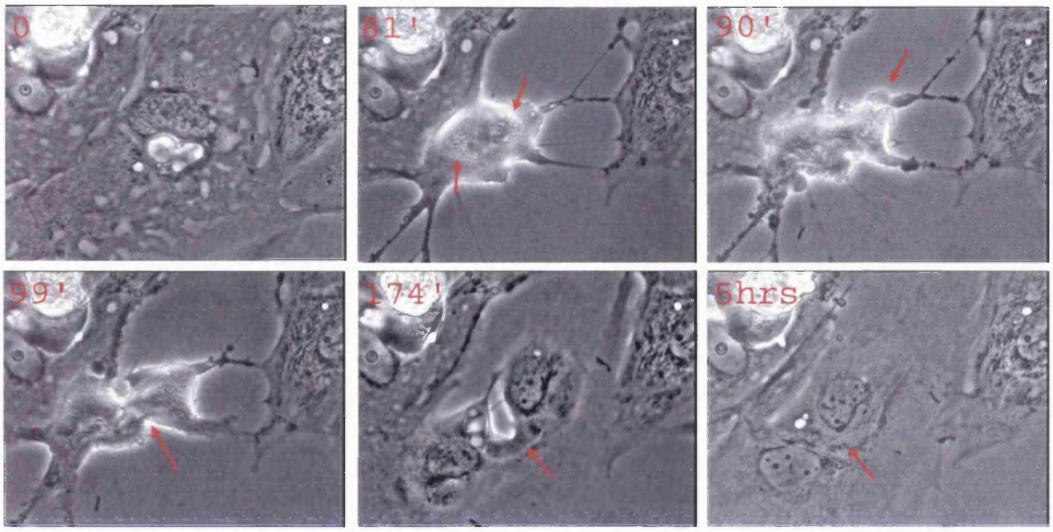

Figure 4.5 A monucleate cardiomyocyte gives rise to a binucleate cell. A cardiomyocyte was followed in real time by time-lapse digital microscopy. Time zero- A contracting cardiomyocyte in prophase. 81 minutes after, the cardiomyocyte is in anaphase (arrows pointing to chromosomes). At 90 minutes, note the blebs, very common in dividing newt cardiomyocytes. Note the cleavage furrow at 99 minutes (see arrow pointing to the furrow). 75 minutes afterwards the daughter cells do not come completely apart as they are still connected by a bridge containing myofibrils (see arrow). Approximately two hours afterwards, the bridge between both cells enlarges becoming clearer that it is a binucleate cell.

resulted in two partially separated cells with distant nuclei (see Fig. 4.4 B, 12d and C, 14d) that eventually came closer (see Fig. 4.4 B, 13d and C, 15d). This was clearer in continuous time-lapse microscopy of mitotic cells, where the formation of a cleavage furrow was always observed, but this furrow did not close in some cells, thereby giving rise to multinucleates (Fig. 4.5). The presence of striated myofibrils was often detected in such cleavage furrows (Fig. 4.5). In total, 31% of the cardiomyocytes gave rise to a multinucleate cell (i.e. 2 or more nuclei) in their first mitosis (Fig. 4.6).

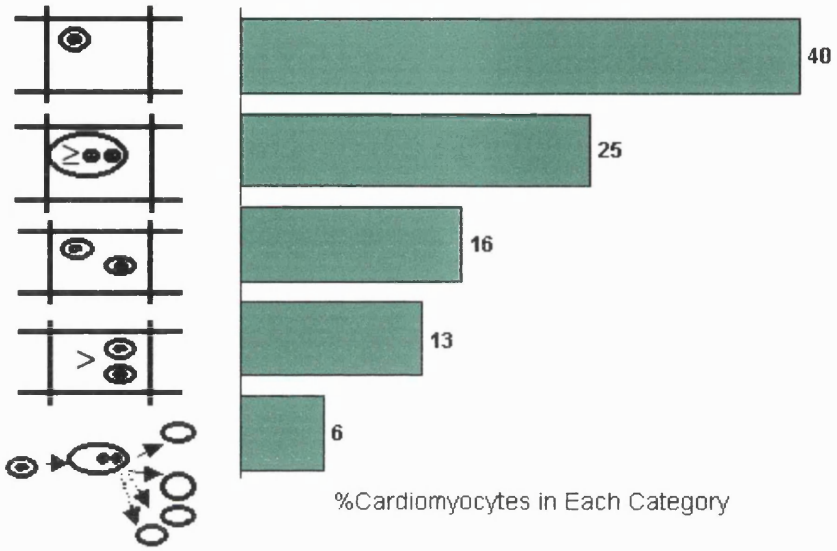

Figure 4.6 Quantitative analysis of the proliferative potential of cardiomyocytes by time-lapse video microscopy. From top to bottom: cells that did not divide, cells that became multinucleated, cells that divided once, cells that divided more than once (the daughter cells might be mono or multinucleate) and cells that became multinucleated and then divided again, undergoing cytokinesis (the outcome of this last division varied). Analysis of the progeny of 195 cells pooled from five independent experiments.

It was observed that approximately 29% of the beating cardiomyocytes were able to go through one or more complete rounds of cell division (i.e. karyokinesis and cytokinesis) giving rise to mononucleate progeny (Fig. 4.7 A and B, Fig. 4.6). Some of these cells gave rise to clones of cells that were not contracting and showed weak, disorganised staining for sarcomeric-MyHC (Fig. 4.8). These clones showed two main patterns of proliferation (Fig. 4.9 A): the first in which both daughter cells continued to divide at around the same time (Fig. 4.9 B zone 1); the second in which only one of the daughter cells continued dividing (Fig. 4.9A zone 2).

Cells that resulted from "fusion-like" processes did not divide. Approximately one fifth of the multinucleate cardiomyocytes formed by incomplete mitosis were able to divide, giving rise to progeny with different number of nuclei (Fig. 4.10 A, B; Fig. 4.9 B zone 3 and Fig. 4.6).

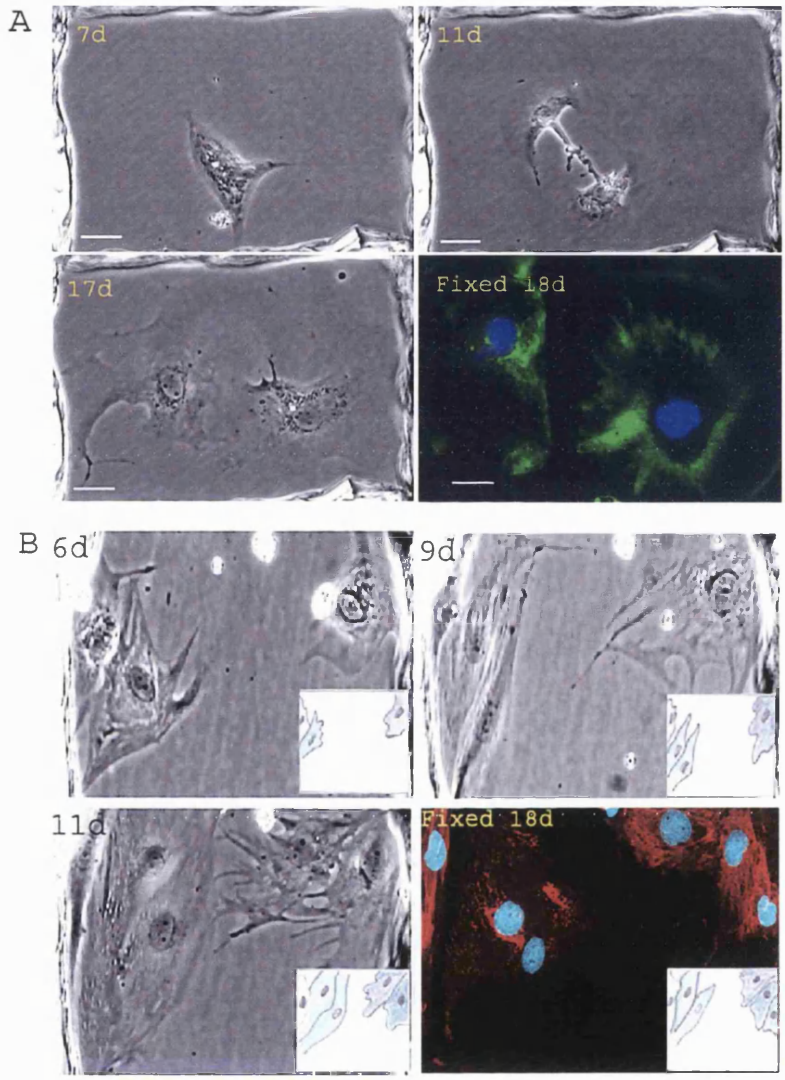

Figure 4.7 Cardiomyocytes can divide and give rise to mononucleate progeny. Description of experiment as in legend of Fig. 4.3. (A and B) Sequence of images in the same square of the grid taken at different days. The number at the top left of the images indicates the days after plating the cells onto laminin. Cells were labelled for sarcomeric MyHC (green in A and red in B) and DNA (blue). Scale bar – 50 μm. (A) A single cardiomyocyte exiting division at 11 days. Note that both daughter cells show myosin staining (green). (B) A schematic figure is shown at the right, lower side of each image. Square with 3 cardiomyocytes at 6 days. At 9 days, the lower cell on the left side of the picture divided and at 11 days the cell in the top right corner divided. When the cells were fixed, at 18 days, one of the daughters of the cell in the right corner had divided.

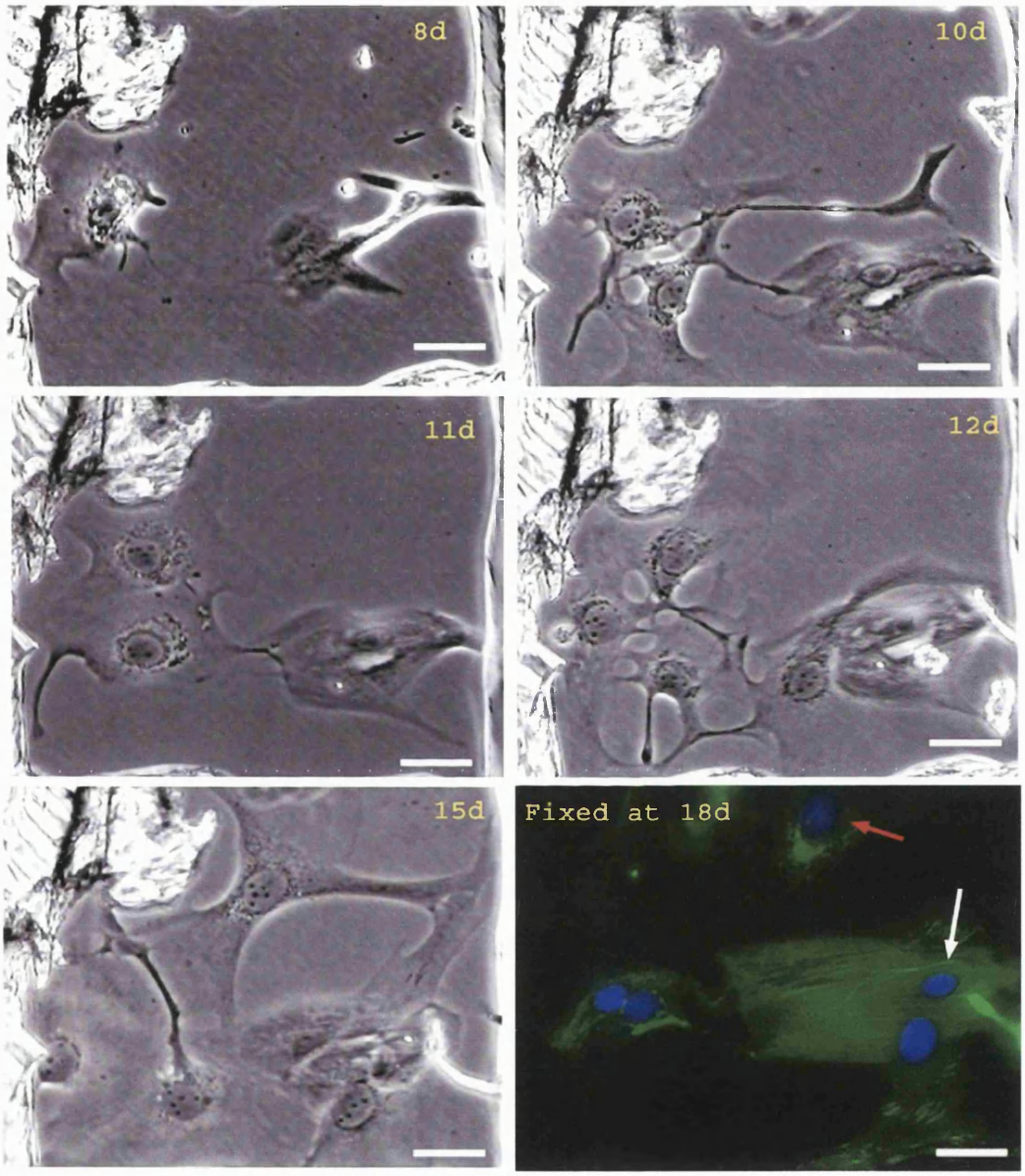

Figure 4.8 Cardiomyocytes can divide more than once. Description of experiment as in legend to Fig. 4.3. Cells were stained for sarcomeric MyHC (green) and DNA (blue). The number on the top right corner of each picture shows the number of days elapsed since the cells were plated onto laminin. The cell on the left at 8 days gives rise to two daughter cells at 10 days. Each of the daughter cells divides once again at 12 days. All the daughter cells stain positive for sarcomeric MyHC (lower, right corner), but this staining is less strong and organised (red arrow) than in the cell that did not divide (white arrow). Scale bar – 50 μm.

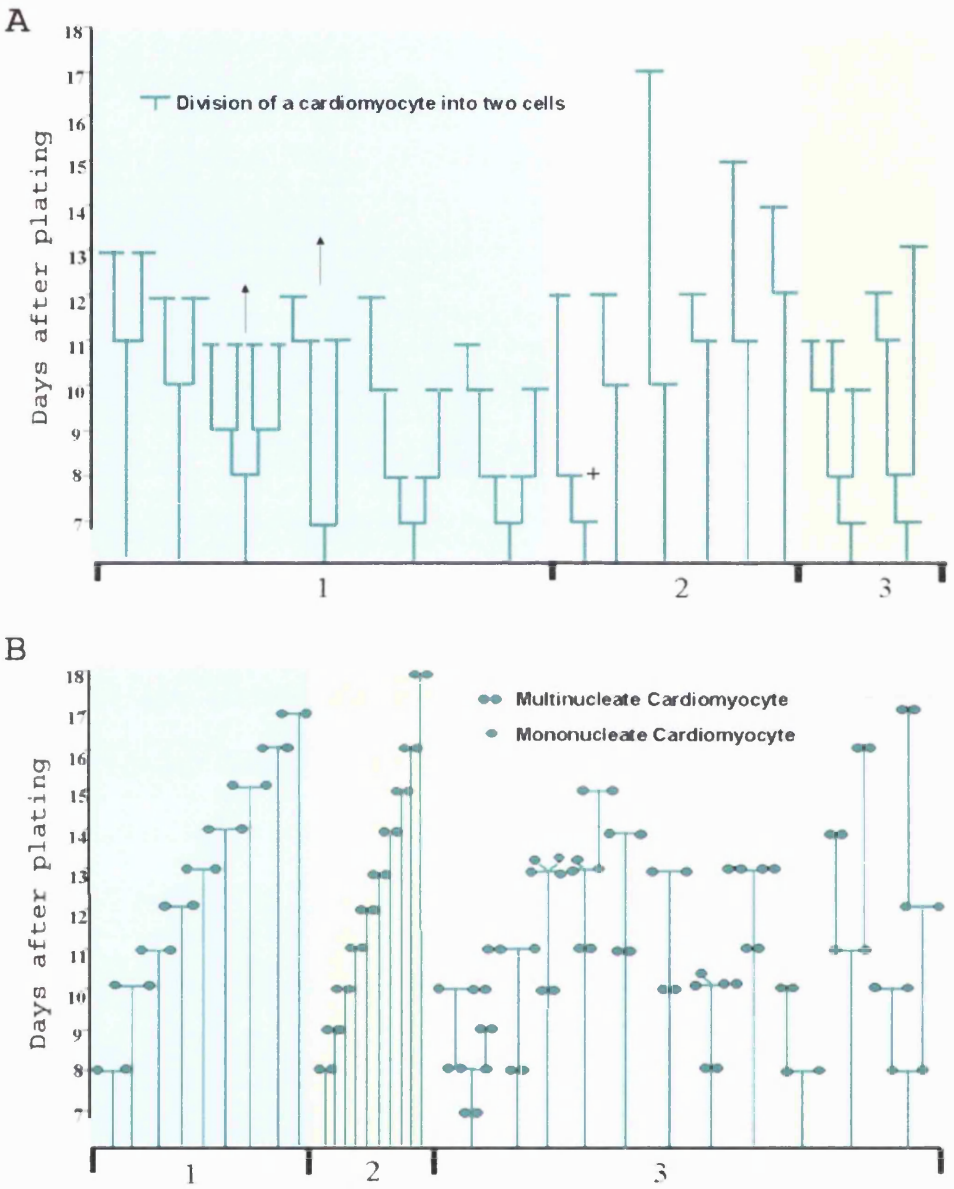

Figure 4.9 Patterns of the proliferation of newt cardiomyocytes. Description of experiment as in legend of Figure 4.3. Pedigrees were constructed from the time-lapse pictures of 3 different cultures to show the diversity of division patterns observed (a total of 79 dividing cells). Cells were grouped into two different graphs, depending on their pattern of cell division. (A) Only cardiomyocytes that divided more than once and gave rise to mononucleate progeny were incorporated in this graph. Zone 1, clones in which the progeny divided at the same time. The arrows in two clones indicate that those clones continue to divide, but using this experimental set-up it was impossible to trace those divisions as accurately as all the others represented here. Zone 2, clones that divided in a more asymmetric way. The cross indicates that a cell died. Zone 3, cells that did not fit into the categories of zone 1 or 2. (B) Cells that divided only once or became multinucleate. Zone 1, cells that divided only once giving rise to two daughter cells. Zone 2 and 3, formation of multinucleate cardiomyocytes at different days after plating. Zone 2, formation of multinucleate cardiomyocytes that did not divide. Zone 3, more complex pedigrees of cardiomyocytes that gave rise to multinucleate cardiomyocytes. Note in zone 3 that binucleate cardiomyocytes can divide, having many different outcomes. Many cells behaved as in zones 1 and 2. Only one example of each of the clones represented in zone 3 was found in the 79 cells represented in this graph.

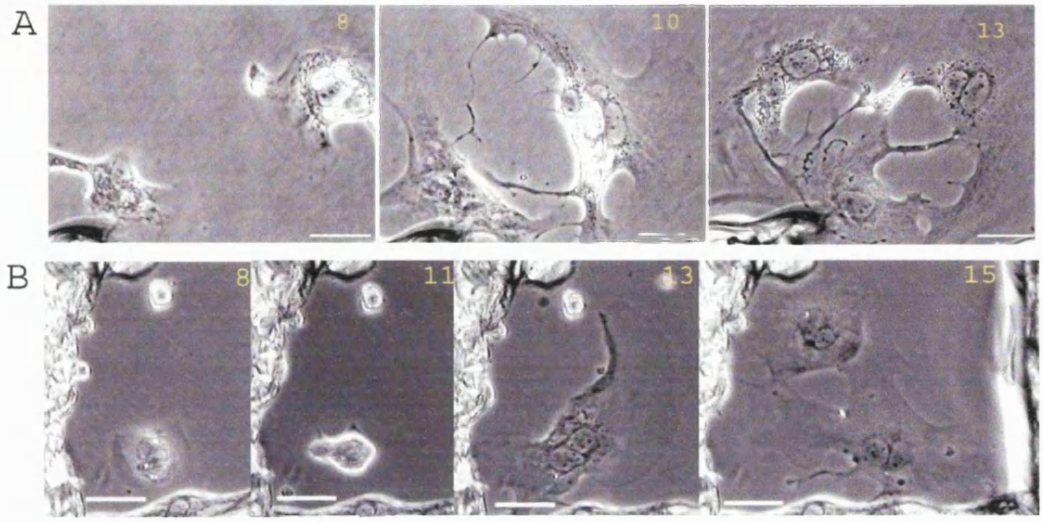

Figure 4.10 Multinucleate cardiomyocytes can divide. Description of experiment as in legend to Fig. 4.3. All the daughter cells stained positive for sarcomeric-MyHC (not shown here). The number on the top right corner of each picture shows the number of days elapsed since the cells were plated. (A) The cardiomyocyte on the right divides at 10 days, giving rise to a multinucleate cell which divides again at 13 days, giving rise to two binucleate daughter cells (see arrows). (B) The cardiomyocyte on the lower part of the image is dividing at 11 days giving rise to a binucleate cell which divides into two daughter cells (15). Scale bar – 50 μm.

Considering the 25 clones in Fig. 4.9 in which cardiomyocytes divided at least once (Fig. 4.9A and Fig. 4.9B, zone 3), the average interval between consecutive cell divisions was approximately 2.2 days, with a minimum of 1 and a maximum of 7 days. In general, all the cells that divided more than once underwent their first division between 7 and 12 days after plating. Some cells took as long as 17 days to enter mitosis (Fig. 4.9).

In summary, 60% of the cardiomyocytes underwent mitosis and approximately half of them were able to finish cytokinesis. 19% of the cardiomyocytes underwent more than 1 mitosis (Fig. 4.6). Overall, there was great variation in the proliferative capacity of individual clones, and in the outcome of the divisions in terms of the number of nuclei per daughter cell (Fig. 4.9 A and B).

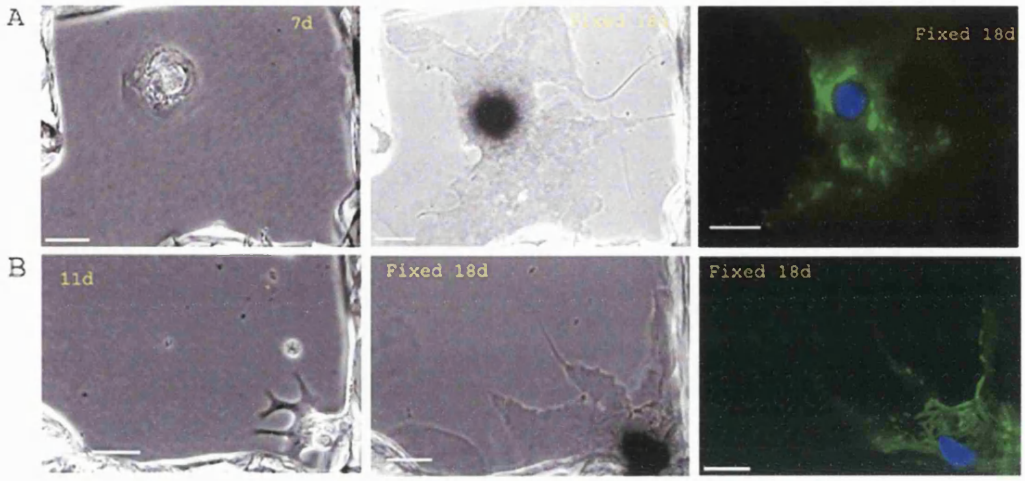

Figure 4.11 S phase entry without mitosis. Cells were plated onto a dish scored with a grid and pictures of the same square in the grid were taken once or twice a day. Cells were cultured in the presence of ^{14}C-thymidine up to 15 days after plating, when the label was removed. At 18 days, the plate was fixed and stained for sarcomeric MyHC (green) and Hoechst (blue). (A and B) Two examples where a single cell was followed in a square of the grid. The cardiomyocytes did not divide, but incorporated ^{14}C-thymidine (black shade over the nucleus in the middle image of A and B), showing that both cells entered S phase without following mitosis.

4.2.2.2 Analysis of the Proliferative Potential: DNA Synthesis and Progression to Mitosis

In order to investigate whether newt cardiomyocytes progress normally from DNA synthesis to mitosis, I performed time-lapse analysis (as described in the previous section) in media labelled with radioactive thymidine. This experiment was set-up to explore whether all the cells that had undergone DNA synthesis had progressed to mitosis. For this experiment, I had to use very small concentrations (0.54 mCi/mmol, 0.01μCi/ml) of labelled ^{14}C-thymidine since I had found that higher doses of ^{14}C-thymidine and ^{3}H-thymidine inhibited mitosis in newt cardiomyocytes (the experiments where this problem was investigated are described in the Materials and Methods section). From day 3 after plating, cells were cultured in media containing ^{14}C-thymidine. At 15 days after plating,

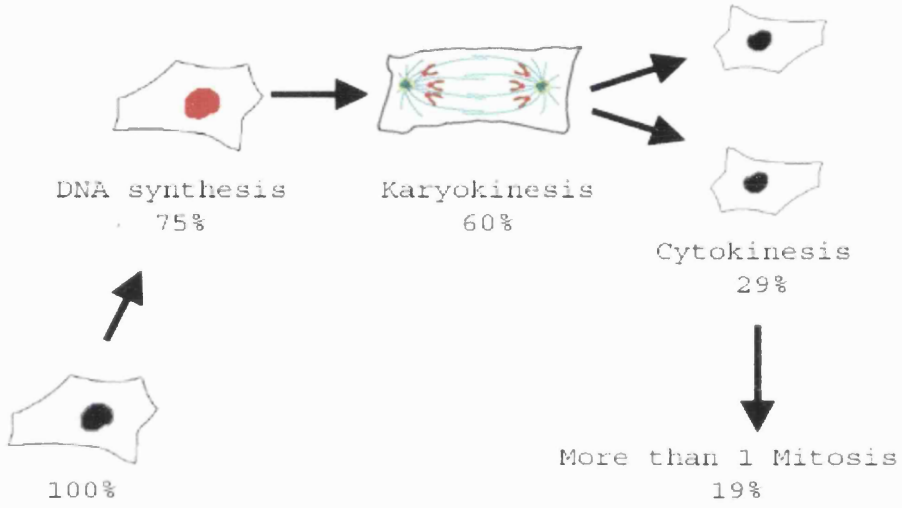

Figure 4.12 Summary of the time lapse results on the proliferative potential of newt cardiomyocytes. The percentages shown are always in relation to the original total of cardiomyocytes (100%). 75% of the cardiomyocytes entered the cell cycle. 60% of the cardiomyocytes entered mitosis and underwent cytokinesis but only 29% finished cytokinesis. 19% of the cells entered a second round of mitosis (including cells which had become multinucleate).

three days prior to fixing them, the cells were washed and given non-labelled media. In these conditions, cardiomyocytes that had incorporated labelled thymidine would have had 3 days[6] to finish mitosis before the end of the experiment. A total of 109 cardiomyocytes were analysed of which 75% entered S phase. Of the cardiomyocytes that entered S phase, 76% were observed to undergo mitosis (see Fig 4.11). An analysis of the dynamics of the entire

[6] The average cell cycle time of newt cardiomyocytes has not been described. The average time of S phase for newt A1 myotubes is 48-72 hrs (Tanaka et al., 1997)..

population observed shows that 15% of the cells that entered S phase did not undergo mitosis (see Fig. 4.12).

Table 4.1 Comparison of the data obtained in this chapter with *in vivo* data from the group of Oberpriller and collaborators .

	Increase in the number of cells	%Binucleates	%Mononucleates with polyploid nucleus
1-Data presented here-*in vitro*	219%	14%	7%
2-(Bader and Oberpriller, 1979)- *in vivo*	256%		
3-(Oberpriller et al., 1989; Oberpriller et al., 1995)-*in vivo*		6%	7%

1-Extrapolation of the data presented in this chapter. Assuming that the mononucleates that divided more than once would have divided an average of three times and that the cells that became binucleate and divided again would have given rise to three cells, one of which was binucleate and two mononucleates; 2-Experiment where the tip of the ventricle was cut, minced and grafted into the heart. The number of cells in the graft was counted and averaged in different animals at 16 and 30 days; 3-The same experimental set-up as in 2. At 45 days the number of binucleates and mononucleate cells with polyploid nucleus in the graft was counted.

4.3. Discussion

This study has for the first time quantitatively examined the proliferative potential of adult newt cardiomyocytes. The data shows that 75% of adult newt ventricular cardiomyocytes enter S phase, 60% of the total undergo mitosis and approximately half of these undergo cytokinesis, during the first 18 days in culture (Fig. 4.12). This ability to divide is clearly distinct from that of adult mammalian ventricle cardiomyocytes, which have rarely been observed to undergo mitosis, and never to undergo cytokinesis (Claycomb, 1991; Claycomb and Bradshaw, 1983; Nag, 1991; Nag and Cheng, 1986; Soonpaa and Field, 1998).

The proliferative potential of newt cardiomyocytes was investigated using two different techniques: lineage tracing and time-lapse microscopy. In both cases, the percentage of cells that underwent one or more complete rounds of mitosis was 35%. Differences were found in the percentage of cells that did not enter mitosis (13% smaller in the lineage analysis) and that became multinucleate (13% larger in the lineage analysis). These could be due to various factors: differences in the duration of the experiments (24 days in the lineage analysis vs. 18 days in the time-lapse), fusion-like processes (which occurred in 7% of cells in the time-lapse and could not be accounted for in the lineage tracing experiment) and difficulty in ascertaining cell boundaries in aged cultures in the lineage tracing experiment. From now on, I will be discussing the results of the time-lapse experiment, which I consider to be more accurate, because each cell and its nuclei were followed individually.

There was large variation in the overall proliferative capacity and in the possible outcome of mitosis of newt cardiomyocytes (Fig. 4.6 and Fig. 4.9). One possible interpretation is that there are two populations of cardiomyocytes in relation to their proliferative potential. The first, and most abundant, is composed of cells that divided only once and subsequently arrested. One important result was the identification of two points at which many of these cells arrested their progression through the cell cycle: the first prior to mitosis, and the second at

closure of the cleavage furrow. After S phase entry and DNA synthesis, 24% of cells did not progress to mitosis and half of the cells that entered mitosis did not finish cytokinesis (see Fig. 4.12). The second, less abundant, population was composed of cells that divided several times, which generated non-contracting progeny with a small amount of sarcomeric-MyHC. The small amount of sarcomeric-MyHC could be a consequence of successive cell divisions in the absence of synthesis and assembly of myofibrils. Alternatively, the loss of sarcomeric-MyHC might reflect a greater degree of dedifferentiation in these cells as a consequence of increased proliferation.

Another interesting observation was that the average interval between consecutive cell divisions was approximately 2.2 days, with a minimum of 1 and a maximum of 7 days (Fig. 4.9). The average timing of 2.2 days (53 hr) is comparable to that found for the cell cycle period of mononucleate cells from the blastema of regenerating limbs of axolotls (Wallace and Maden, 1976)

How do the results described in this chapter relate to *in vivo* observations? An extrapolation[7] of the data presented in Fig. 4.6 suggests that, at the end of 18 days, there should be a 219% increase in the total number of cells, and that 14% of these should be binucleate and 7% mononucleate with a polyploid nucleus (Table 4.1). In the experiments of Oberpriller *et al.* (1989) 6% of the cells in the graft were binucleate and 7% were mononucleate with a polyploid nucleus, 45 days after grafting the minced ventricular myocardium (Oberpriller et al., 1989; Oberpriller et al., 1995). Using the same protocol, there was a 256% increase in the number of cells in the graft between 16 and 30 days after grafting the minced ventricular myocardium, a time interval where cells were actively proliferating (Bader and Oberpriller, 1979). A comparison of the extrapolation of my observations and the

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[7] Assuming that mononucleates that divided more than once would have divided an average of three times and that the cells that became binucleate and divided again would have given rise to three cells, one of which was binucleate and two mononucleates.

results obtained *in vivo* (Table 4.1) suggests that the blocks to cell cycle progression described *in vitro* might reflect the events that occur *in vivo* following injury to the myocardium. Additionally, the proliferating clones of cells, which after one or two rounds of division express very little sarcomeric-MyHC, might contribute significantly to the increase in cell mass in the repaired ventricle. Certainly, the *in vivo* studies claimed that there was partial disorganisation of myofibrils at the peak of cell proliferation but that later there was reorganisation of the myofibrils (Bader and Oberpriller, 1979). It would be interesting to subculture the clones of proliferating cardiomyocytes in media with a low percentage of serum in order to investigate whether they are able to reassemble myofibrils and resume beating. In conclusion, the present observations at the single cell level are able to explain the results at the population level in the newt-injured myocardium.

What conclusions can be drawn from the blocks to cell cycle progression found in the newt cardiomyocytes? In mammalian cardiomyocytes, the G2/M checkpoint and the ability to undergo cytokinesis, in addition to entry into S phase, have long been recognised as critical boundaries for cardiomyocyte cell cycle progression. Cardiac myocytes become polyploid and/or multinucleate after birth (Brodsky, 1991). In adult rat cardiac myocytes, 15% have a G2/M nuclear content (Poolman et al., 1998), while mammalian neonatal cardiomyocytes reactivate DNA synthesis after transfection with E2F-1, and accumulate in G2/M (Agah et al., 1997b; Kirshenbaum et al., 1996). Finally, *Rb -/-* stem cell derived cardiomyocytes enter the cell cycle but become multinucleate due to inability to complete cytokinesis (Schneider, 1995). The molecular regulation of these blocks in cardiomyocytes is still not clearly understood. The fact that different mammals have different proportions of diploid cells, mononucleate cells with polyploid nuclei, and multinucleate cells in the heart (see Chapter 1 for more details), brings an additional level of complexity. It has been suggested that the block to cytokinesis might be due to the inability of

differentiated cardiomyocytes to disassemble their myofibrils during mitosis, hence these structures might hinder the ability to close the cleavage furrow (Borisov and Rumyantsev, 1991). Part of data obtained from the present study support this view (Fig. 4.4 and 4.5). On the other hand mammalian hepatocytes, which do not have myofibrils, have similar blocks to cell cycle progression after birth (see Chapter 1; Uryvaeva, 1985). Why different cell types become multinucleate and polyploid after birth is still unknown and remains as an interesting problem.

The existence of a block after entry into S phase and at cytokinesis in adult newt cardiomyocytes suggests that the regulation of both the differentiated state and cell cycle progression may be fundamentally similar in newt and mammalian cardiomyocytes, possibly relying on a different regulation of the same pathways.

The present finding that the dissociated culture of newt cardiomyocytes has different classes of cells based on their ability to progress through the cell cycle (i.e. cells that are able to go through complete cell division cycles and cells that display a blockade in M phase entry and cytokinesis) suggest new experiments to address the complex control of cardiac muscle cell proliferation. It would be interesting to use single cell libraries and microarray technology to compare gene expression of cells that have different proliferation capabilities. Additionally, the fact that in newt cardiomyocyte cultures a significantly high percentage of the cells undergo mitosis makes them attractive to study the regulatory events associated with mitosis in a muscle cell. The combined use of fluorescently-tagged myofibrillar proteins with drugs that regulate assembly and disassembly of the cytoskeleton could be a feasible approach to study the formation of binucleate cells.

-Chapter 5-

Molecular Regulation of Entry Into S Phase in Newt Cardiomyocytes

5.1 INTRODUCTION

Rather little is known about the molecular mechanisms involved in the response of the adult amphibian cardiomyocyte to injury. Identification of the stimuli and the intracellular signalling pathways regulating cell cycle re-entry and entry into S phase by newt cardiomyocytes should contribute to our understanding of the plasticity of the differentiated state in urodele cells; and this should also give insight into the mechanisms preventing proliferation of adult mammalian cardiomyocytes.

In Chapter 3, I showed that serum enhances cell cycle re-entry in cultured adult cardiomyocytes. The effect of some growth promoting factors present in serum has been studied on newt cardiomyocytes (Oberpriller et al., 1995; Soonpaa et al., 1992; Soonpaa et al., 1994). Those studies were performed in media containing 10% FBS, resulting in high background responses. The maximum effect observed was a two-fold increase in the percentage of cells in S phase, achieved with Endothelial Cell Growth Supplement (ECGS), an extract of bovine pituitary glands that contains FGF-1. Purified factors such as PDGF and FGF-1 induced entry into S phase of less than 30% over the control (Oberpriller et al., 1995; Soonpaa et al., 1992; Soonpaa et al., 1994). The establishment of new assays is therefore necessary to access the activity of growth factors on newt cardiomyocytes.

The cultured skeletal myotube has been an informative system for investigating the plasticity of the differentiated state in newt cells. Newt myotubes, in contrast to their mammalian counterparts, respond to elevated serum concentrations by entering and traversing S phase (Tanaka et al., 1999; Tanaka et al., 1997). As referred to in Chapter 1, cultured newt myotubes, as their mammalian counterparts, are refractory to a variety of families of growth factors, such as PDGF, EGF and FGF, which are active on their mononucleate precursors (Tanaka et al., 1999). Crude thrombin preparations have been shown to be able to induce S phase by the myotubes and to be of significantly higher specific activity

than serum sources (Tanaka et al., 1999). It has been shown that thrombin acts indirectly on the myotubes through serum proteolysis, by generating an activity (Fa) (Fig. 5.1), which is resistant to inhibition of thrombin proteolytic activity (Tanaka et al., 1999). This activity has been purified significantly from the crude thrombin preparations but its identity remains unknown. It is interesting that this activity acts on the newt myotubes in the absence of serum but not on its mononucleate precursors or its mammalian counterparts (Tanaka et al., 1999). Responsiveness to this factor may be a property of the urodele differentiated state.

A requirement for Fa might be important to restrict cell cycle re-entry to the injury/clot site. In fact, thrombin proteolytic activity is not present in the normal limb, and is elevated in the distal limb stump at a time when implanted A1 myotubes and endogenous myofibers have been observed to enter S phase (Kumar et al., 2000; Tanaka et al., 1999). It is likely that a similar mechanism might operate in the newt heart. After amputation and mincing of the tip of the newt ventricle, DNA synthesis of cardiomyocytes was significantly higher in the minced muscle and limited within a radius of 500μm of the wound (Oberpriller et al., 1989).

The fact that Fa does not induce cell cycle re-entry in mammalian myotubes (Tanaka et al., 1999) suggests that cell-intrinsic factors could be also involved in regulating the plasticity of urodele myotubes. Proteins of the pRb-family of pocket proteins (pRb, p107 and p130) are inhibitors of the transition from the G1 to S phase of the cell cycle. These proteins are important in the control of differentiation and establishment of the post-mitotic arrest, in the maintenance of a stable differentiated state and in re-entry to the cell cycle in mammalian differentiated cells (see Chapter 1). Inactivation of pRb by phosphorylation occurs in cell cycle re-entry from the differentiated state in hepatocytes (Albrecht et al., 1998) and smooth muscle cells (Rao, 1999). Mammalian wild-type myotubes do not inactivate pRb in response to serum. The

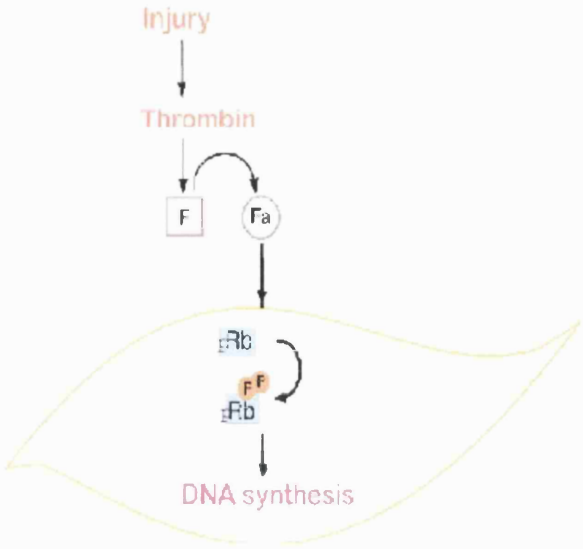

Figure 5.1 Entry into S phase by newt myotubes. Schematic diagram of the activation of S-phase re-entry by newt myotubes in the context of injury. A major aspect of wound healing is activation of the coagulation cascade, resulting in the conversion of prothrombin to thrombin. In mammals, this induces the conversion of fibrinogen to fibrin polymers and formation of a clot. In newts, thrombin activation, in addition, leads to cell-cycle re-entry from the differentiated state. This involves the conversion of a latent activity (F to Fa) within serum, which can stimulate newt myotubes to undergo S phase (Tanaka et al., 1999). Entry into S phase by newt myotubes is dependent on pRb phosphorylation (Tanaka et al., 1997).

importance of pRb in the establishment and maintenance of the post-mitotic arrest of mammalian myotubes has been shown by the fact that *Rb* homozygous-null myotubes are able to enter S phase in response to serum (Novitch et al., 1996; Schneider et al., 1994) and inactivation of pocket proteins is necessary for cell cycle re-entry by the myotubes induced by the E1A protein (Mal et al., 2000). In contrast to mammalian myotubes, treatment with elevated serum concentrations results in phosphorylation of the retinoblastoma protein in their newt counterparts (Tanaka et al., 1997). Different regulation of the pRb pathway is then

a candidate mechanism to explain the different responsiveness to serum in urodele-differentiated cells (Fig. 5.1).

The pocket proteins may also play an important role in the maintenance of the differentiated state in mammalian cardiomyocytes. Overexpression of E2F can induce DNA synthesis in neonatal and adult cardiomyocytes (Agah et al., 1997b; Kirshenbaum et al., 1996). The pathway leading to DNA synthesis in newt myotubes (Fig. 5.1) is therefore a candidate to regulate the plasticity of adult newt ventricular cardiomyocytes. In this chapter I investigate whether pocket protein phosphorylation is necessary for entry into S phase in newt cardiomyocytes and whether Fa promotes DNA synthesis in these cells.

5.2 RESULTS

5.2.1 The Status of pRb Phosphorylation in Serum Stimulated Cardiomyocytes

pRb is hypo-phosphorylated in quiescent mammalian cells and in newt myotubes maintained in low serum. It becomes phosphorylated when the cells are stimulated to enter S phase (Tanaka et al., 1997). In order to investigate the presence and phosphorylation state of pRb in newt cardiomyocytes after serum stimulation, I decided initially to approach this issue at the single cell level, by immunocytochemistry. This method, in contrast to a biochemical approach, should allow a direct link between pRb phosphorylation and S phase entry, as assessed by BrdU incorporation, in newt cardiomyocytes.

The mammalian pRb protein has 16 consensus CDK phosphorylation sites (Ser/Thr-Pro motifs), and a number of differentially phosphorylated pRb forms seem to exist in cells (Mittnacht, 1998). The antibodies I used were a kind gift from Dr. Sybille Mittnacht (ICR, London). These antibodies specifically recognise phosphorylated Serine-608 (S-608; a residue that is conserved from human to newt, Fig. 1.3.2, Chapter 1) in Western blots (Dr. Sybille Mittnacht personal communication). This residue is predominantly unphosphorylated in cells in the G_0/G_1 compartment, becomes phosphorylated prior to entry into S phase, and remains phosphorylated throughout the rest of the cell cycle (Zarkowska et al., 1997). The functional role of S-608 phosphorylation in the progression to S phase has not yet been elucidated.

I tested the suitability of these antibodies for immunocytochemistry in newt myotubes. These cells are known to have hypophosphorylated pRb in low serum media and phosphorylated pRb in the presence of high serum (Tanaka et al., 1997). Myotubes were cultured in serum free medium or with 10% FBS for four days. Controls performed in parallel samples showed that as expected myotubes were entering S phase in 10%, but not in the media without serum.

There were positive cells for pRb antibody staining in both conditions (see Fig. 5.2 for an example). Control experiments with no primary antibody did not show any staining. One possible explanation for the positive staining of newt myotubes with the tested antibodies in both media with and without serum is that S-608 was phosphorylated in both conditions. An alternative explanation is that the antibodies may not be specific for phosphorylated S-608 in immunocytochemistry. In any case, the antibodies did not seem appropriate for this approach.

After the failure of this approach, I decided to investigate the phosphorylation state of endogenous pRb in newt cardiomyocytes using the more traditional biochemical approaches of immunoprecipitation and Western blotting. This approach has the advantage of investigating the net phosphorylation state of the protein (not just phosphorylation of a single amino-acid residue), since it detects overall changes in mobility on SDS gels. A mixture of two antibodies, SK70 and XZ56, was previously used for pRb detection in newt myotubes (Tanaka et al., 1997). SK70 is an affinity purified polyclonal rabbit antibody, prepared against a portion of the pocket region of newt pRb, that appears to preferentially recognise more highly phosphorylated forms of newt pRb (Tanaka et al., 1997). XZ56 is a mouse monoclonal antibody that recognises human pRb and cross-reacts with less phosphorylated forms of newt pRb (Hu et al., 1991; Tanaka et al., 1997). When extracts of newt myotubes maintained in low serum were immunoprecipitated with SK70 and XZ56, to obtain all forms of pRb, and then detected by Western blotting with SK70, the pRb migrated as a single band at 110 kD, indicating that the protein was not phosphorylated. In contrast, 50% of pRb from myotubes maintained in high serum for 4 d had retarded mobility (Tanaka et al., 1997).

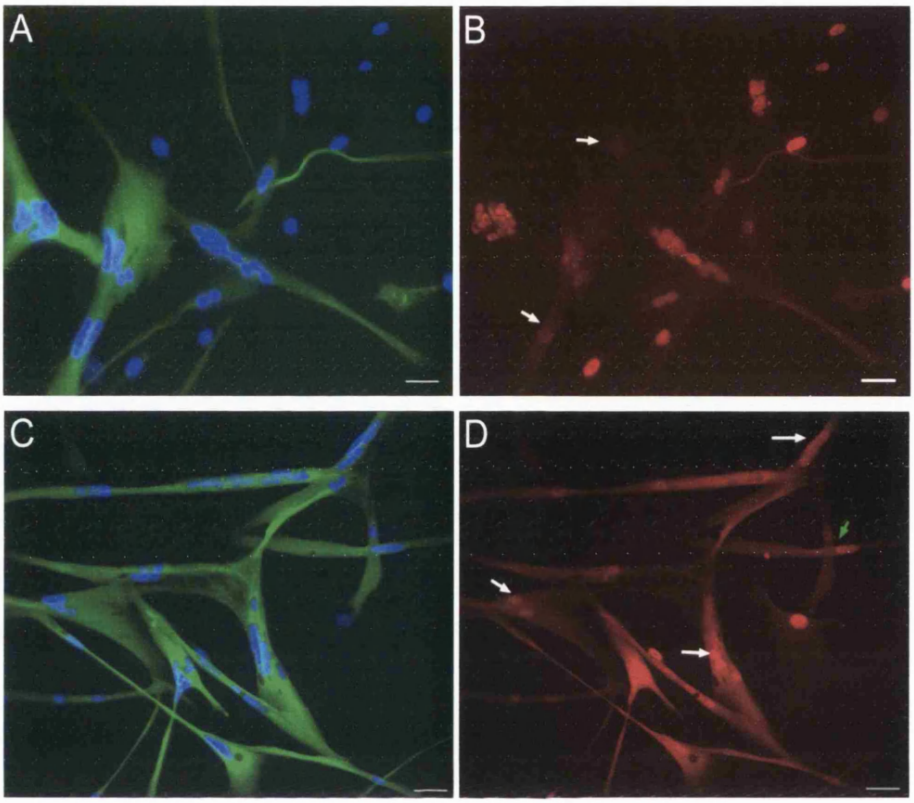

Figure 5.2 Evaluation of a single cell assay for phosphorylated pRb. Newt skeletal myotubes were purified and maintained in 10% serum containing media (A and B) or in media without serum (C and D). Both samples were fixed after 4 days and stained for sarcomeric-MyHC (green), DNA (blue) and pRb (red). Both samples were processed at the same time and pictures of pRb staining were acquired with the same parameters. Staining for pRb seems more distinct and nuclear-localised in the stimulated sample (B vs. D). White-arrows show that staining for Rb is faint in some of the stimulated nuclei (B) and strong in some of the non-stimulated nuclei (D). The same myotubes can have both positive and negative nuclei (green arrow). A clear difference in pRb staining of stimulated and non-stimulated sample was not found. Scale bars 100 μm.

Experiments were performed[8] using the same protocol in extracts of stimulated newt cardiomyocytes, 10 days after plating the cells in media containing 10%FBS. A pRb band was found which had the same retarded

[8] These experiments were performed by Dr. András Simon, a post-doctoral research fellow in this laboratory.

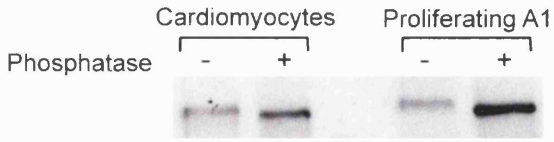

Figure 5.3 Preliminary results on pRb phosphorylation in newt cardiomyocytes. Extracts of cardiomyocytes plated in 10% FBS and of cycling A1 mononucleate cells were immunoprecipitated with SK70 and XZ56, to obtain both phosphorylated and non-phosphorylated forms of pRb, and then detected by Western blotting with SK70 as described in Materials and Methods. Upon treatment with phosphatase the band migrated faster, showing that most of the pRb in the extracts was phosphorylated.

mobility as the one found in cycling A1 mononucleate cells (see Fig 5.3). When treated with phosphatase the protein migrated as a single 110 kD band (see Fig 5.3). This result was obtained very recently. I am now improving the resolution of the Western blots so that I can confirm this result and also analyse the phosphorylation state of pRb in extracts from non-stimulated cardiomyocytes.

5.2.2 p16 Inhibits Entry into S phase by Newt Cardiomyocytes

In order to test if pRb phosphorylation was directly involved in newt cardiomyocyte DNA synthesis, I asked whether expression of the CDK 4/6 inhibitor, p16, would inhibit the response. The p16 protein can only inhibit entry to S phase or colony formation in cells that express pRb-family proteins (Bruce et al., 2000; Dannenberg et al., 2000; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995; Sage et al., 2000). As no newt CDI has yet been cloned, I used the human p16 sequence, which has been shown to inhibit S-phase entry when expressed in newt myotubes (Tanaka et al., 1997; Velloso et al., 2000). In order to express p16 in the cardiomyocytes I had to transfect these cells.

5.2.2.1 Transfection of Newt Cardiomyocytes

Infection by adenoviruses and microinjection of plasmid DNA have proven to be effective techniques for transfection of mammalian cardiomyocytes (Bartoli

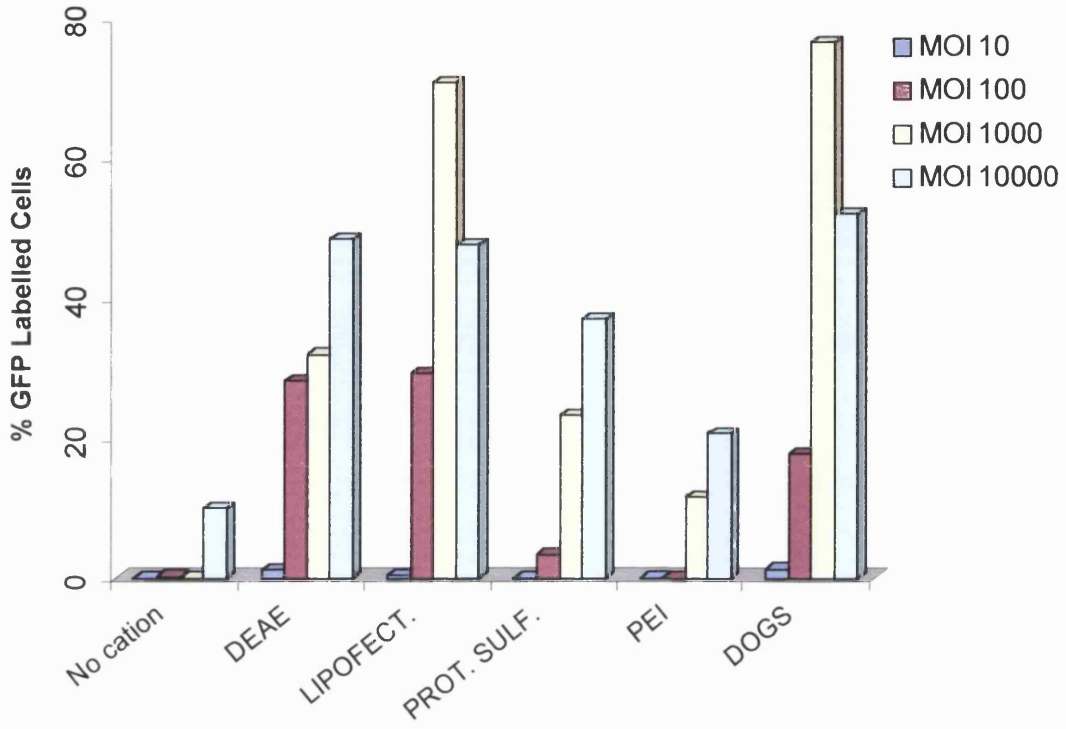

Figure 5.4 Cationic-enhanced adenoviral delivery of genes into newt cardiomyocytes. Percentage of GFP-positive cells 4 days after infection. Cells were infected 4 days after plating. Adenovirus were incubated with polycations and cationic lipids prior to infecting the cells as described in Materials and Methods. DEAE-DEAE dextran; LIPOFECT-Lipofectamine; PROT.SULF. - Protamine Sulfate; PEI- polyamine polyethyenimine and DOGS- lipopolyamine dioctadecylamidoglycylspermine. A minimum of 200 cells were scored for each condition. Note that Lipofectamine and DOGS were the most effective enhancers of infection at a MOI of 1000.

and Claycomb, 1997; Kirshenbaum et al., 1993; Zhou et al., 2000) and I investigated both approaches for newt cardiomyocytes.

Adenoviruses have been widely used for gene delivery in replicating and non-replicating cells, including mammalian cardiomyocytes (Benihoud et al., 1999; Kirshenbaum, 1997; Zhou et al., 2000). These viruses enter the cell via receptor-mediated endocytosis and remain separated from the host's genome, eliminating the problems of mutational and genetic instability of infected cells (Benihoud et al., 1999). I used a recombinant-adenovirus, kindly provided by Dr. Michael Themis (Imperial College, London), which belongs to the serotype 5

(Ad5) and is a non-replicating form, since it has a deletion of the E1 early gene (E1A + E1B), necessary for replication. The virus contains the Green Fluorescent Protein (GFP) gene under the control of the eukaryotic CMV promoter.

In the first experiment less than 5% of newt cardiomyocytes expressed GFP after infection (results not shown). It has been reported that efficiency of infection by adenovirus can be improved after incubation of the virus with cationic lipids or cationic polymers (Arcasoy et al., 1997; Dodds et al., 1999; Lanuti et al., 1999; McKay et al., 2000). This modification was very successful and 70% of the cardiomyocytes expressed GFP (see Fig. 5.4) when a Multiplicity of Infection[9] (MOI) of 1000 was used together with lipofectamine or lipopolyamine dioctadecylamidoglycylspermine (DOGS). Lipofectamine had less visible toxic effects than DOGS, as cells appeared healthier and there was less cell debris. The expression of GFP was stably maintained at 20 days after co-infection with lipofectamine, as it was detectable in 70% of the cells. This efficiency of transfection is comparable to those obtained in mouse cardiomyocytes with adenovirus (in these cells the use of cationic molecules is not necessary; Kirshenbaum, 1997; Zhou et al., 2000).

As a good compromise between efficiency of transfection and cell toxicity was attained using lipofectamine, the experiment was repeated with this cationic-lipid. In order to determine whether DNA synthesis was impaired after infection, the cells were incubated with BrdU for 18 hours. The percentage of transfected cells was once more high (approximately 80% for MOI 1000), but there was marked inhibition of DNA synthesis (see table 5.1). This effect could be due to the lipofectamine, the virus, high levels of GFP expression, or to any combination of the three factors. I decided to discontinue the experiments using adenoviruses, because preliminary results indicated that microinjection of cells do not have the same inhibitory effects.

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[9] Number of infectious viral particles per cell.

Table 5.1 Lipofectamine-enhanced infection of cardiomyocytes with adenovirus inhibits entry into S phase

	% BrdU-labelled cardiomyocytes
Control (no virus or lipofectamine)	23.4 ± 4
MOI 100	1.7 ± 0.7
MOI 1000	1.8 ± 0.1

Cells were infected 4 days after plating. Eighty per cent of the cells were expressing GFP at the MOI of 1000. Cells were given a BrdU pulse of 18 hours, 7 days after infection. Results shown are the average of the counts of two dishes plus or minus the difference between each experimental value and the mean. Approximately 300 cells were counted in each dish.

Microinjection of adult rat cardiomyocytes has been successfully achieved (Bartoli and Claycomb, 1997). One of the problems of microinjecting an excitable cell such as a cardiac myocyte is contraction of the myofibrils after exposure of cytoplasm to extra-cellular calcium, resulting in cell retraction and detachment. To avoid myofibril contraction, 2,3-butanedione monoxime (BDM) was used in the medium as it inhibits cross-bridge interaction (Bartoli and Claycomb, 1997). Initially, cells were microinjected with Texas-Red dextran to investigate whether the injection procedure would impair S-phase entry. Cells were injected 5 days after plating. Injection at earlier time points was not possible, as cells were not properly adhered leading to cell death after injection. Seven days after injection, cells were exposed to BrdU for 18 hours. No significant inhibition of DNA synthesis was observed in the injected population (see Table 5.2), and hence

microinjection of plasmid DNA was chosen as the method for transfecting newt cardiomyocytes.

Using the same microinjection protocol described above, cells were microinjected with an expression plasmid coding for human p16, kindly provided by Dr. Gordon Peters (ICRF, London). After 7 days, the cells were exposed to BrdU for 18 hours, fixed and stained for BrdU, p16 and sarcomeric-MyHC. Incorporation of BrdU was markedly reduced in cardiomyocytes expressing p16 as compared to cardiomyocytes in the same plate that were not expressing that CDI (Figure 5.5 and Table 5.3.). This was not due to non-specific inhibition of re-entry after microinjection since expression of a marker (alkaline phosphatase, a cytoplasmic phosphatase active only under alkaline conditions) did not significantly inhibit cell cycle re-entry in the cardiomyocytes (Figure 5.6 and Table 5.3). These results show that prevention of pocket protein inactivation by CDK phosphorylation inhibits DNA synthesis in newt cardiomyocytes.

5.2.3 Thrombin Proteolysis of Serum Does Not Induce DNA Synthesis in Newt Cardiomyocytes

The next approach was to test whether thrombin-activated serum would promote DNA synthesis in newt cardiomyocytes, as shown for newt skeletal myotubes (Tanaka et al., 1999). Establishing an assay for factors that promote DNA synthesis in newt cardiomyocytes is a difficult task since the steps necessary for cell purification and plating involve the use of serum[10]. This is not desirable, as serum itself promotes DNA synthesis (as shown in Chapter 3), and hence the background would be elevated.

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[10] It is our own experience the newt heart fibroblasts will not adhere without serum and the cardiomyocytes take longer to adhere and spread without serum (see Chapter 3).

Table 5.2 Injection of dextran does not impair DNA synthesis in cardiomyocytes.

% Brdu-labelled dextran- positive cardiomyocytes	% BrdU-labelled dextran-negative cardiomyocytes
23.3±7.7	34.9±6.7

Cells were injected with Texas-Red-dextran as described in Materials and Methods. Seven days later, cells were given an 18 hour BrdU pulse. The percentage of BrdU positive injected (dextran positive) and non-injected cardiomyocytes in the same plate was counted. Results shown are the average of the counts of four separate experiments plus or minus the average of the difference between each experimental value and the mean. A total of 348 cardiomyocytes were counted as positive for dextran.

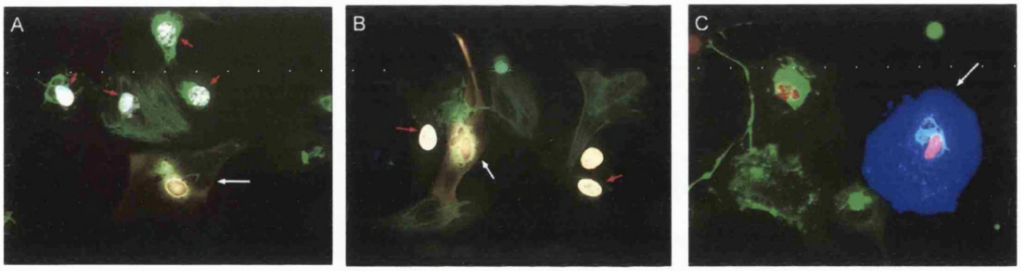

Figure 5.5 Expression of human p16 inhibits DNA synthesis. Newt cardiomyocytes were microinjected with a plasmid coding for the human p16 (A and B), or with a control plasmid coding for alkaline phosphatase (C). A BrdU pulse was given to the cells after 7 days, and cells were fixed and processed for BrdU (light blue in A and B, red in C), sarcomeric MyHC (green), p16 (red in A and B) and alkaline phosphatase (dark blue in C). p16 was always found both in nuclei and cytoplasm of expressing cells (white arrows in A and B). Note that p16-expressing cardiomyocytes are BrdU negative, in contrast to some of the other cells in the field (red arrows). Note that the alkaline phosphatase-expressing cardiomyocyte (white arrow in C) is BrdU positive. The total number of nuclei was counted under phase-contrast optics.

Table 5.3 Expression of human p16 in newt cardiomyocytes inhibits DNA synthesis

Injected constructs	*% Brdu labelled cardiomyocytes*	
	Injected cardiomyocytes	*Non-injected cardiomyocytes*
p16	1.6 ± 2	25.1 ± 7.8
Alkaline Phosphatase	24.3± 8.3	31.7 ± 1.7

Separate plates of cardiomyocytes were microinjected with an expression plasmid encoding either the CDK inhibitor p16 or, as a control, human placental alkaline phosphatase (a cytoplasmic phosphatase active only under alkaline conditions) at a concentration of 50 μg /ml. Entry into S phase was measured as described in legend of figure 5.6. The results presented are the average of three independent experiments, plus or minus the average of the difference between each experimental value and the mean. A total of 223 cardiomyocytes expressed p16, and 220 cardiomyocytes expressed alkaline phosphatase. A block of entry into S phase is evident upon expression of p16, whereas the alkaline phosphatase control has no major effect. The same effect was observed at higher doses of injected DNA.

Two different protocols were tested with respect to the percentage of serum used for plating cells (Fig.5.6 A and B). Similar protocols have been used for mammalian cardiomyocytes (Zhao et al., 1998). In both assays cells were given a BrdU pulse at 4 days after stimulation. The cells were stimulated with four different concentrations of thrombin in the presence of 1% serum. This concentration of FBS was below the threshold levels for S phase in the absence of

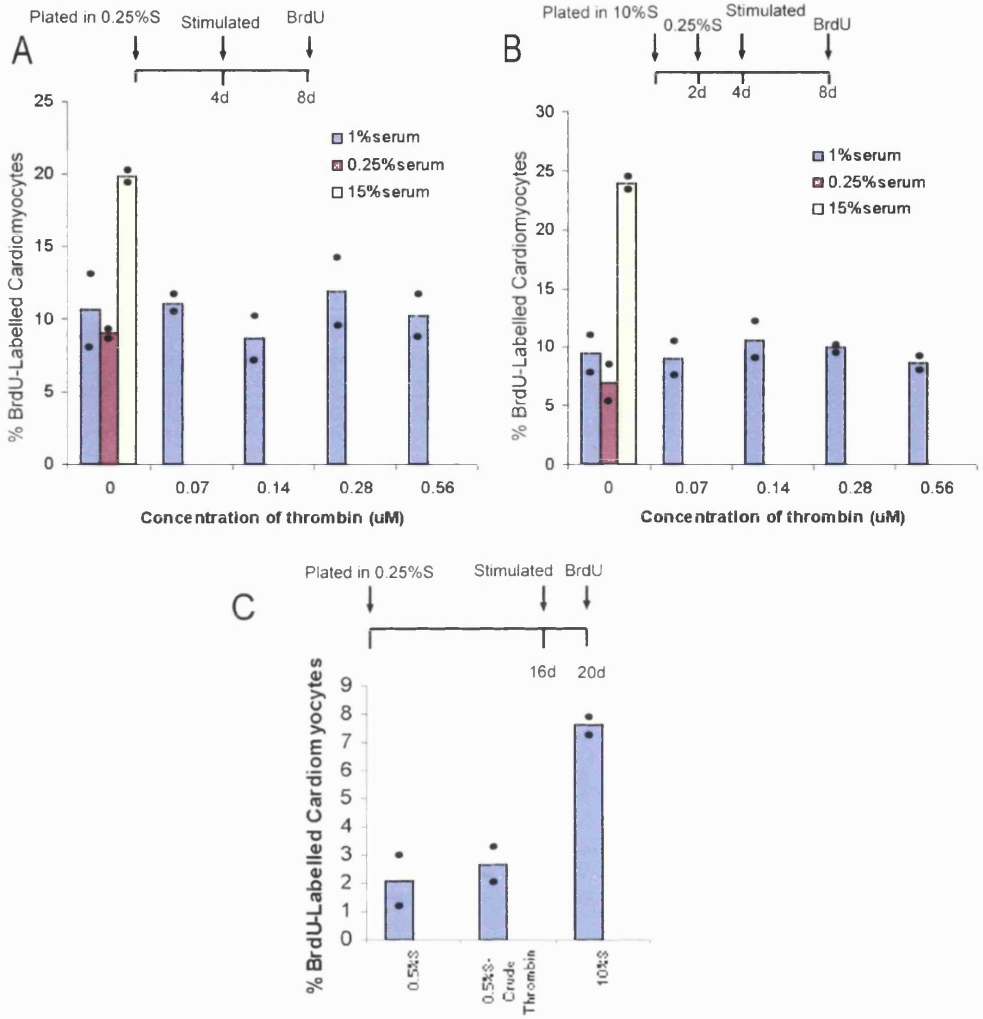

Figure 5.6 Thrombin mediated activation of serum and crude thrombin do not enhance entry into S phase in newt cardiomyocyte cultures. The protocol used for each experiment is represented above the respective graph. Approximately 300 cells were counted in each dish. Experiments shown in A and B were performed three times. Experiment shown in C was performed twice. (A and B) Cells were stimulated in the presence of 1% FBS and different concentrations of purified thrombin (the activity of the thrombin was controlled as described in Materials and Methods). (C) Cells were stimulated with 100 μg/ml of crude thrombin (the cell cycle re-entry activity of this batch of crude thrombin had previously been tested in newt myotubes) in the presence of 0.5% serum.

thrombin, but in the presence of the concentrations of thrombin used here it was very potent in inducing DNA synthesis in myotubes (Tanaka et al., 1999). Using this type of assay a clear distinction can be observed between the negative control (0.5% Serum) and the positive control (15% Serum; Fig. 5.6 A and B), but I could not observe any effect over the background after thrombin treatment (Fig. 5.6 A and B). Additionally, no effect was observed with preparations of crude thrombin that had high S phase entry activity in newt myotubes (Fig. 5.6 C; experiments in newt myotubes were performed by Dr. Andras Simon in the laboratory (not shown). These preparations of crude thrombin contain thrombin and the putative ligand generated by thrombin proteolysis of serum (Tanaka et al., 1999). As such, the assay for crude thrombin could be done in media containing a low concentration of FBS. These last results were obtained by plating the cells in 0.25% FBS-containing media and stimulating them 16 days afterwards, with crude thrombin or 10%FBS. This change in the protocol was added in order to reduce the background response of cardiomyocytes.

5.3 Discussion

The work presented in this chapter has established for the first time that inactivation of pRb-family proteins by phosphorylation plays a pivotal role in cell cycle re-entry by newt cardiomyocytes. The evidence for this is that expression of the CDI protein p16 prevents DNA synthesis in serum stimulated newt cardiomyocytes. Preliminary results indicate that pRb is phosphorylated upon serum stimulation, although further experiments are necessary to confirm it. Additionally, this work has revealed that Fa, which results from thrombin proteolysis of serum and promotes DNA synthesis in newt myotubes, does not induce entry into S phase by newt cardiomyocytes. This suggests that different signalling pathways might operate in the two cell types.

pRb regulation in newt cardiomyocytes

Cell cycle re-entry by newt cardiomyocytes was inhibited by expression of the CDI p16 (Table 5.3). When I started this work it was thought that there was a linear pathway involving p16, CDK4/cyclin D and pRb (see Chapter 1). Two reports had shown that that the presence of functional pRb was required for growth suppression by p16 (Lukas et al., 1995; Medema et al., 1995). However, recent findings have shown that p16-mediated cell cycle arrest can also occur in mouse embryonic fibroblasts lacking *Rb* (Bruce et al., 2000). In these cells, the p16-induced arrest is not mediated exclusively by pRb, but depends on the non-redundant functions of two other pRb-family members: p107 and p130 (Bruce et al., 2000). Additional work in mouse embryonic fibroblasts lacking the three pocket proteins has shown that at least one of them is required for p16-mediated cell cycle arrest (Dannenberg et al., 2000; Sage et al., 2000). Could the effect of expression of p16 in newt cardiomyocytes be mediated by any of the other pRb-family members? Although this hypothesis cannot be discarded, to date no other pRb family members have been cloned in amphibia. In addition, preliminary results indicate that pRb is phosphorylated in serum-stimulated cardiomyocytes (Fig. 5.3), suggesting that pRb plays an important role in regulating entry into S

phase in those cells. It will be important to analyse the phosphorylation state of pRb in non-stimulated cardiomyocytes to establish this conclusively. The expression of a constitutively active pRb (pRb molecule with no phosphorylation sites) is another possible strategy to show that this member of the pocket protein family can prevent cell cycle re-entry (Hamel et al., 1992; Tanaka et al., 1997).

Factors that induce DNA synthesis in newt cardiomyocytes

Thrombin treated serum did not induce DNA synthesis in newt cardiomyocytes (Fig. 5.6), arguing against the hypothesis that a single pathway (see Fig. 5.1) is responsible for the plasticity of all differentiated cell types in the newt.

What could be the factors inducing S phase entry by the newt cardiomyocytes? In this work, I have established assays to test for candidate factors. Using these assays a 2-4 fold increase in the number of cells re-entering the cell cycle was observed when cells were stimulated with high concentrations of serum (10% and 15% FBS; Fig. 5.6). This difference should be sufficient to detect the activity of candidate factors when tested in low serum conditions, and represents a major improvement to assays performed previously on these cells, where factors were tested in the presence of 10% FBS (Oberpriller et al., 1995; Soonpaa et al., 1992; Soonpaa et al., 1994). Further refinement of the assay might also be possible as I have observed that newt cardiomyocytes survive in media without serum, in the presence of 1% BSA (results not shown). Candidate factors to test are those shown to be released upon injury and those shown to stimulate the division of embryonic and neonatal mammalian cardiomyocytes, such as IGF-1, FGF-1 and 2, and neuregulin (see Chapter 1). Using the same assay as described in Fig. 5.7 for thrombin, I have tested PDGF activity on newt cardiomyocytes, but no effect was found (results not shown).

Further studies on the molecular regulation of the plasticity of the differentiated state in newt cardiomyocytes

One of the important achievements of this work was the establishment of a protocol to transfect newt cardiomyocytes without major inhibition of DNA

synthesis (Table 5.2 and 5.3). The use of microinjection techniques should allow further study of the molecular regulation of the plasticity of the differentiated state in newt cardiomyocytes. Additionally, it may be possible to modify the technique of transfection of cardiomyocytes by adenoviral infection in order to avoid inhibition of entry into S phase. This should make the biochemical manipulation of transfected newt cardiomyocytes feasible.

Implications of this work

The fact that pocket protein phosphorylation is necessary for cell cycle re-entry by newt cardiomyocytes has several implications. The role of pocket proteins in maintaining the post-mitotic arrest of newt cardiomyocytes is in accordance with results obtained for neonatal and adult mammalian cardiomyocytes, wherein inactivation of pocket proteins by viral oncoproteins and E2F induces cell cycle re-entry (Agah et al., 1997b; Kirshenbaum et al., 1996; Kirshenbaum and Schneider, 1995). Additionally, cardiomyocytes differentiated in culture from *Rb-/-* stem cells have been found to undergo DNA synthesis, in contrast to their wild type counterparts (Schneider, 1995). These results suggest that the stability of the differentiated state in newt and mammalian cardiomyocytes might be regulated in a similar manner, but newt cells are able to undermine that stability by re-utilisation of the intracellular pathways used for normal cell proliferation. Additionally, together with the work in newt skeletal myotubes (Tanaka et al., 1997), and in mammalian hepatocytes (Albrecht et al., 1998) and VSMCs (Rao, 1999), this work points to the importance of pocket protein regulation in cell cycle re-entry from the differentiated state.

This study has also shown that there are differences in the regulation of the plasticity of the differentiated state in at least two cell types in the newt. In fact, different extracellular factors may be responsible for triggering cell cycle re-entry in newt cardiomyocytes and newt myotubes. One possibility is that there is not a singular injury/plasticity pathway in urodele amphibians, and different types of cells may be stimulated by different extracellular factors. Alternatively, there

might be a regeneration-associated pathway specific to some regenerative domains, such as limbs and lens. The study of this pathway in these urodele-regenerating domains could be very informative. Results obtained by Dr. Yutaka Imokawa in this laboratory show that thrombin proteolytic activity is localised to the dorsal iris after removal of the lens. The cells that give rise to the regenerating lens originate exclusively in this region. With the experimental tools developed with this work it should be feasible to study the intracellular players and the extracellular factors involved in newt cardiomyocyte cell cycle re-entry, and whether they have any resemblance to other differentiated newt cells.

-Chapter 6-

SUMMARY

This study has, for the first time, quantitatively examined the proliferative potential of the adult newt cardiomyocyte at the single cell level, and addressed the molecular regulation of the entry into S phase. The primary cell culture of newt cardiomyocytes described here shows a profile of entry into S phase similar to the one observed after injury to the newt ventricle *in vivo* (Bader and Oberpriller, 1979; Bader and Oberpriller, 1978). My analysis of the proliferative potential at the single cell level showed that after serum stimulation, the majority of the cardiomyocytes enter S phase, and display different proliferative behaviours, namely arresting before mitosis, undergoing acytokinetic mitosis or going through one or more rounds of complete mitosis. The blocks described in the progression through mitosis may explain the appearance of polyploid and multinucleate ventricular cardiomyocytes after injury (Oberpriller et al., 1989). Additionally, the cells that were observed to go through one or more rounds of mitosis may account for the majority of the increase in cardiomyocyte number *in vivo*, and the consequent regeneration of the heart.

The plasticity of the newt cardiomyocyte described here contrasts with that of its adult mammalian counterpart. DNA synthesis and mitosis are rare events in the latter, and complete cytokinesis was never documented (MacLellan and Schneider, 2000; Soonpaa and Field, 1997; Soonpaa and Field, 1998). This plasticity does not seem to be dependent on a urodele-specific growth factor since I have shown that mammalian serum can induce cell cycle progression in these cells. A hypothesis that may be put forward to explain the plasticity of the newt ventricular cardiomyocyte is that it reflects a widespread plasticity of the differentiated state in newt cells. In this context there may be a molecular program that is triggered only in the context of injury, unlocking the cells from the post-mitotic arrest. The prototypical examples of plasticity in newt cells are

the cell cycle re-entry and dedifferentiation of skeletal myotubes (Echeverri et al., 2001; Kumar et al., 2000; Lo et al., 1993; Tanaka et al., 1997), and the transdifferentiation of pigmented epithelial cells from lens and retina (Eguchi, 1998; Kodama and Eguchi, 1995). This plasticity is thought to be important for the generation of the progenitor cells that contribute to the amazing regenerative ability of urodele amphibians (Brockes, 1997; Brockes, 1998a). The existence at the cellular level of a complex regeneration program has been recently suggested after the discovery of the drug myoseverin (Rosania et al., 2000). Myoseverin not only triggers the dedifferentiation of mammalian C2C12 myotubes in culture but also induces the transcription of a variety of genes known to be involved in wound healing and tissue regeneration (Rosania et al., 2000).

Several of the aspects studied here show that there are common aspects of plasticity in newt differentiated cells. The first is that the ability to synthesise DNA seems to be a common feature of differentiated cells, such as the skeletal myotube, the RPE and, as I have shown in this thesis, the cardiomyocyte. The second is that re-entry into the cell cycle in these cells is triggered either by injury *in vivo* or by serum, the clotted fraction of blood, in culture. The fact that serum may be more than just a complex set of growth factors that promote proliferation, has been recently shown in the analysis of the fibroblast response to serum (Iyer et al., 1999). In that case, serum induced in fibroblasts the transcription of a complex set of genes involved not only in proliferation, but also in the wound healing response (Iyer et al., 1999). Serum might thus possess the priming signals that induce differentiated newt cells to respond to injury and contribute to regeneration. The third aspect that may be common to the plasticity of differentiated newt cells, with need for further verification in the cardiomyocytes, is the requirement for pocket protein phosphorylation for S phase entry. This last aspect indicates that S phase entry from the differentiated state may use the same pathways as proliferating mammalian cells, albeit with a different regulation.

In this work, several divergent aspects of the response to serum of newt-differentiated newt cells were also highlighted. First, while newt myotubes arrest in G2 after S phase (Tanaka et al., 1997), I have shown that although some cardiomyocytes may arrest after S phase entry, the majority enter M phase with or without completion of cytokinesis, and may go through one or more rounds of cell division. Second, newt myotubes, in culture, are induced to undergo S phase by Fa, a factor resulting from the thrombin mediated proteolytic activation of serum (Tanaka et al., 1999). Common growth factors, such as PDGF, do not have cell cycle re-entry activity in myotubes (Tanaka et al., 1999). Thrombin has also been shown to be present in regenerating limb blastemas and localised to the dorsal margin of the lens, at a time where the plasticity of the differentiated cells is evoked ((Tanaka et al., 1999), Dr. Yutaka Imokawa, unpublished results). Fa, however, does not induce S phase entry in primary cultured newt cardiomyocytes. This could be a consequence of primary culture, since the exposure to clotted blood upon harvesting of the ventricle, or the dissociation of the cells, could change the responsiveness of the cells. Alternatively, it could be that the plasticity of different cell types in the newt is regulated differently, possibly depending on the ontogeny of each cell type. In this context it would be important to study further the factors existent in serum that promote S phase entry in the newt cardiomyocyte. Additionally, further work on the molecular regulation of plasticity in several differentiated newt cell types should be done in order to understand whether there is a common pathway in the response to serum. The use of microarrays to study gene expression may be one way to go forward in this research, although the newt is somewhat problematic for this approach at present.

The existence of a block after entry into S phase and at cytokinesis in adult newt cardiomyocytes shows that cell cycle progression of a percentage of the newt cardiomyocytes resemble that of their neonatal mammalian counterparts.

This regulation raises important questions such as whether adult newt cardiomyocytes are as differentiated as their mammalian counterparts, or possibly retain features of neonatal cells. The fact that adult newt cardiomyocytes as neonate but not adult mammalian cardiomyocytes are diploid is evidence in favour of the latter. On the other hand adult newt cardiomyocytes as their adult mammalian counterparts do not proliferate in the absence of injury, which is evidence against that hypothesis. I have started to address this question, together with Dr. Elizabeth Ehler, by looking at the expression of isoforms of proteins from the myofibrils, but much remains to be done. In this respect it would be important to compare the expression of other proteins, such as ANF, and also to compare the physiology of newt and mammalian cardiomyocytes both at embryonic and adult stages.

I have begun to address the molecular regulation of the S phase entry in the newt cardiomyocyte. The ability to phosphorylate pocket proteins may be one of the differences that allow newt cardiomyocytes, but not their mammalian counterparts, to respond to injury by entering S phase. However, this difference may not account for the progression through M phase and cytokinesis since inactivation of pocket proteins in mammalian cardiomyocytes leads to a G2 arrest (Agah et al., 1997a; Akli et al., 1999; McLellan and Schneider, 1999). Further study of the regulation of mitosis and cytokinesis in the newt cardiomyocyte should provide important insights into why mammalian cardiomyocytes are blocked in G2. Furthermore the newt cardiomyocyte culture provides an intrinsic control, since 15% of the newt cardiomyocytes arrest in G2. A complementary approach is to study the regulation of cell cycle progression in the adult *Xenopus laevis* cardiomyocyte. The use of the eggs of this organism as a model to study cell cycle control, and the use of the embryos to study heart development and cardiomyocyte differentiation (Mohun and Ming Leong, 1999) have created many molecular tools to address the problem.

Heart disease is a major cause of death. In the aftermath of a heart attack the damaged area is replaced by a scar, which impairs cardiac performance. Ultimately, doctors have to resort to the complex process of finding a heart donor for heart transplantation. Ideally, one should be able to replace the damaged heart tissue with new cardiomyocytes. Recently, much focus has been given to the use of cell transplantation and stem cell technology to repair the damaged mammalian heart among other tissues. Successful transplantation of cells depends on many factors such as survival, maturation and electromechanical coupling of the donor cells. Many types of cells, such as immortalised atrial cardiomyocytes, skeletal muscle cells, bone marrow stem cells, and embryonic, neonatal and adult cardiomyocytes have been transplanted into damaged mammalian hearts (Kessler and Byrne, 1999; Orlic et al., 2001; Reinlib and Field, 2000). Transplantation of these cells into the mammalian heart has had different results, depending on the cell type, where and when the cells were implanted. The recent implantation of a particular population of bone marrow cells led to improvement of the cardiac funtion (Orlic et al., 2001). The implantation was performed immediately after heart damage, and the animals were analysed 9 days afterwards. It is still not known what are the long-term results of this experiment, whether there may be a risk of cell overgrowth.

An alternative, or complementary approach to cell transplantation is the stimulation of the division of endogenous cells. In the newt, repair of the heart is achieved both by division of resident cardiomyocytes and formation of a scar (Oberpriller and Oberpriller, 1991; Oberpriller et al., 1995). The work presented in this thesis suggests that the regulation of the differentiated state may be fundamentally similar in newt and mammalian cardiomyocytes, possibly relying on a different regulation of the same pathways, such as the pocket protein pathway. These similarities may underlie the observation that a few mammalian cardiomyocytes enter the cell cycle after injury, and that atrial cardiomyocytes can

undergo DNA synthesis and mitosis. Further study of the regulation of the plasticity of newt differentiated cells should help us to design ways of manipulating the extracellular environment, and/or the cells, in order to evoke similar responses in mammals. An advantage of this approach may be that in contrast to implanted non-differentiated cells which have to differentiate into the proper cells, cardiomyocytes may be able to coordinate cell function with cell division, as do hepatocytes during liver regeneration. Additionally, this strategy may avoid the risks of over-proliferation of cells since the control of cell number seems to be so effectively regulated in newts, within the regenerative territories (Brockes, 1998b).

-Chapter 7-

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