Regulation of Epithelial Cell Transformation
and Survival by Raf Activation

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Signal Transduction Laboratory
Imperial Cancer Research Fund, London

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Abstract

Oncogenic Ras genes are activated by point mutation in about 30% of human tumours, being one of the most frequent mutations in human cancers. Activation of the Raf/MAPK pathway by Ras plays a major role in the establishment of cell transformation allowing cells to evade apoptosis and negative cell cycle control. In many advanced human tumours of epithelial origin, Ras was found to co-operate with TGFβ in promoting tumour progression and cells undergo an epithelial to mesenchymal transition, termed EMT, in which the epithelial phenotype, characterised by strong cell-cell adhesion and cell polarity, is lost. The acquisition of a mesenchymal phenotype is characterised by reduced cell-cell contacts and induction of invasive growth. In order to analyse the contribution of the Raf/MAPK pathway on changes in the epithelial cell-morphology during cell transformation, an inducible Raf-1 fusion protein was stably expressed in MDCK cells, which allowed to monitor the progression of cellular events.

Activation of Raf was sufficient to cause disruption of cell-cell junctions. Furthermore, sustained activation of Raf was sufficient to cause EMT, thereby inducing down-regulation of E-cadherin expression. Raf activation promoted invasive growth in collagen gels, which was dependent on the establishment of autocrine TGFβ signalling, whose secretion was induced by Raf. During EMT, cells were rendered insensitive to anti-proliferative TGFβ effects. Furthermore, expression of the TGFβ effector Smad3 was down-regulated in MDCK cells which have undergone EMT, and re-expression of Smad3 rendered these cells sensitive to growth inhibition by TGFβ.

Apoptosis induced by TGFβ in MDCK cells was prevented by short-term Raf activation at a time point at which cells were still susceptible for TGFβ-induced growth arrest. The mechanism by which short-term Raf activation prevents TGFβ-induced apoptosis in MDCK cells differs from the one observed in MDCK cells which had undergone EMT due to sustained Raf activation. While short-term activation of Raf did not prevent release of cytochrome c from mitochondria, it was abolished by sustained Raf activation, presumably due to expression of Bcl-XL. The anti-apoptotic potential of Raf was not restricted to TGFβ-induced apoptosis, but protected epithelial cells from a variety of pro-apoptotic stimuli, suggesting a more general survival mechanism mediated by Raf.
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<tr>
<td>A</td>
<td>alanine</td>
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<tr>
<td>aa</td>
<td>amino acid residue</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>carboxy-</td>
</tr>
<tr>
<td>C.elegans</td>
<td>Caenorhabditis elegans</td>
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<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>Dulbecco’s modified Eagles media</td>
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<td>Escherichia coli</td>
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<tr>
<td>ECL</td>
<td>enhanced chemi-luminescence</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDTA</td>
<td>ethylene-diamine-tetraacetic acid</td>
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<tr>
<td>EGF</td>
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<td>ELISA</td>
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GAP  GTPase activating protein
GDP  guanosine diphosphate
GEF  guanine exchange factor
GFP  green fluorescent protein
Grb2  growth factor receptor-bound protein 2
GTP  guanosine triphosphate
HCl  hydrochloric acid
HGF/SF  hepatocyte growth factor/scatter factor
HRP  horse radish peroxidase
HSP  heat shock protein
IAP  inhibitor of apoptosis
Ig  immunoglobulin
JNK  Jun N-terminal kinase
kD  kilo Dalton
l  litre
LB  Luria Bertani broth
M  methionine
m  milli
M  molar
MAPK  mitogen-activated protein kinase
MDCK  Madin-Darby canine kidney
MEK  MAPK/ERK kinase
MEKK  MEK kinase
mRNA  messenger ribonucleic acid
N  amino-
P  proline
PAGE  poly-acrylamide gel electrophoresis
PAK  p21 activated protein kinase
PCNA  proliferating cell nuclear antigen
PI3K  phosphoinositide 3-kinase
PKB  protein kinase B
PKC  protein kinase C
pRb  retinoblastoma protein
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<td>PVDF</td>
<td>polyvinylfluoride</td>
</tr>
<tr>
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<td>SDS</td>
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<td>SOS</td>
<td>son of sevenless</td>
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<td>T</td>
<td>threonine</td>
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<tr>
<td>TCF</td>
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<td>tumour necrosis factor</td>
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<td>ultraviolet</td>
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<td>Xenopus laevis</td>
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<tr>
<td>Y</td>
<td>tyrosine</td>
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<td>μ</td>
<td>micro</td>
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Chapter 1

1 Introduction

1.1 The multistep process of cancer

The development of cancer is based on dynamic changes in the genome, generated by activating mutations in proto-oncogenes whose gene products are responsible for promoting cell growth or inactivating mutations in tumour suppressor genes resulting in loss of function. These defects occur in genes responsible for the control of cell proliferation and homeostasis. This leads to the establishment of cancer typical features, such as insensitivity to anti-growth signals, evasion of apoptosis, growth factor independence, limitless replication of the cell and sustained angiogenesis leading to tumour progression, invasion of tumour cells and metastasis. Tumourigenesis is a multistep process acquired over time by alterations in the genome. Genetic studies have suggested that at least 4 to 7 mutations have to manifest for progression to a fully malignant tumour (Renan, 1993); (Kinzler and Vogelstein, 1996). A proliferating cell population is particularly susceptible to accumulation of multiple mutations since growth permits clonal expansion of mutant cells.

During the multistep process of epithelial cell transformation, cells acquire certain functional capabilities which allow them to evade cellular control mechanisms. This includes resistance towards apoptosis and the evasion of anti-proliferative signals which eventually can result in cell invasiveness, a prerequisite for metastasis formation. In about 30% of human tumours of epithelial origin the ras oncogene has acquired activating mutations, which can promote cell transformation in the presence of co-operating oncogenes (Hanahan and Weinberg, 2000).

1.2 Ras signalling

1.2.1 Ras superfamily

The Ras proteins are members of the Ras-like superfamily of small GTPases consisting of more than 80 members in the mammalian system. They are divided into sub-groups depending on structure and function. The members of the different families are listed in Table
1.1. Cross-talk occurs between Ras subfamily members. For example, like Ras, activated mutants of R-Ras proteins have been shown to promote growth transformation and alter differentiation. Furthermore, they use some common Ras-guanine nucleotide exchange factors (RasGEFs) and can interact with many common effectors. Therefore, the effectors may be activated in a co-ordinated fashion by the same extracellular machinery having possible distinct and overlapping roles with Ras (Graham et al., 1994). Moreover there is evidence for an interrelationship between Ras, Rap and Ral, linking at multiple levels (reviewed in (Reuther and Der, 2000)).

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<td>Ras</td>
<td>Ras</td>
<td>Ha-Ras, Ki-Ras, N-Ras 1A, 1B, 2A, 2B, 4A, 4B A, B R-Ras, TC21 (R-Ras2) R-Ras3 (M-Ras) Rheb Rin</td>
<td>(Bos, 1997)</td>
</tr>
<tr>
<td></td>
<td>Rap</td>
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<td></td>
<td>Ral</td>
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<td>R-Ras</td>
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<td></td>
<td>Rheb</td>
<td></td>
<td>(Lee et al., 1996)</td>
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<tr>
<td></td>
<td>Rin</td>
<td>Rin, Rit</td>
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<tr>
<td>Rho</td>
<td>Rho</td>
<td>A, B, C 1, 2, 3</td>
<td>(Hotchin and Hall, 1996)</td>
</tr>
<tr>
<td></td>
<td>Rac</td>
<td>Cdc42, G25K</td>
<td>(Scita et al., 2000)</td>
</tr>
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<td></td>
<td>Cdc42</td>
<td>TC10</td>
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<td></td>
<td>TC10</td>
<td>RhoG</td>
<td>(Van Aelst and D'Souza-Schorey, 1997)</td>
</tr>
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<td></td>
<td>RhoE</td>
<td>RhoE, Rho8/Rnd3, Rnd1/Rho6, Rnd2/Rho7</td>
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<td></td>
<td>RhoD</td>
<td>RhoD</td>
<td>(Moss and Vaughan, 1998)</td>
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<td></td>
<td>TTF</td>
<td>TTF</td>
<td>(Mattaj and Englmeier, 1998)</td>
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<tr>
<td>Rab</td>
<td>Rab</td>
<td>&gt; 40 members in mammalian family</td>
<td>(Novick and Zerial, 1997)</td>
</tr>
<tr>
<td>ADP Ribosylation Factor (ARF)</td>
<td>Class I</td>
<td>ARF1, 2, 3</td>
<td></td>
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<td></td>
<td>Class II</td>
<td>ARF4, 5</td>
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<td>Class III</td>
<td>ARF6</td>
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<tr>
<td>Ran</td>
<td>Ran</td>
<td>Ran</td>
<td>(Finlin and Andres, 1997)</td>
</tr>
<tr>
<td>Rad</td>
<td>Rad</td>
<td>Rad, Gem, Kir, Rem</td>
<td></td>
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Table 1.1: Members of the mammalian Ras-like GTPase superfamily.
1.2.2 Ras family

Three human ras genes have been identified encoding four 188-189 amino acid proteins of 21 kD in size. They are H-Ras, N-Ras and K-Ras, with K-Ras existing in two splice variants, K-Ras4A and K-Ras4B. The latter is the predominant form of K-Ras found in mammalian cells. Ras proteins which share a high degree of sequence identity (85%) have the ability to transform NIH3T3 fibroblasts and other cell types (Barbacid, 1987); (Bourne et al., 1990). However, the conservation of the three genes throughout vertebrate evolution argues for distinct roles for each. The strongest evidence comes from gene targeting experiments where embryonic lethality is seen in K-Ras, but not in H-Ras or N-Ras knockout mice (Umanoff et al., 1995); (Johnson et al., 1997) or in H-Ras/N-Ras double knockout mice (Esteban et al., 2001). This reflects a unique function of K-Ras in development or an exclusive expression of K-Ras in specific tissues during development. Furthermore, recent reports have pointed out differences in how the different Ras proteins are transported to the plasma membrane and where they localise. The C-terminus of all Ras proteins contains two signal sequences that promote their binding to the plasma membrane. The C-terminal CAAX tetrapeptide sequence (A is an aliphatic amino acid, X is methionine or serine) is the recipient of three post-translational modifications: farnesylation, AAX proteolysis and carboxymethylation (Gutierrez et al., 1989); (Fujiyama and Tamanoi, 1990); (Hancock et al., 1990); (Hancock et al., 1991). This promotes localisation of Ras to the plasma membrane. A second signal sequence upstream of the CAAX motif, the hypervariable domain, shows the greatest divergence among the Ras proteins (4% aa identity). It is modified by palmitoylation in H-Ras, N-Ras and K-Ras4A, which is required for the completion of localisation of Ras to the plasma membrane (Hancock et al., 1989); (Choy et al., 1999). Interestingly, farnesyltransferase inhibitors, developed as anti-Ras drugs, are effective inhibitors of H-Ras and N-Ras function, but they do not block K-Ras function (Cox and Der, 1997); (Oliff, 1999).

1.2.3 Ras as a molecular switch

Ras proteins act as molecular switches, cycling between an active GTP-bound state and inactive GDP-bound state, thereby functioning as important relays in the transduction of signals from receptor tyrosine kinases (RTK). Ras is anchored via C-terminal lipid modifications to the inner surface of the plasma membrane, and like heteromeric G proteins, Ras is activated by GTP-GDP exchange. This is regulated by Guanosine nucleotide exchange
factors (GEFs) (Prive et al., 1992), which promote the formation of active Ras by regulating the exchange of GDP for GTP. The formation of the inactive Ras-GDP is promoted by GTPase activating proteins (GAPs) (Bollag and McCormick, 1991), which increase the intrinsic GTPase activity of Ras (Figure 1.1). Recruitment of GEFs to the plasma membrane activates Ras signalling (Quilliam et al., 1995). The major mammalian GEFs in Ras signal transduction are SOS1 and 2 (Bowtell et al., 1992), RasGRF1 and RasGRF2, and RasGRP (Farnsworth et al., 1995); (Fam et al., 1997). So far the role of other putative Ras GEFs, such as Vav or SmgGDS is still unclear.

Figure 1.1. The Ras molecular switch. Guanidine nucleotide exchange factors (GEFs) promote GTP binding while GTPase activating proteins (GAPs) enable GTP hydrolysis. Thus Ras serves as a molecular switch, cycling between an active Ras GTP complex and an inactive Ras GDP complex.

The two GAPs which have been identified and show catalytic activity towards Ras are p120GAP (Trahey and McCormick, 1987); (Adari et al., 1988); (Vogel et al., 1988) and NF1 (Ballester et al., 1990); (Martin et al., 1990). The enzymatic activity of GAPs and GEFs responds to extracellular stimuli such as growth factors (Lowy et al., 1991). Most receptor tyrosine kinases do not bind to GRFs directly but do so via adapter proteins, such as the mammalian protein growth factor receptor-bound protein2 (Grb2), which links EGF receptor to SOS, thereby regulating Ras activation (Buday and Downward, 1993).

Activating mutations of Ras usually operate by rendering the Ras protein insensitive to GAP action. Since the mutants have reduced intrinsic GTPase activity, they remain GTP-bound
Ras genes become constitutively activated when point mutated at specific amino acid residues. The most common mutations in human tumours arise at codon 12 (Bos, 1989), but point mutations are also found at codon 13 and 61 (Lowy and Willumsen, 1993). Mutants that act in a dominant negative fashion, such as RasN17, have a very low affinity for guanosine nucleotides. They may act by sequestering RasGEFs and thus preventing other Ras molecules from utilising GEF exchange activity.

1.2.4 Activation of Ras by extracellular stimuli

Activation of Ras occurs in response to a wide variety of stimuli, such as growth factors, cytokines, hormones and neurotransmitters. These stimuli signal to transmembrane receptors, such as RTKs, non-receptor tyrosine kinase-associated receptors and G-protein coupled receptors (GPRC) and cytokine receptors. One of the best characterised Ras activators is epidermal growth factor (EGF) receptor (Satoh et al., 1990). This is depicted in Figure 1.2. The EGF receptors are single transmembrane domain proteins that dimerise and transphosphorylate upon binding to their ligand.

**Figure 1.2. Model for the activation of Ras by the receptor tyrosine kinases (RTK).** Tyrosine phosphorylation of RTK, such as the EGF receptor, promote its association with the SH2 domain containing adapter protein Grb2. Recruitment of Grb2-SOS to the receptor complex catalyses RasGTP exchange. Association of membrane-bound Ras with Raf induces activation of the MAPK cascade. PI3K can be activated via Ras or by direct binding of its p85 subunit to the receptor complex. P, tyrosine phosphorylation.
These phosphorylated residues function as binding sites for the Src-homology2 (SH2) domains of Grb2 (Lowenstein et al., 1992). Grb2 is bound to the C-terminus of the guanosine exchange factor SOS via its SH3 domain, which in turn activates membrane associated Ras by catalysing Ras-GTP exchange (McCormick, 1993); (Moodie and Wolfman, 1994). This allows Ras-GTP to interact with and activate a variety of effector proteins.

Other growth factor receptors use similar mechanisms for Ras activation. For example the SOS-Grb2 complex binds to the insulin receptor via another adaptor protein, Shc, and the insulin receptor substrate (IRS-1) (Skolnik et al., 1993). A similar though more complex mechanism is used by FGF receptors (reviewed in (Olson and Marais, 2000)). The G-protein coupled receptor (GPCR) which consists of α1 and βγ subunits activates RasGRF. This involves calcium (Ca2+) association of RasGRF-associated Calmodulin. Receptor activation (GPCR and RTK) of Phospholipase C (PLC), leading to the production of DAG and the release of intracellular calcium, may stimulate RasGRP activation of Ras (reviewed in (Reuther and Der, 2000)). An increase in GEF activity is just one mechanism by which Ras is activated. Phorbol ester treatment of T cells leads to Ras activation without involving a change in guanine nucleotide exchange activity and instead coincides with down-regulation of RasGAP activity (Downward et al., 1990). Furthermore, in adipocytes, Ras activity is regulated by PI3K-mediated inhibition of GAP activity (DePaolo et al., 1996).

1.2.5 Biological effects of Ras

Ras is a key regulator of many cellular processes, including proliferation and cell survival, which are prerequisite for cellular transformation. Evidence for the transforming potential of Ras came from studies showing that expression of activated mutant ras induced transformation in immortalizes rodent fibroblasts (Chang et al., 1982); (Shih and Weinberg, 1982). These cells show reduced growth factor dependency, loss of contact inhibition, anchorage independent growth and the ability to promote tumour formation when injected in athymic mice (Barbacid, 1987).

However, unlike immortalized cell lines, activated ras alone is not sufficient to transform normal primary cells but requires the presence of a second co-operating oncogene (e.g. E1A, Myc) or loss of a tumour suppressor gene (pRb, p16INK4a or p53) to achieve full transforming potential (reviewed in (Hanahan and Weinberg, 2000)). Many established cell
lines have acquired genetic lesions apart from the immortalisation process (Newbold and Overell, 1983). For example, immortalised mouse embryo fibroblasts (MEFs) were found to have lost function of the tumour suppressor genes p53 or p19ARF (Zindy et al., 1998). The requirements for multiple genetic defects correlates with the observed multistep nature during tumour development. Often the oncogenes that co-operate with Ras in primary cells are co-mutated with ras in spontaneous tumours. Therefore, it appears that undergoing growth arrest, senescence, apoptosis or differentiation in response to inappropriately activated Ras may function as a mechanism of tumour suppression. These inhibitory mechanisms need to be overcome in order for Ras to stimulate mitogenesis and promote tumour formation.

Activating mutations in the ras allele occurs in 30% of all human tumours (reviewed in (Bos, 1989)). The highest mutation rate was found in tumours from exocrine pancreas, with more than 80% of the tumours harbouring a mutated k-ras gene (Almoguera et al., 1988). About 50% of human colon carcinomas bear mutations in the k-ras oncogenes (Vogelstein et al., 1988) and the incidence of ras-gene mutations is about 50% in follicular adenomas and undifferentiated carcinomas of the thyroid (Lemoine et al., 1989). This leads to constitutive activation of the Ras effector pathways (Medema and Bos, 1993). The presence of activated forms of Ras in human tumours shows an intriguing predilection for k-ras mutations (Bos, 1989), with mutations in h-ras being quite rare. This indicates that k-ras DNA might be more susceptible for mutations, or that activated K-Ras protein has unique biological properties which make it more capable of tumour induction than the other Ras family members (reviewed in (Ellis and Clark, 2000)). The ras gene is the most widely mutated human proto-oncogene, reflecting the multiple effects that Ras signalling can have on pleiotropic activation of diverse biological responses such as cell proliferation or growth arrest, senescence or differentiation and apoptosis or cell survival. The exact outcome of Ras activation appears to depend on both the cell type and the magnitude and duration of the Ras activity as well as the sum of positive and negative signals (Kauffmann-Zeh et al., 1997); (Marshall, 1995). Ras proteins act through a number of effectors, a key effector being the serine/threonine kinase Raf-1 (Egan et al., 1993); (Vojtek et al., 1993), which activates the MEK/ERK MAP kinase pathway (Moodie and Wolfman, 1994). Ras binds to at least two other types of effector proteins: the lipid kinase phosphoinositide 3-OH kinase (PI3K) (Rodriguez-Viciana et al., 1994); (Downward, 1998) and members of the Ral-guanine nucleotide exchange factor (RalGEF) family (Urano et al., 1996) (Figure 1.3). In addition, several other potential effectors for Ras have been reported but their biochemical and
biological properties are poorly understood (Katz and McCormick, 1997); (Campbell et al., 1998).

![Ras activation of multiple effector-mediated signalling pathways](image)

**Figure 1.3** Ras activation of multiple effector-mediated signalling pathways. Raf, PI3K and RalGDS represent the best-characterised downstream effectors of Ras function. Some of the interactions between the different Ras effector pathways are shown. These interactions are discussed in the text.

1.3 Ras/Raf/MAPK pathway

1.3.1 Raf as a Ras effector

Originally, Raf-1 was discovered as the v-Raf oncoprotein in mouse sarcoma virus 3611, which has the ability to induce tumour formation in mice (Jansen et al., 1983); (Rapp et al., 1983). Raf-1 functions as a serine/threonine specific kinase and three mammalian Raf-1 proteins have been identified: Raf-1, A-Raf and B-Raf (Bonner et al., 1984); (Huleihel et al., 1984).
They are encoded by three different genes which are located on different chromosomes. While Raf-1 is ubiquitously expressed, A-Raf and B-Raf exhibit more restricted expression profiles, being predominantly expressed in neuronal and urogenital cells (Storm et al., 1990); (Wadewitz et al., 1993). Furthermore, oncogenic Ras leads to differential regulation of the Raf family members (Marais et al., 1997). The different phenotypes of the Raf knockout mice strongly suggest that these proteins are non-redundant and serve distinct functions (Hagemann and Rapp, 1999). A-Raf null mice generate intestinal and/or neurological defects, depending on the genetic background (Pritchard et al., 1996). B-Raf null mice have neuroepithelial differentiation defects as well as defects in maturation and maintenance of endothelial cells (Wojnowski et al., 1997). They die in utero due to vascular haemorrhage due to apoptotic death of differentiated endothelial cells. The phenotype of Raf-1 knockout mice is dependent on the genetic background. Targeted disruption of theraf-1 gene in an inbred background results in a lethal phenotype. In an outbred background Raf-1 knockout mice die shortly after birth: they show a general growth retardation and developmental defects most apparent in placenta, skin and lung, indicating a general role for Raf-1 in development (Wojnowski et al., 1998).

The initial finding that placed Raf downstream of Ras was genetic: Raf was found to be essential for Ras signalling in eye development in D. melanogaster (Dickson et al., 1992) and for vulval development in C. elegans (Han et al., 1993). Raf-1 was subsequently shown to be necessary for Ras signalling in mammalian cells since dominant negative Raf-1 blocked Ras induced gene transcription (Bruder et al., 1992) and cell proliferation (Kolch et al., 1991). Direct binding of Raf-1 to Ras was demonstrated in yeast-two-hybrid experiments and using in vitro binding assays. Specifically, the N-terminal part of Raf-1 binds directly to the effector domain of Ras-GTP (Moodie et al., 1993); (Van Aelst et al., 1993); (Vojtek et al., 1993); (Wame et al., 1993); (Zhang et al., 1993). Moreover, Raf-1 can reproduce many of the cellular responses of Ras in mammalian cells. For example, both Ras and Raf-1 can activate ERK (extracellular signal-regulated kinase), and dominant negative versions of both can block growth factor induced ERK activation (de Vries-Smits et al., 1992); (Schaap et al., 1993). Furthermore, the Ras V12G37 effector domain mutant, which can no longer bind to Raf-1, is defective in ERK activation and this impairment can be complemented by mutations in Raf-1 which restore an interaction with RasV12G37 (White et al., 1995). Raf-1 has been shown to mimic some effects of activated Ras. For example, activated Ras or Raf-1 can induce cell cycle arrest prior to immortalisation in fibroblasts (Hirakawa and Ruley, 1988).
and in Schwann cells (Ridley et al., 1988). These observations provide strong evidence that Raf-1 is a genuine effector of Ras signalling, responsible for the activation of the ERK-MAPK pathway, with Raf-1 being able to elicit many (but not all) of the Ras phenotypes (reviewed in (Katz and McCormick, 1997); (Marshall, 1999)).

1.3.2 Raf activation

1.3.2.1 Ras-Raf interaction

Activation of Raf-1 seems to be a complex multistep process which involves many components, with the mechanism still remaining to be fully characterised (reviewed in; (Kolch, 2000)). A current model for Raf-1 activation is depicted in Figure 1.4. Raf-1 is composed of two functional domains, the amino-terminal regulatory domain, comprising the conserved regions CR1 (aa 62-194) and CR2 (aa 254-269) and the C-terminal kinase domain, termed CR3 (aa 330-627). The N-terminal domain of Raf-1 suppresses its catalytic activity and deletion of part of this domain results in a constitutively active Raf-1 mutant (reviewed in (Morrison and Cutler, 1997)).

Figure 1.4. Schematic depiction of Raf-1. Sites of Raf-1 phosphorylation are shown. RBD, Ras binding domain; CRD, cysteine-rich domain; CR, conserved region. Numbers indicate the amino acid residue numbers.
In its inactive state, Raf-1 is located in the cytosol in a multi-protein complex of 300-500 kD (Wartmann and Davis, 1994). Ras is known to play a key role in activating Raf-1 by directly binding to and recruiting Raf-1 from the cytoplasm to the plasma membrane (Leevers et al., 1994); (Stokoe et al., 1994). Signalling that activates Ras results in the formation of Ras-Raf-1 complexes. It has been found recently that K-Ras activates Raf-1 more efficiently than H-Ras (Yan et al., 1998). Conversely, H-Ras is a better activator of PI3K than K-Ras (Laezza et al., 1998). Mutations in either Ras or Raf-1 which block their interaction or which inhibit Ras function also prevent Raf-1 activation (Marais et al., 1995); (Luo et al., 1997). However, the discovery that purified recombinant Ras is not sufficient to activate Raf-1 in vitro implies that other components are involved (Traverse et al., 1993); (Zhang et al., 1993). Furthermore, cytosolic Ras, which can be generated by blocking membrane localisation with farnesyltransferase inhibitors, is still able to bind to Raf-1, but these cytosolic complexes are inactive (Kikuchi and Williams, 1994); (Lerner et al., 1995); (Okada et al., 1996). It has been revealed that Ras-GTP can not activate Raf-1 unless Ras-GTP is membrane bound and additional factors are present (Dent et al., 1994); (Stokoe and McCormick, 1997); (Tamada et al., 1997).

Ras can interact with two domains in the Raf-1 amino-terminus: the Ras-binding-domain (RBD; aa2-140) and the cysteine-rich-domain (CRD; aa 139-186) (Brtva et al., 1995); (Drugan et al., 1996). These domains have a low affinity for Ras-GDP, but a high affinity for Ras-GTP. The RBD alone is sufficient for translocation of Raf-1 from the cytosol to the membrane, but the CRD is required for efficient activation (Hu et al., 1997); (Luo et al., 1997); (Roy et al., 1997). This is consistent with the finding that fusion of the CAAX motif of Ras to Raf-1 (Raf-CAAX) locates Raf-1 to the plasma membrane, but results only in partial Raf-1 activation. Additional stimulation with growth factors and Ras activation can further activate RafCAAX (Stokoe et al., 1994); (Leevers et al., 1994), which seems to be mediated by direct binding of Ras to Raf-CAAX, as well as by other Ras-initiated signalling processes (Mineo et al., 1997); (Sun et al., 2000). It has been shown that artificially induced dimerisation of Raf-1 can cause its activation, but the physiological significance is still unclear (Luo et al., 1995); (Farrar et al., 1996).

1.3.2.2 Raf activation requires phosphorylation

Phosphorylation plays a crucial role in activation of Raf-1 (reviewed in (Kolch, 2000)). It has been shown that Raf-1 becomes hyperphosphorylated in response to upstream signalling and
Raf-1 activity can be abolished by protein phosphatases (reviewed in (Keyse, 2000)). Both serine/threonine and tyrosine phosphorylation may have a significant role in Raf-1 activation. Autophosphorylation on T268 appears to be required for Raf-1 activation. The binding of dimeric 14-3-3 proteins to Raf-1 is also essential for regulating its activity and seems to be linked to the regulation of phosphorylation of Raf-1 (Fantl et al., 1994); (Freed et al., 1994). Essential 14-3-3 binding sites in Raf-1 are formed by phosphorylation of S259 and S621, and dephosphorylation of Raf-1 disrupts 14-3-3 binding (Tzivion et al., 1998). The binding of 14-3-3 to both sites appears to inhibit Raf-1 activity, whereas binding of 14-3-3 proteins only to the C-terminal S621 is essential for Raf-1 activity (Rommel et al., 1996); (Clark et al., 1997). Furthermore, removal of 14-3-3 by competition with synthetic phosphopeptides disabled both basal and induced Raf-1 activity (Tzivion et al., 1998). Re-addition of recombinant 14-3-3 could revive Raf-1, but only if it had been activated previously. This demonstrates that 14-3-3 plays an important role in stabilising both the inactive and the activated conformations of Raf-1. Importantly, Ras interferes with the interaction between the N-terminal of Raf-1 and 14-3-3, which leads to a conformational change in Raf-1 and relocation of 14-3-3 dimer to the C-terminus (Rommel et al., 1996). This stabilises the activated Raf-1 associated with Ras-GTP at the plasma membrane and allows phosphorylation of substrates (Tzivion et al., 1998). Mitogen induced activation of Raf-1 requires dephosphorylation of S259, which is carried out by protein phosphatase 2A (PP2A) (Abraham et al., 2000). Inhibition of PP2A prevents both dephosphorylation and activation of Raf-1. It is thought that upon activation, Raf-1 is recruited to the plasma membrane by Ras-GTP, which results in the displacement of 14-3-3 from S259 and allows the required dephosphorylation of S259 by PP2A. The presence of S621 is required for full kinase activation by stimulatory factors and the continuous presence of 14-3-3 at this site is necessary for retaining activity once the kinase is activated, but the exact role of S621 phosphorylation is as yet unclear. In addition, complete Raf-1 activation appears to require phosphorylation at one or both tyrosine residues Y340 and Y341, as mutations of each of these sites blocks activation by Ras (Fabian et al., 1993); (Marais et al., 1995); (Dent et al., 1995). Phosphorylation of these tyrosines appears to be performed predominantly by Src family kinases (Fabian et al., 1993); (Mason et al., 1999). However, B-Raf does not contain tyrosine residues at the positions equivalent to Y340 and Y341. These positions are occupied by aspartic acid residues (D447 and D448), which are responsible for the higher kinase activity of B-Raf compared to Raf-1 (Pritchard et al., 1995). In addition, phosphorylation on
S338 mediates activation of Raf-1. It has been found that this phosphorylation can be indirectly regulated by Ras through activation of PI3K, which activates PAK3, and this in turn phosphorylates S338 (King et al., 1998); (Mason et al., 1999). Another positive regulator of Raf-1 appears to be PKCζ, which binds to and phosphorylates Raf-1. This interaction is mediated by 14-3-3 \textit{in vitro} and \textit{in vivo} (van Dijk et al., 1997); (Van Der Hoeven et al., 2000). Other PKC isoforms are able to activate Raf-1, such as PKCα which phosphorylates S499 in NIH3T3 cells (Kolch et al., 1993) and PKCε which was found to activate Raf-1 when it was pre-activated by Ras (Ueffing et al., 1997). Marais et al. found that Raf-1 activation by signals that activate PKC is mediated through Ras-GTP activation and the formation of Ras-GTP/Raf-1 complexes, but differs from activation by receptor tyrosine kinases as it is not blocked by dominant negative RasN17 (Marais et al., 1998). The impact of Ras signalling on PKC activation of Raf-1 remains to be elucidated.

1.3.2.3 Raf regulation by heatshock proteins and KSR

Raf-1 can associate with chaperones such as heat shock proteins HSP90 and HSP50 (also known as cdc37) (Grammatikakis et al., 1999), thereby stabilising both the active and inactive form of Raf-1 (Schulte et al., 1995). Furthermore, there is evidence that Hsp50 not only stabilises Raf-1 but also enables the interaction with v-Src kinases and MEK as both are found in complexes with Hsp50 and Hsp90 (Stewart et al., 1999). Another regulator of Raf-1 seems to be the kinase suppressor of Ras (KSR) (reviewed in (Kolch, 2000)). KSR was identified by different groups as a protein which suppresses the phenotypes caused by activated Ras (reviewed in (Downward, 1995)). This might occur by binding of KSR to the Raf-1 downstream effector kinase MEK which leads to inhibition of MEK activation (Denouel-Galy et al., 1998). However, KSR was found to enhance Raf-1 activity either by direct phosphorylation of Raf-1 (Xing and Kolesnick, 2000) or by association with Raf-1 at the plasma membrane (Michaud et al., 1997). Furthermore, KSR might function as a scaffolding protein for Raf, MEK and ERK and interaction with each of them can have both positive and negative effects depending on the KSR expression level (Cacace et al., 1999).

1.3.3 The Raf/MEK/ERK pathway

Activated Ras controls one of the key signalling pathways regulating cell proliferation, differentiation and survival, the Raf-1/MEK/ERK pathway, also referred to as mitogen activated protein kinase (MAPK) pathway (Figure 1.4). This MAPK module comprises a
highly conserved cascade of three dual specificity protein kinases: a MAPK kinase kinase (MAPKKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates a MAPK. The MAPK cascade is evolutionarily highly conserved in all eukaryotes and plays a key role in the regulation of gene expression (reviewed in (Carrington and Johnson, 1999)).

Upon activation of the Ras effector Raf-1, a MAPKKK, the MAPKKK MEK1 and MEK2 are phosphorylated on serine and threonine residues. Subsequently, MEKS activate the MAPKs ERK1 and ERK2 by phosphorylation of threonine and tyrosine residues within a T-X-Y motif, thereby initiating nuclear translocation of ERKs. ERK1 and ERK2 are proline-directed protein kinases, as they phosphorylate only serine and threonine residues followed by one or more proline residues (Catling et al., 1995). Transcription factors are major phosphorylation targets for ERKs (reviewed in (Treisman, 1996)), but other targets including cytoskeletal proteins, kinases, phosphatases and enzymes have been identified. Recently, multiple potential ERK-MAPK substrates were indentified in human erythroleukaemia cells using a functional proteomics approach, indicating a function for ERK-MAPK in DNA repair, membrane trafficking, translation and cytoskeletal regulation (Lewis et al., 2000).

In mammalian cells, ERK1 and 2 phosphorylate several proteins belonging to the Ets family of helix-turn-helix transcription factors, including the ternary complex factors (TCFs) Elk1, SAP1 and 2, and Ets-1 and 2 (reviewed in (Wasylyk et al., 1998)). Elk-1 forms a complex with the serum-response factor (SRF) and binds to the serum response DNA element (SRE), which is present in many promoters of immediate early genes, including the c-fos promoter. Furthermore, ERKs can directly activate the transcription factors c-Fos (Chen et al., 1996), c-Myc (Alvarez et al., 1991) and p53 (Milne et al., 1994). Phosphorylation of c-Fos in response to ERK leads to enhanced c-Fos protein stability (Okazaki and Sagata, 1995), and dominant negative ERK mutants block transcription by AP-1 (Frost et al., 1994).

ERKs can also phosphorylate the serine/threonine protein kinases p90Rsk1 (ribosomal S6kinase) and Rsk2, also known as MAPK activated protein kinase (MAPKAPK) 1 and 1b, respectively. p90Rsk1 can phosphorylate and activate several transcription factors, such as c-Fos, CREB (cAMP response element binding protein), CREB-binding protein (CBP) and serum response factor (SRF) (Treisman, 1996). Furthermore, ERK activated p90Rsk1 was also found to phosphorylate and thereby inactivate the pro-apoptotic protein Bad (Bonni et al., 1999). Activation of p90Rsk1 appears to be not only controlled by ERKs, because full activation also requires phosphorylation by PDK1 (Nebreda and Gavin, 1999). In
addition, ERKs can activate MAPKAPK2 and 3 and Mnks1 and 2 (Ludwig et al., 1996), (Waskiewicz et al., 1997). MAPKAP2 and MAPKAP3 phosphorylate HSP27, which might function as an actin binding protein (Clifton et al., 1996). Mnk1 and Mnk2 phosphorylate elongation initiation factor 4E (EIF4E), implying a role in translational control (Waskiewicz et al., 1997). Furthermore ERKs were found to mediate cell motility by phosphorylation of myosin light chain kinase (MLCK) (Klemke et al., 1997).

The duration and intensity of MAPK activation seems to be important in the downstream effector activation (Marshall, 1995). It has been shown that activated Raf-1 can elicit either a mitogenic response or cell cycle arrest in NIH3T3 fibroblasts, depending on the level of pathway activation (Sewing et al., 1997). Indeed, it has been demonstrated that the duration of MAPK signalling determines the repertoire of Fos and Jun proteins upregulated. The choice of substrates for ERK1 and ERK2 are potentially controlled by a number of mechanisms including cell-type specificity and expression levels. Positive feedback loops can also occur within the cascade. For example ERKs can phosphorylate MEK1, Raf-1 and KSR, and MEK1 can phosphorylate Raf-1 through an ERK dependent pathway (reviewed in (Kolch, 2000)). Docking domains, such as DEF and KIM, which respectively act as ERK specific and MAPK specific binding sites, function as guide for the appropriate MAPK to its phosphorylation target. The ERK specific motif DEF (docking site for ERK, FxFP) consists of an SP or TP phosphorylation site in the proximity of an ERK binding site (FXFP). The general MAPK binding site KIM (kinase interactive motif) is located more remotely from the phosphorylation site (Jacobs et al., 1999). Some ERK substrates contain both docking domains, although they do not seem to co-operate in ERK binding. However, they might serve to integrate signals for different MAPK on a joint substrate.

Physical interaction between components of the MAPK cascade seem to be mediated by scaffold proteins, bringing defined kinases together into a protein complex for specific functions. In addition to KSR, other non-enzymatic scaffold proteins have been reported in mammalian cells. These include JIP-1 (JNK-interacting protein-1) (Whitmarsh et al., 1998) and MPI (MEK partner1) (Schaeffer et al., 1998). Recently a Raf-1 kinase inhibitor protein (RKIP) has been identified (Yeung et al., 1999), which can selectively disrupt interactions between Raf-1 and MEK, resulting in suppression of Raf-1 induced transformation (Yeung et al., 2000). It is possible that more proteins with similar function will be found in mammalian cells, and that such proteins will provide a mechanism for retaining that specificity of MAPK modules in response to specific signals, even when the components are shared between
pathways.

1.3.4 Other MAPK cascades

Also other MAPK cascades are activated in mammalian cells in addition to the Ras/Raf-1/MEK/ERK cascade. This includes the JNK (c-jun N-terminal kinase) and the p38MAPK pathways (Kyriakis and Avruch, 1996), which are depicted in Figure 1.5. Both JNK and p38MAPK cascade can be activated in response to extracellular stress stimuli, such as DNA damaging agents, oxidative stress or pro-inflammatory cytokines, such as TNFα and IL-1, and protein synthesis inhibitors (cycloheximide), and are therefore classified as stress activated protein kinases (SAPK). Although many JNK and p38 activating stimuli are pro-apoptotic, the biological outcome is highly divergent and seems to be dependent on the cell type and the cellular context.

**Figure 1.5. Mammalian MAPK signalling pathways.** Each pathway contains a central signalling module of MAPKKK, MAPKK and MAPK. Small GTPases link transmembrane signalling to activation of the MAPK cascade.
Each member of the MAP kinase cascade also has many upstream activators and downstream targets. They can be activated in a Ras dependent manner, independently of Raf-1 (Minden et al., 1994) as well as being targeted by members of the Rho family of GTPases. Furthermore, TAK1, a MAPKKK, was found to activate JNK (Shirakabe et al., 1997) and p38 (Hanafusa et al., 1999) upon different pro-apoptotic stimuli. Although once considered as linear signalling pathways, the MAPK modules are now realised to be part of a much larger signalling network.

1.3.5 Other Raf effectors

Although the MEK/ERK pathway is a major effector of Raf-1, there is accumulating evidence that there are other Raf-1 effectors. It has been shown that Raf-1 might function as a direct link between mitogenic signalling and cell cycle machinery. Raf-1 was found to directly interact with the tumour suppressor protein pRb in response to growth factor stimulation, thereby phosphorylating and inactivating pRb (Wang et al., 1998). Another report showed that Raf-1 might directly phosphorylate and thereby activate the phosphatase Cdc25A (Galaktionov et al., 1995), possibly by interaction of both proteins with 14-3-3 proteins (Conklin et al., 1995). Recently it has been illustrated that Raf-1 can colocalise with vimentin filaments in NIH3T3 cells, thereby mediating phosphorylation via Raf-1 associated kinases, possibly including casein kinase 2 (CK2). This leads to depolymerisation of vimentin filaments, which is not prevented by MEK inhibitors (Janosch et al., 2000). Moreover, CK2 activation by Raf-1 is implicated in activating the NF-κB pathway (Lin et al., 1999). Interaction between Raf-1 and the anti-apoptotic protein Bcl-2 have been described in over-expression studies (Wang et al., 1996), but the physiological relevance is still unclear.

1.3.6 Additional Ras effector pathways

Alternative Ras effectors include members of the PI3K family (Rodriguez-Viciana et al., 1996) and the RasGDS family (Kikuchi et al., 1994). Experiments using effector domain mutants of Ras gave rise to differential biological effects reflecting the activation of specific Ras effectors in Ras mediated cellular responses. RasV12G37, which activates RasGDS, reduces the dependence on serum growth factors but does not change cell morphology. Mutants that activate Raf-1 (RasV12S35 and RasV12E38) induce enhanced growth properties and reduced anchorage dependence and morphological transformation. RasV12C40 activates PI3K, which leads to growth promoting and cytoskeletal alterations and protection against
apoptosis (White et al., 1995); (Rodriguez-Viciana et al., 1997). These mutants strongly synergise in transformation assays upon co-expression. This implies that multiple downstream pathways from Ras are required for full transformation. Furthermore, several other candidate effector proteins have been identified so far, such as AF-6 (Watari et al., 1998), PKCζ (Diaz-Meco et al., 1994), MEKK1 (Russell et al., 1995), Rin1 (Ham et al., 1995) and Nore1 (Vavvas et al., 1998), but their biological functions are as yet poorly defined. The function of AF-6, at least in epithelial cells, seems to be associated with the formation of tight junctions by binding to ZO-1, which is impaired in the presence of activated Ras (Yamamoto et al., 1997).

1.3.7 Ras/PI3K pathway

The downstream effector phosphoinositide-3-kinase (PI3K) comprises a family of related proteins which are important in a wide range of biological activities, including cell proliferation, cytoskeletal remodeling, cell migration and cell survival (Rodriguez-Viciana et al., 1996); (Vanhaesebroeck et al., 1997). Ras is important for growth factor stimulation of PI3K activity and the role of Ras in PI3K activation may be to localise PI3K to the plasma membrane and bring it into contact with its lipid substrate. Ras directly interacts with PI3K via its catalytic subunit p110 to stimulate its lipid kinase activity, which leads to phosphorylation of the 3-position of phosphoinositides (Leevers et al., 1999). PI3K, however, also functions as a protein kinase (Dhand et al., 1994); (Hunter, 1995).

The nine members of the PI3K family can be grouped into three classes according to the mode of activation, structure and substrate specificity. The best characterised ClassI kinases are composed of the regulatory subunit p85, containing the Ras binding domain, and the catalytic subunit p110. They generate most of the PI-3,4,5-trisphosphate (PI(3,4,5)P) in vivo but also PI-3,4-bisphosphate (PI(3,4)P2. Both lipids are important for the activation of Akt, PDK1 and some PKC isoforms. ClassII kinases are known to generate mainly PI(3,4)P2 in a calcium-dependent manner and ClassIII kinases increase the level of phosphatidylinositol (PI)-3-phosphate (PI(3)P).

Oncogenic Ras is a much more potent activator of PI3K than growth factor activated Ras (McCormick, 1999). It is well established that oncogenic Ras suppresses apoptosis through the PI3K pathway, mainly through the activation of the serine/threonine kinase Akt, also known as PKB (protein kinase B). This is an important hallmark of tumour progression, as
activation of this pathway promotes survival of epithelial cells after detachment from the extracellular matrix, which normally would induce apoptosis (Downward, 1998). Recently it has been shown that PI3K functions as an essential anti-apoptotic effector in the proliferative response to primary human thyroid epithelial cells to oncogenic Ras (Gire et al., 2000). It was suggested that PI3K might activate the NF-κB pathway, which is implicated in regulating cell-survival (Burow et al., 2000); (Jones et al., 2000); (Scheid and Woodgett, 2000).

Furthermore, PI3K can also activate Rac and this Rho-GTPase family member in turn is known to regulate actin reorganisation, gene expression and cell cycle progression (Downward, 1998). Whereas growth-factor induced Ras signalling activates Rac via PI3K, sustained signalling by oncogenic Ras was found to down-regulate Rac activity via the Raf-1/MAPK pathway in epithelial MDCK cells. This increases Rho activity and might contribute to an epithelial-to-mesenchymal transition (EMT) (Zondag et al., 2000). However, activation of Rac was found to reduce Rho activity in fibroblasts (Sander et al., 1999), indicating that the effects of Rac and Rho may be cell type specific.

1.3.8 Ras/RalGDS pathway
Ras can also activate a family of GEFs for the Ral small GTPases (RalGEFs). A member of this family is RalGDS, which in turn activates the ubiquitously expressed Ras family member Ral (Feig et al., 1996). Several reports show that redistribution of RalGDS from the cytosol to the plasma membrane may be important for activation, perhaps by binding to Ral at the membrane (Wolthuis et al., 1998). Stimulation of a variety of receptors, such as G-protein coupled receptors and RTKs, induces rapid activation of endogenous Ral. There is evidence for a role of RalGDS in promoting cell growth and Ras transformation (Wolthuis and Bos, 1999), but the role of Ral and RalGDS in Ras function needs to be further elucidated.

1.4 HGF/SF signalling

1.4.1 HGF/SF and its receptor c-Met
Hepatocyte growth factor/scatter factor (HGF/SF) is a polypeptide cytokine and member of the plasminogen-related growth factor family, whose structure and mechanism of activation
resembles those of the protease plasminogen. In contrast to plasminogen, HGF/SF lacks enzymatic activity (Donate et al., 1994). HGF/SF is secreted as an inactive proform, which can bind with high affinity to its receptor c-Met, but is unable to activate it (Naka et al., 1992). The proform is converted into the bioactive form upon proteolytic cleavage, which results in the formation of a heterodimer, covalently linked by a disulfide bond. There is evidence that the proteases urokinase plasminogen activator (uPa) and tissue plasminogen activator (tPA) can cleave the proform in vitro (Naldini et al., 1992).

The effects of HGF/SF are mediated through a receptor tyrosine kinase (RTK) c-Met, a proto-oncogene (Naldini et al., 1991); (Bottaro et al., 1991), which is a transmembrane domain protein that dimerises upon binding to HGF/SF. This results in an auto-/- transphosphorylation of tyrosine residues in the kinase domain (Ponzetto et al., 1993); (Bardelli et al., 1999). The phosphorylated residues Y 1349 and Y 1356 function as binding sites for the SH2 domain of the regulatory subunit (p85) of PI3K, which in turn gets activated (Ponzetto et al., 1994). Furthermore c-Met can directly bind and activate the proto-oncogene product Src and PhospholipaseCγ (Fixman et al., 1995). In addition, adapter proteins can bind to the c-Met receptor, including the Grb2/SOS complex inducing Ras signalling and Gab1, which activates PI3K. Furthermore, Gab1 seems also to activate MAPK (ERK1 and ERK2) via interaction with another adaptor Shp2 (Schaeper et al., 2000).

1.4.2 Biological effects of HGF/SF

HGF/SF has been discovered independently as a mitogenic factor (HGF) for hepatocytes (Michalopoulos et al., 1984); (Nakamura et al., 1984); (Russell et al., 1984) and a motogenic stimuli (SF) (Stoker and Perryman, 1985). HGF/SF causes dissociation of epithelial cells, termed scattering, which is characterised by cell migration and reduced intercellular contacts. For example monolayers of subconfluent MDCK cells, which grow in epithelial islands, break up into isolated, scattering cells when treated with HGF/SF (Stoker and Perryman, 1985). HGF/SF promotes morphogenic alterations through the formation of branching tubular structures in epithelial cells, which were cultured in three-dimensional collagen matrices (Montesano et al., 1991); (Khwaja et al., 1998), in mammary gland (Yang et al., 1995) and in metanephric organ cultures (Woolf et al., 1995). Branching morphogenesis involves combined effects on cell-cell adhesion, cell-matrix adhesion, proteolytic remodeling of the extracellular matrix (ECM) and cell proliferation, but thereby retaining epithelial features including an apical-basolateral polarity (Santos et al., 1993).
During mouse embryogenesis, c-Met is expressed in the epithelial cells of various organs, whereas HGF/SF is expressed in the adjacent mesenchyme (Sonnenberg et al., 1987). In the developing kidney, HGF/SF and c-Met are regulating tubulogenesis and branching morphogenesis (Santos et al., 1994). The genetic inactivation of HGF/SF or its receptor in mice results in embryonic lethality because of the absence of muscles that derive from migrating myogenic progenitor cells, which are normally generated by an epithelial-to-mesenchymal transition (Bladt et al., 1995). These migrating cells cannot be observed in these null embryos (Heymann et al., 1996). Intriguingly, transfection of NIH3T3 mesenchyme cells with c-Met leads to establishment of an autocrine loop with their constitutively expressed HGF/SF and mediates a complete mesenchymal-to-epithelial transition (Tsarfaty et al., 1994).

HGF-mediated epithelial morphogenesis is based on the orchestration of a number of cellular effects, including proliferation, motility, degradation of extracellular matrix components and survival (Gumbiner, 1992). In transformed cells, these integrative effects appear to be responsible for invasiveness (Rosen et al., 1994). This complexity of HGF/SF signalling is based on the co-operation of multiple signalling pathways. It has been found that the motility response to HGF/SF in MDCK cells is dependent on PI3K (Royal and Park, 1995) as well as on Ras and Rac/Rho activation (Ridley et al., 1995), whereas the growth response requires activation of the Ras/Raf-1/MAPK pathway via the Grb2-SOS complex (Ponzetto et al., 1996). HGF/SF was found to induce expression of urokinase-plasminogen activator (uPA) and uPA receptor in MDCK cells, both of which are implicated in the remodeling of ECM required for cell invasion (Pepper et al., 1992).

HGF/SF signalling also appears to play a role in human tumours. Overexpression of c-Met has been observed in carcinomas derived from follicular epithelium as well as in ovarian carcinomas and pancreatic cancer (Di Renzo et al., 1994); (Di Renzo et al., 1995) and is often found in liver metastasis. HGF/SF stimulates the motility and invasiveness of carcinoma cells (Rosen et al., 1994) and was found to induce angiogenesis (Grant et al., 1993), which is required for growth and metastasis of solid tumours. Recently a direct genetic link between c-Met and human cancer has been revealed by the identification of activating germline and somatic mutations in the c-met gene in hereditary papillary renal carcinomas (HPRC) and childhood hepatocellular carcinomas (Park et al., 1999).
1.5 Cell Cycle Regulation

The eukaryotic cell cycle can be divided into four stages: G1, S, G2 and M (Figure 1.6). At G1 the cell integrates mitogenic and growth inhibitory signals, thereby deciding cell fate: to proceed, pause or exit the cell cycle. G1 is followed by S-phase during which cellular DNA is replicated. Having completed DNA replication, cells enter a second growth phase, termed G2. During G2 cells duplicate functional elements required for cell division. Following G2 cells undergo mitosis (M), during which chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells, thus completing the cell cycle. Withdrawal from the cell cycle can occur through depletion of mitogenic factors resulting in quiescence (G0) or alternatively when a cell undergoes differentiation. Cell cycle progression is controlled by multiple mechanisms and it has become clear that tumourigenesis involves the disruption of these controls (reviewed in (Sherr and Roberts, 1999)).

![Figure 1.6. Stages of the cell cycle in vertebrates.](image)

1.5.1 G1 to S-phase transition

At a certain point in late G1 phase, termed restriction point (Pardee, 1989), cell cycle signals culminate in a "decision" whereby the cell is committed to complete the cell cycle and enter S-phase or to remain in a quiescent non-proliferative state, depending on mitogenic signals. In
the presence of growth factors, G1 to S-phase transition occurs (Figure1.6). This is controlled by members of a conserved family of serine/threonine kinases, the cyclin dependent kinases (CDKs). During G1 CDK activity requires binding to G1 cyclins (cyclinD1-3, cyclinE and cyclinA) (reviewed (Sherr and Roberts, 1995)), phosphorylation at two central tyrosine residues by CDK activating kinases (CAK) (reviewed (Kaldis, 1999)) and dephosphorylation at inhibitory sites in the amino-terminus by Cdc25 phosphatases (Galaktionov et al., 1996). In addition, CDK activity is also negatively regulated by at least two families of CDK inhibitors (CKIs) to prevent unappropriate cell cycle (reviewed in (Sherr and Roberts, 1999)).

CDK4 and CDK6 bind to the tissue specific expressed D-type cyclins (D1, D2, D3), forming an active kinase complex (Matsushime et al., 1992); (Meyerson and Harlow, 1994). CyclinE forms active complexes only with CDK2 (Dulic et al., 1992); (Koff et al., 1992). CyclinA, which is required for both entry into S-Phase and onset of mitosis, binds and activates CDK2 and CDK1. Cyclin A/CDK2 complexes are predominantly formed in late G1 and S-phase, whereas cyclinA/CDK1 is activated in G2 (Pagano et al., 1992). Mitogen-dependent accumulation and activation of cyclinD/CDK4 or cyclinD/CDK6 complexes trigger the initial phosphorylation of the retinoblastoma (pRb) protein, a tumour suppressor and member of the family of pocket-proteins which also include the related proteins p130 and p107 (Ewen, 1998). In the hypophosphorylated state, pRb binds and inactivates transcription factors, such as members of the E2F family, thereby repressing transcription of responsive genes, whose products are essential for DNA synthesis (reviewed in(Nevins, 1998)). In addition, pRb recruits transcriptional repressors such as histone deacetylases (Ferreira et al., 1998). Hyperphosphorylation of pRb disrupts this interaction, which leads to inactivation of pRb and release of E2F transcription factors (Mittnacht et al., 1997); (Harbour et al., 1999). This enables transcription of E2F-responsive genes, the products of which are necessary for S-phase entry, including the expression of cyclinE (Ohtani et al., 1995). Formation of CyclinE/CDK2 complexes occurs, which then phosphorylate pRb on additional sites. This hyper-phosphorylation leads to complete pRb inactivation and thus increases E2F release, further enabling activation of E2F responsive genes, including cyclinA (Sherr and Roberts, 1995). CyclinA/CDK2 complexes then maintain this level of pRb phosphorylation beyond G1. The activity of the cyclinE/CDK2 complex peaks at the G1 to S-transition, after which cyclinE is degraded and replaced by cyclinA. Accumulation of cyclinA occurs prior to S-phase entry (Dulic et al., 1992) and cyclinA associated kinase activity is required for
completion of S-phase and entry into M-phase. Once pRb is inactivated, D-type cyclins are no longer required (Lukas et al., 1996). This shift in pRb phosphorylation from mitogen-dependent cyclinD/CDK complexes to mitogen-independent cyclinE/CDK2 complexes accounts in part for the loss of dependency on extra-cellular growth factors at the restriction point.

1.5.2 The CDK inhibitors (CKIs)

The catalytic activities of CDKs are constrained by CDK inhibitors (CKIs). These inhibitors can be divided into two classes based on their structure and CDK target: the CIP/KIP family of proteins and the family of INK4 (inhibitors of CDK4) proteins (reviewed in (Sherr, 2000). The more broadly acting Cip/Kip inhibitor family (CDK interacting protein/ Kinase inhibitory protein) consists of p21Cip1, p27Kip1, and p57Kip2. These proteins share a characteristic inhibitory domain in their amino-terminus and act as inhibitors for CDK2 complexes \textit{in vivo} but they have been found to bind and inhibit all cyclin/CDK complexes \textit{in vitro} (Xiong et al., 1993). In quiescent cells, Cip/Kip protein levels are elevated. In response to mitogenic signals the cells enter the cell cycle and cyclinD1 expression is increased. This leads to accumulation of cyclinD/CDK4 and cyclinD/CDK6, which require Cip/Kip proteins for their assembly, stability and nuclear import (Soos et al., 1996); (Cheng et al., 1998), while the bound CDK remains catalytically active. However, under certain conditions \textit{in vitro}, Cip/Kip proteins were found to inhibit cyclin D-dependent activities (Kato et al., 1994). Recent reports have shown that a single molecule of p21Cip1 is sufficient to inhibit the kinase activity of cyclinE/CDK2 and cyclinA/CDK2 complexes but not cyclinD/CDK4 (Hengst and Reed, 1998). In proliferating cells, p27Kip1 proteins are almost completely associated with cyclinD/CDK complexes, which allows assembly of cyclinD/CDK4 complexes. This promotes CDK-dependent phosphorylation of pRb and cyclinE/CDK2 activation, mediating cell cycle progression into S-phase (reviewed in (Sherr and Roberts, 1999)). Upon S-phase entry, cyclinE/CDK complexes antagonise the action of its own inhibitor: it phosphorylates unbound p27Kip1, which is then targeted for ubiquitination and proteosomal degradation (Sheaff et al., 1997). Mitogen withdrawal results in rapid cyclinD degradation and the previously sequestered Cip/Kip proteins are released, inhibiting cyclinE/CDK2 activity and thus leading to cell cycle arrest (Reynisdottir and Massague, 1997).

The members of the INK4 family p16INK4a, p15INK4b, p18INK4c and p19INK4d contain
multiple ankyrin repeats, allowing them to specifically inhibit the kinase activity of the cyclinD associated CDKs, CDK4 and CDK6. The signals which lead to the synthesis of INK4 proteins are poorly understood, but it has been found that p15INK4b is induced by TGFβ resulting in G1-arrest (Hannon and Beach, 1994). In general, the INK4 proteins compete with D-type cyclins for binding to their CDKs (Parry et al., 1999); (McConnell et al., 1999). Various studies suggest that INK4 proteins disrupt cyclinD/CDK/Cip/Kip complexes and sequester the CDKs into binary CDK/INK4 complexes. As a result, cyclinD and Cip/Kip inhibitors are liberated. Unbound cyclinD proteins are rapidly degraded by the ubiquitin-dependent proteasome pathway (Diehl et al., 1997), whereas the released Cip/Kip inhibitors bind to and inactivate cyclinE/CDK2 complexes, leaving pRb hypophosphorylated and E2F function repressed.

In the absence of functional pRb protein the disruption of cyclinD/CDK complexes and release of Cip/Kip proteins is not sufficient to inhibit cyclinE/ckdk2 activity, as it is normally under pRb-E2F control. This has been shown in pRb null cells, where cyclinE/CDK activities are elevated and transition into S-phase occurs (Medema et al., 1995). Disruption of the pRb pathway occurs frequently in human cancers (reviewed in (Roussel, 1999)). This can result from deletions or loss-of-function mutations in the pRb or p16INK4 loci. As mitogen-activated signals converge in the pRb pathway, the result of these alterations is an overactivated proliferation pathway, independent of mitogenic requirements.

1.5.3 Regulation of cell cycle components by Ras

Depending on the level of activity, Ras can be both proliferative and growth inhibiting. Growth factor induced activation of Ras is required for inactivation of pRb during G1- to S-phase transition (Taylor and Shalloway, 1996); (Mittnacht et al., 1997); (Downward, 1997) as illustrated using Ras inhibitory antibodies or dominant negative RasN17 where cells arrest in G1 and growth factor stimulated cells do not leave G0 to re-enter the cell cycle (Mulcahy et al., 1985); (Feig and Cooper, 1988).

Ras mediated proliferation can be regulated via multiple mechanisms. Mitogenic activation of the Ras/Raf/ERK pathway induces cyclinD1 transcription, which leads to inactivation of pRb proteins (Filmus et al., 1994); (Albanese et al., 1995). D-type cyclins connect extracellular signalling pathways to the cell cycle machinery, and as such they are growth factor sensors. CyclinD1 induction also seems to be necessary for Ras-induced anchorage independent
growth (Liu et al., 1995). Alternatively, cyclinD transcription can also be induced by the APC/β-catenin/TCF/LEF1 pathway (Morin, 1999); (Tetsu and McCormick, 1999) or by the extracellular matrix through integrin signalling (Bohmer et al., 1996). In epithelial cells and fibroblasts, Ras activity can also increase translation of CyclinD1 and stabilise the protein (Aktas et al., 1997). Constitutively active MEK, Raf-1 or PI3K individually can all stimulate expression of cyclinD1 promoters, but maximal stimulation of cyclinD requires the cooperative action of several Ras effectors following serum stimulation of quiescent fibroblasts (Kerkhoff and Rapp, 1997); (Gille and Downward, 1999). Furthermore PI3K also regulates the stability of cyclinD1 protein, through activation of Akt/PKB, which blocks phosphorylation of cyclinD1 by GSK-3β and therefore inhibits cyclinD1 degradation (Cross et al., 1995); (Diehl et al., 1998). It has been shown that the PI3K downstream effector p70S6kinase can also activate cyclinD transcription and translation (Hashemolhosseini et al., 1998). Elevated levels of cyclinD1 protein, but only low level amplification of the cyclinD1 gene are found in mouse skin tumours as well as many human tumours (Bianchi et al., 1993); (Robles and Conti, 1995). Ras can also down-regulate p21Kip1 in late G1 (Takuwa and Takuwa, 1997). This seems to involve both activation of Raf/MAPK and the PI3K signalling pathways, mediating protein stability (Weber et al., 1997); (Treinies et al., 1999). ERK is able to phosphorylate p21Kip1 in vitro, which abrogates p21Kip1 binding to CDK2 complexes (Kawada et al., 1997). PI3K has been shown to stabilise cyclinD protein, possibly by allowing cyclinD to titrate out p21Kip1 and thus targeting p21Kip1 for degradation. PI3K can also activate Rho, which might be a key regulator of p21Kip1 degradation, as inhibition of Rho activity abrogated growth factor mediated p21Kip1 degradation (reviewed in (Marshall, 1999)).

A large body of work exists pointing out that Ras is not only a growth promoting oncogene but can also negatively function on the cell cycle in vitro (reviewed in (Lloyd, 1998)). It has been shown that expression of oncogenic Ras in fibroblasts leads to cell cycle arrest through induction of p53 or activation of the CDK inhibitor p16. Activation of Ras or Raf-1 can also induce transcription of p21Cip, thus leading to G1 cell cycle arrest in a p53-dependent manner (Lloyd et al., 1997). This can be suppressed by the activation of Rho, allowing Ras to drive cells into S-phase (Olson et al., 1998). Furthermore, p16 null MEFs and p53 null MEFs no longer undergo growth arrest in response to Ras (Serrano et al., 1997). Although p16 is upregulated by Ras, there is currently no evidence which Ras effector pathway is involved in this increased expression. Increased p16 leads to growth arrest or senescence
depending on the cell type, a potentially important mechanism for preventing clonal expansion of cells with an activated \textit{ras} oncogene. It seems that the exact outcome of Ras activation appears to depend on the cell type and the biological context of the cell. Moreover, the cellular effects of Ras will be determined by the magnitude and duration of the Ras signal, as well as the influence of co-operation with other activated oncogenes.

1.6 Apoptosis

1.6.1 Morphological changes during apoptosis

Programmed cell death, also known as apoptosis, is an essential mechanism in the elimination of cells during development and in homeostasis of multi-cellular organisms in order to control cell number (Hengartner, 2000). During vertebrate life, most newly formed but unwanted lymphocytes in the thymus undergo apoptosis. Apoptosis is also required to eliminate neutrophiles which are continuously produced in the bone marrow. Apoptosis also plays an important role in a variety of diseases, including cancer, autoimmune disease and many neurodegenerative disorders.

The process of controlled killing by activation of an intracellular death programme was proposed in 1972, when Kerr and co-workers observed that large numbers of cells die after hormone withdrawal from hormone-dependent tumours (Kerr et al., 1972). Dying cells show characteristic morphological changes, including membrane blebbing, DNA fragmentation and chromatin condensation, cell shrinkage and eventually disassembly of the cell into membrane-enclosed vesicles, termed apoptotic bodies. Apoptotic bodies are eliminated by phagocytosis performed by neighboring cells, thereby preventing an inflammatory response that would result from the release of intracellular components. At the cell surface phosphatidyl-serine is externalised, which promotes recognition of the apoptotic cell for phagocytosis (Homburg et al., 1995); (Martin et al., 1995). In contrast, cells which die by an uncontrolled process, termed necrosis, swell and the organelles lose their integrity. The plasma membrane ruptures and the intracellular contents are released, which can elicit a damaging inflammatory response.

The first genetic evidence for apoptosis came from studies in \textit{C.elegans:} during development, apoptosis normally occurs in 131 cells resulting in an adult worm with 1090 cells. This made
C. elegans a model organism for studying the core components of the cell death machinery (reviewed in (Meier et al., 2000)).

1.6.2 Caspases

Studies in C. elegans led to identification of genes required for apoptosis, the ced genes. One of them is called ced-3 and loss of function mutations in ced-3 resulted in survival of all 131 doomed cells (Ellis and Horvitz, 1986). CED-3 is the prototype of a family of cysteine proteases, termed caspases, with specificity for aspartic acid residues C-terminal to the cleavage site in their substrates (Yuan et al., 1993). Caspases are highly conserved throughout evolution. To date fourteen mammalian caspases have been identified, of which eleven are of human origin and three of murine origin (reviewed in (Wolf and Green, 1999)) (Figure 1.7).

![Phylogenetic tree of caspases in vertebrates](image)

**Figure 1.7. Phylogenetic tree of caspases in vertebrates.** Members of the caspase family have distinct roles in apoptosis and inflammation. The family includes three murine homologs: caspase-11,-12 and -14 (caspase-14 is not depicted here). In apoptosis, caspases can be divided functionally into initiator caspases and effector caspases (more details are discussed in the text).

Whereas caspase-2, -3 and -6 to -10 are involved in the apoptotic process, caspase-1 and murine caspase-11 function predominantly as cytokine processors. Less is known about caspases-4, -5 and -12 to -14. Since they show a higher degree of sequence similarity to caspase-1, they are grouped with the cytokine processors, but there are indications at least
that caspase-12 might be involved in apoptosis.

1.6.2.1 Structure of caspases

Caspases are synthesised as enzymatically inert zymogens composed of three domains (reviewed in (Earnshaw et al., 1999)). An N-terminal prodomain, a large domain (~20kD) containing the active site cysteine within a conserved QACXG motif and a C-terminal small domain (~10kD). Caspase prodomains range in length from 23 aa for caspase-6 and -7 to 219 aa for caspase-10. The prodomain is separated from the p20 subunit by an aspartate cleavage site. The large and the small subunits are separated by a linker region containing one or two aspartate cleavage sites. Based on their structural and functional differences the apoptotic caspases can be divided into two groups. The first group comprises caspases with large prodomains which are involved in the initiation of the apoptotic response and are therefore named initiator caspases. Two types of protein-protein interaction motifs have been identified in their prodomains: caspase-8 and -10 contain two death effector domains (DED), while caspase-2 and -9 contain caspase recruitment domains (CARDs). These domains enable them to bind adapter molecules containing similar domains, a process which is required for their activation. The second group comprises caspases with short prodomains. Since they are activated by initiator caspases, they have been termed effector caspases.

1.6.2.2 Activation of caspases

Caspases are ubiquitously expressed in most cells as proenzymes or zymogens with low intrinsic enzymatic activity. This allows rapid activation of procaspases in response to a pro-apoptotic stimulus. All procaspases are activated by proteolytic cleavage either through an autocatalytic process or through other caspases. A first proteolytic cleavage in the linker region divides the procaspase into a large and small caspase subunit and a second cleavage removes the N-terminal prodomain. The active caspases are heterotetramers composed of two identical small and two identical large subunits with two active sites (Figure 1.8) (reviewed in (Earnshaw et al., 1999)).
Figure 1.8. Schematic representation of the proteolytic activation of caspases. Caspases are synthesised as single-chain precursors. Activation proceeds by cleavage of the N-terminal prodomain and cleavage between the large and small subunits. The catalytically active caspase is a heterotetramer.

The spectrum of caspases required to be activated for commitment to apoptosis in the same cell type can vary depending on the apoptotic stimulus. Currently, two major pathways are described, by which different initiator procaspases can be activated. One is dependent on a cell surface stimulus and therefore termed extrinsic pathway: the apoptotic signal is transduced through the binding of an extracellular death ligand to its death receptor (reviewed in (Ashkenazi and Dixit, 1998)). This results in the activation of procaspase-8, which is mediated by recruitment to an adapter protein. The other major pathway for procaspase activation occurs via intrinsic stimuli as a consequence of cellular stress such as irradiation, cytotoxic drugs, and growth factor withdrawal. This involves release of cytochrome c from mitochondria, which is required for activation of the adapter protein bound initiator caspase-9.

Once initiator caspases are activated, they target and activate effector caspases, thereby initiating a complex amplification cascade. Initiator caspase-8 and -9 have been shown to be able to activate most effector caspases in vitro. In turn, activated effector caspases are able to cleave and activate the initiator caspases via positive feedback amplification. The situation in vivo is that the subset of caspases activated by a distinct caspase are more limited. The caspases need to be present in sufficient quantity in the correct location at the appropriate time to activate a substrate. However, complex feedback loops of caspase activation do appear to
operate in vivo. One non-caspase protease has been identified which can activate caspases: granzyme B from cytotoxic T cells is an aspartate-specific serine protease which can activate procaspase-3 and -7 (Zhou and Salvesen, 1997); (Stennicke et al., 1998). Distinct apoptotic signals are able to induce the amplifying apoptotic caspase cascade, which eventually converges in the same biochemical and morphological changes. However, caspases involved in a specific apoptotic response seem to vary depending on the cell type and on the stimulus.

1.6.2.3 Cellular substrates of caspases

Once active, caspases can cleave a variety of intracellular substrates, including structural components, protein kinases and components of the DNA repair machinery. This results in execution of cellular survival pathways and destruction of cell structure and commitment to cell death (reviewed in (Earnshaw et al., 1999)).

An important feature of apoptosis is the disassembly of the nucleus. The inhibitor of the nuclease CAD (ICAD) is cleaved by caspase-3 (Liu et al., 1997), which activates the nuclease CAD (caspase activated deoxyribonuclease) responsible for DNA fragmentation necessary for chromatin condensation (Enari et al., 1998). Mediators of DNA repair, such as poly(ADP-ribose)polymerase (PARP), DNA-polymerase kinases (DNA-PKs) and hsRAD51 are among the earliest proteins to be cleaved in the apoptotic cell, which prevents DNA repair allowing accumulation of damaged DNA. Cleavage of major structural nuclear proteins, including the laminins, are essential for disassembling the nuclear structure required for nuclear shrinkage (Rao et al., 1996). Caspases can disrupt cytoskeletal integrity as well as intercellular contacts by cleaving a number of cell adhesion proteins including the cytoskeleton proteins actin (Mashima et al., 1995); (Kayalar et al., 1996) the intermediate filament cytokeratin18 (Caulin et al., 1997) and the adherens junction components β-catenin (Brancolini et al., 1997) and γ-catenin (Herren et al., 1998). Caspases also target and inactivate several proteins involved in cytoskeleton-associated cell survival pathways, such as focal adhesion kinase (FAK) (Crouch et al., 1996), Akt and Raf-1. However, Raf-1 and Akt are not directly cleaved by caspases, suggesting the activation of other proteases by caspases (Widmann et al., 1998). Protein phosphatase 2A (PP2A) (Santoro et al., 1998), which normally down-regulates MAPK, is activated through caspase cleavage. Furthermore, cell-cycle regulators can be cleaved during apoptosis, such as the pRb protein and the p53 inhibitory protein Mdm-2. However, several of the key programs to alter cell morphology, such as cell shrinking and the emission of pro-engulfment signals, are still poorly understood.
Some caspase substrates are involved in regulating a positive feedback loop for caspase activation. For example, caspase-3 was found to cleave anti-apoptotic proteins Bcl-2 and Bcl-X. This results in the release of their C-terminal fragments, promoting caspase activation. Similarly, caspase-8 cleaves and activates Bid, a pro-apoptotic Bcl-2 family member. Release of its C-terminal fragment induces release of cytochrome c from mitochondria, triggering further caspase activation (Li et al., 1998); (Luo et al., 1998).

1.6.3 Death receptors

Mammals have developed a mechanism that enables the organism to directly target specific cells for apoptosis. This kind of instructive apoptosis is important for a rapid response, especially in the immune system. Cell surface receptors, so called death receptors, transmit apoptotic signals initiated by their death ligands. Death receptors belong to the tumour necrosis factor (TNF) receptor superfamily, which share a similar cysteine rich extracellular domain (reviewed in Ashkenazi and Dixit, 1998)). The death receptors contain a homologous cytoplasmic sequence, termed death domain (DD) (Tartaglia et al., 1993), which is similar in structure to DED or CARD domains. The best characterised death receptors are Fas (also called CD95 or Apo1) and TNF receptor-1 (TNFR1) (Gruss and Dower, 1995); (Nagata, 1997). Additional receptors are death receptor 3 (DR3) (also termed Apo-3), DR4 and DR5 (also called Apo2, TRAIL-R2). The ligands that activate these receptors are structurally related molecules belonging to the TNF superfamily. Fas ligand (FasL) binds to Fas, TNFα and lymphotoxinα bind to TNFR1, Apo-3 ligand (Apo-3L) binds to DR3, and TRAIL (also termed Apo-2L) binds to DR4 and DR5.

Fas/CD95 was originally identified to mediate the removal of activated mature T-cells at the end of the immune response. Meanwhile, it is also found to be expressed in a number of other non-lymphoid cell types and tumour cells (reviewed in Schulze-Osthoff et al., 1998)). Binding of heterotrimeric FasL to pre-associated Fas trimers (Siegel et al., 2000) leads to recruitment of the cytoplasmic adapter protein FADD (Fas-associated death domain protein, also called Mort1) (Figure 1.9). FADD interacts through its DD with the DD of Fas. Via its DED, FADD binds to the DEDs of procaspase-8 (reviewed in Ashkenazi and Dixit, 1998)). Subsequently, this leads to aggregation of multiple caspase-8 molecules and autoproteolytical activation of caspase-8 (Muzio et al., 1998); (Yang et al., 1998). This multi-protein complex is termed death inducing signalling complex (DISC) (Muzio et al., 1996). Procaspase-8/FADD interaction can be prevented by c-FLIP (FADD like ICE inhibitory protein), a
Figure 1.9. Model of the death receptor signalling pathway. The apoptotic signal is initiated by direct ligand-mediated trimerisation of death receptors at the cell surface. Recruitment of adapter proteins (e.g., FADD) induces activation of the initiator caspase-8, which subsequently results in activation of a caspase cascade leading to apoptosis.

catalytically inactive caspase-8 like protein with two DEDs (Irmler et al., 1997). FLIP probably competes with procaspase-8 for binding to FADD and binding of FLIP to FADD prevents apoptosis.

Beside FADD, other cytoplasmic proteins were found to bind to Fas (Nagata, 1997). One of them is Daxx (death domain associated protein) which is suggested to mediate Fas induced apoptosis in a FADD-independent manner, leading to activation of the stress activated JNK (Yang et al., 1997). The same study showed that expression of dominant negative Daxx at least partially blocks Fas induced apoptosis. This was only observed in Fas cross-linking experiments, but not in response to FasL (Villunger et al., 2000). Since Daxx knockout mice are embryonic lethal due to extensive apoptosis, the physiological role for Daxx remains unclear (Michaelson et al., 1999). Interestingly, UV-induced apoptosis is strongly attenuated in Fas-resistant keratinocytes and UV was also found to directly oligomerise and thereby activate death receptors such as Fas and TNFR1 (Rehemtulla et al., 1997); (Aragane et al., 1998).

In addition to its cytotoxicity, TNFα also plays a crucial role in the promotion of inflammation. Since TNFα receptors are ubiquitously expressed, most cell types respond to TNF stimulation. Binding of TNFα to TNFR1 activates the transcription factors NF-κB.
and AP-1, leading to expression of pro-inflammatory genes (Tartaglia et al., 1991). In some cell types, TNFα also induces apoptosis through TNFR1, but only if protein synthesis is blocked, suggesting the existence of survival factors. Expression of those survival factors is probably controlled through NF-kB and JNK/AP-1, since inhibition of either pathway sensitises cells to TNFα-induced apoptosis (Beg and Baltimore, 1996); (Van Antwerp et al., 1998) TNF binding results in trimerisation of the TNFR1 and recruitment of the adapter protein TRADD (TNFR-associated death domain), which binds via its DD to the DDs of the receptor. This initiates recruitment of several other proteins: Binding of FADD to the TRADD/receptor complex leads to aggregation and autoactivation of procaspase-8 (Chinnaiyan et al., 1996); (Varfolomeev et al., 1996). Binding of RIP (receptor-interacting protein) which also contains a DD is followed by association with TRAF2 (TNFR-associated factor-2). This multi-protein complex stimulates the activation of NF-κB and of JNK/AP-1 pathways. RIP and TRAF2 activate the NF-κB kinase NIK, which in turn activates members of the IKK family (inhibitor of κB (IκB) kinase). IKK phosphorylates IκB, which then gets ubiquitinated and degraded via the proteasomal pathway. This releases and activates NF-κB transcription factor (Mercurio et al., 1997). The pathway from TRAF2 and RIP to JNK involves a cascade that includes the MAPK MEKK1 (MAP/ERK kinase kinase-1), JNKK (JNK kinase) and JNK (Natoli et al., 1997). One study showed that TRAF2 can bind to the caspase inhibitors cIAP1 and cIAP2 (cellular inhibitor of apoptosis) (Shu et al., 1996), but the physiological relevance is unclear. FADD is essential for TNFα induced apoptosis, since cells from FADD null mice are resistant to TNFα-induced apoptosis (Yeh et al., 1998).

1.6.4 Mitochondria and apoptosis

1.6.4.1 Formation of the apoptosome

Cytochrome c release from mitochondria into the cytosol plays a crucial role in amplification of many types of apoptotic signals, including cellular stress (irradiation, cytotoxic drugs, ceramides), signals from death receptors and growth factor withdrawal. This includes cytochrome c, which is essential for the initiation of a caspase cascade by causing formation of the apoptosome (reviewed in (Green and Reed, 1998); (Desagher and Martinou, 2000)).

Cytochrome c is normally located in the intermembrane compartment of the mitochondria, where it serves an essential function in the respiratory chain. During apoptosis, cytochrome c release from the mitochondria leads to oligomerisation of the adapter protein Apaf-1
(apoptotic protease activating factor 1), the mammalian homologue of *C.elegans* CED-4. Binding of cytochrome c to Apaf-1 in the presence of ATP or dATP leads to conformational changes, which unmask the CARD domain of Apaf-1. Via CARD-CARD interaction, procaspase-9 is recruited into this protein complex (Li et al., 1997), forming the apoptosome (Cain et al., 1999). Aggregation of procaspase-9 facilitates its autoactivation, which then leads to activation of downstream caspases, such as effector caspase-3 and caspase-7. In the absence of an apoptosis signal, the CARD of Apaf-1 is not exposed and therefore unable to bind to procaspase-9.

Cytochrome c release appears to be essential for apoptosis: upon growth factor deprivation in neurons or *c-myc* expression in fibroblasts, injection of neutralising antibodies to cytochrome c is sufficient to protect cells from apoptosis (reviewed in (Desagher and Martinou, 2000)). Mice deficient for Apaf-1 die *in utero* because of severe defects in tissues where developmental apoptosis is required, leading to embryonic death (Cecconi et al., 1998); (Yoshida et al., 1998). Beside cytochrome c, other proteins are also released from the mitochondria. This includes apoptosis inducing factor AIF and some procaspases, which can be released during apoptosis (Susin et al., 1999); (Susin et al., 1999). However, the mechanism by which mitochondrial proteins are released into the cytosol remains to be determined.

### 1.6.5 Bcl-2 proteins

There is a large body of evidence that release of mitochondrial proteins is controlled by members of the Bcl-2 family. The founder of this family, the *bcl-2* protooncogene was originally identified at a translocation breakpoint (t14;18) in human B-cell lymphomas. As a result of this translocation, Bcl-2 was under the control of the immunoglobulin heavy chain enhancer and therefore constitutively expressed. This led to protection from apoptosis and the accumulation of aberrant B-cells giving rise to malignancy (Tsujimoto et al., 1985).

Over time a large number of Bcl-2 family members have been identified, which can be divided into three groups, based on structural and functional differences. Members of group I, such as Bcl-2 and Bcl-X<sub>L</sub> are anti-apoptotic. They contain four short conserved Bcl-2 homology (BH) domains (BH1-BH4) and a C-terminal hydrophobic sequence, which localises the protein predominantly to the outer surface of mitochondria, but also to the endoplasmatic reticulum (ER) membrane and outer nuclear membrane (Akao et al., 1994);
(Krajewski et al., 1993). In contrast, members of group II and III have pro-apoptotic activity (reviewed in (Adams and Cory, 1998)). Members of group II, including Bax and Bak, are similar in structure to Bcl-2 and Bcl-XL. They also contain the C-terminal hydrophobic tail, but only three BH domains. Group III consists of a large and diverse group of proteins including Bid, Bad and Bim, whose only common feature is the presence of a BH3 domain. These “BH3-only” proteins are essential initiators of apoptosis (Huang and Strasser, 2000).

The principal mechanism by which Bcl-2 family proteins regulate apoptosis is probably by controlling cytochrome c release. Many tumours have been found to express the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL. Bcl-2 and Bcl-XL are bound to the outer surface of the mitochondria where they prevent cytochrome c release (Kluck et al., 1997); (Yang et al., 1997), possibly by dimerising with pro-apoptotic members of the Bcl-2 family, resulting in their inhibition (Oltvai et al., 1993). Once the apoptotic process gets past the mitochondria, Bcl-2 and Bcl-XL have no protection effect (Moriishi et al., 1999).

1.6.5.1 Pro-apoptotic Bcl-2 proteins

Pro-apoptotic Bcl-2 members were found to localise to the cytosol or cytoskeleton prior to a death signal. Upon an apoptotic stimuli they are activated and translocate to the mitochondria, where they can dimerise with and neutralise Bcl-2 or Bcl-XL and induce cytochrome c release (reviewed in (Desagher and Martinou, 2000)). The pro-apoptotic Bcl-2 proteins are differentially activated and mediate cytochrome c release via different mechanisms. For example, cytosolic Bid is specifically cleaved by activated caspase-8, releasing a C-terminal truncated fragment (tBid), which is then targeted to the mitochondria (Li et al., 1998); (Luo et al., 1998). There it binds to Bax and triggers Bax oligomerisation and cytochrome c release (Desagher et al., 1999). Activation of Bax leads translocation to the mitochondria and a conformational change allows oligomerisation and insertion into the outer mitochondrial membrane where it can form a tetrameric pore to enable cytochrome release(Eskes et al., 2000). In its inactive state, Bad is phosphorylated, and associated to 14-3-3 proteins. Phosphorylation of Bad can be mediated by several kinases implicated in cell survival, such as Akt/PKB, p90Rsk and PAK. Upon activation, Bad gets dephosphorylated, which allows Bad to translocate to mitochondria, where it dimerises with Bcl-XL or Bcl-2, thereby antagonising their anti-apoptotic activities and inducing cytochrome c release (reviewed in (Downward, 1999)).

The mechanisms by which cytochrome c release is mediated are unclear at present. Various
mechanisms have been suggested, some of which involve swelling of the mitochondria and subsequent mechanical rupture of the outer membrane. Matrix swelling is an osmotic effect proposed to be caused by the opening of a permeability transition pore (PT) in the inner membrane (Marzo et al., 1998); (Narita et al., 1998) or by hyperpolarisation of the inner membrane (Vander Heiden et al., 1997). However in many instances of apoptosis, mitochondrial swelling is not observed (Martinou et al., 1999); (Mancini et al., 1997) and several studies have reported that PT-related changes in the mitochondrial membrane potential either fail to occur or occur only downstream of the activation of effector caspases (Yang et al., 1997); (reviewed in (Desagher and Martinou, 2000)). There is evidence that cytochrome c release is mediated by pro-apoptotic Bcl-2 family members in a process that induces permeability of the outer membrane, but leaves the inner membrane structural and functional intact (reviewed in (Ahsen et al., 2000)).

1.6.6 Cross-talk between mitochondria-dependent and -independent pathways

The death receptor pathway appears not to require the involvement of mitochondria, and Bcl-2 should have no effect on this process. However, a number of studies have demonstrated that Bcl-2 can inhibit death receptor-triggered apoptosis. There is a large body of evidence showing that cross-talk and integration between the death-receptor pathway and mitochondrial pathway often occurs (Figure 1.10) This has led to the hypothesis that two different mechanisms exist: one, in which death receptor signalling can bypass the mitochondrial pathway and apoptosis is induced directly via DISC formation, which is not blocked by Bcl-2, and a second mechanism in which death receptor signalling is blocked by Bcl-2, implying mitochondria involvement (Scaffidi et al., 1998). The mechanism linking death receptor signalling to mitochondria was shown to involve ability of caspase-8 to cleave Bid, the pro-apoptotic member of the Bcl-2 family. The truncated form of Bid (tBid) is then able to promote cytochrome c release and the formation of the apoptosome, and Bid deficient mice are resistant to Fas induced apoptosis in hepatocytes (Yin et al., 1999). Furthermore, Fas-induced apoptosis is markedly reduced in Apaf-1 null mouse embryo fibroblasts (MEFs) (Ceconi et al., 1998). Other stimuli, such as cytotoxic agents or irradiation, appear to rely on the apoptotic function of mitochondria, since Apaf-1 null and caspase-9 null MEFs are resistant to these stimuli (Ceconi et al., 1998); (Hakem et al., 1998); (Kuida et al., 1998); (Yoshida et al., 1998).
Figure 1.10 Cross-talk between the apoptotic pathways. Apoptotic stimuli elicit signals which may converge on a common apoptotic pathway, resulting in effector caspase activation. The action of Bcl-2 family members may determine the balance between cell survival and cell death.

1.6.7 IAPs

Inhibitors of apoptosis proteins (IAPs) were first discovered in baculovirus through their ability to inhibit apoptosis of insect cells upon viral infection (Uren et al., 1998); (Miller, 1999). They were found to suppress caspase activation and apoptosis induced by a variety of stimuli, including TNF, FasL, staurosporine, etoposide and growth factor withdrawal (reviewed in (Deveraux and Reed, 1999)). The human members of the IAP family, X-linked IAP (XIAP), cIAP1, cIAP2, neuronal IAP (NIAP), KIAP (Lin et al., 2000), livin (Kasof and Gomes, 2000) and survivin contain one to three BIR motifs (baculovirus IAP-repeat). Some IAP members also contain additional protein-protein interaction domains, such as a
RING and a CARD domain. IAPs appear to inhibit apoptosis through direct interactions with caspases. XIAP, which is the most potent inhibitor, can specifically bind and inhibit caspase-3 and -7 and -9 in vitro, thereby blocking the apoptotic process (Deveraux et al., 1997); (Deveraux et al., 1998). Caspase binding and inhibition is mediated by the BIR domains. The RING domain was found to function as a ubiquitin ligase, promoting the degradation of XIAP and possibly of any caspase which it is bound to (Yang et al., 2000). However, it remains to be elucidated whether IAPs normally inhibit active caspases or whether they prevent caspase activation by blocking zymogen processing. There is evidence that XIAP inhibits procaspase-3 activation by blocking the second cleavage of the caspase to remove the prodomain (Deveraux et al., 1998). A novel protein termed Smac or DIABLO was found to antagonise mammalian IAPs (Du et al., 2000); (Verhagen et al., 2000). Smac/DIABLO is released from mitochondria together with cytochrome c during apoptosis. It promotes caspase activation by binding to IAPs and preventing their inhibition of caspsaes (Ekert et al., 2001).

Furthermore, some IAPs are not restricted to caspase inhibition, but appear also to be involved in regulation of the cell cycle, since survivin has been found to be involved in cell cycle progression. In addition, cIAP1 and cIAP2 were found to associate with TRAFs in the TNFR signalling complex, but the physiological relevance of this observation is not clear.

1.6.8 Anoikis

Untransformed adherent epithelial and endothelial cells normally undergo apoptosis when detached from the extracellular matrix. In addition, fibroblasts are also sensitive to anoikis when deprived of soluble growth factors. This phenomenon is called anoikis (Frisch and Francis, 1994) and it ensures that cells displaced from their natural environment are eliminated. Anoikis is an important regulatory event during embryogenesis and normal tissue turnover. Tumour cells are resistant to anoikis and are characterised by their ability to grow in the absence of contacts with the extracellular matrix. Integrins, which are the major extracellular matrix receptors, provide the necessary survival signals. Various signalling pathways downstream of integrin engagement have been implicated in mediating anchorage dependent survival. Matrix binding to integrin receptors was found to activate focal adhesion kinase (FAK) or it can lead to activation of receptor tyrosine kinases, which are responsible for the regulation of downstream survival pathways, including the PI3K and Raf/MAPK pathway (Khwaja et al., 1997); (Le Gall et al., 2000); (Rytooma et al., 2000). Treatment of tumour cells with FAK antisense oligonucleotides also induces apoptosis (Xu et al., 1996).
while over-expression of constitutively active FAK prevents anoikis (Frisch et al., 1996). In detached MDCK cells anoikis involves induction of cytochrome c release from mitochondria and formation of the apoptosome, which is strongly reduced by a broad specificity inhibitor zVAD-fmk, suggesting that caspase activation upstream of mitochondria is required (Ryтомaa et al., 2000). Recently it was demonstrated that anoikis induced by the disruption of FAK function involves the FADD and caspase-8 apoptotic pathway, suggesting that a death receptor mediated apoptotic pathway might be activated (Xu et al., 2000). Another report showed that although FADD and caspase-8 are involved in anoikis, the ligand dependent activation of the death receptors Fas, DR4 and DR5 are not implicated (Frisch, 1999); (Rytomaa et al., 1999). Interestingly, expression of Fas is down-regulated in ras transformed mouse mammary epithelial cells (Peli et al., 1999) and anoikis does not occur in epithelial cells expressing activated ras or src oncogenes (Frisch and Ruoslahti, 1997). Furthermore, upregulation of the Fas inhibitor c-Flip by the Raf/MAPK pathway is implicated in protecting endothelial cells from anoikis (Aoudjit and Vuori, 2001). MAP kinase has been indirectly implicated in integrin mediated cell survival as integrin engagement activates MAP kinase via Shc and recruitment of Grb2/SOS (Wary et al., 1996). The β1 integrin and α4β6 integrins are able to directly activate the Raf-1/MAPK pathway via Ras (Wary et al., 1996); (Mainiero et al., 1997) and expression of a constitutively active MEK1 in fibroblasts was found to be sufficient for anchorage-independent survival (Cowley et al., 1994); (Mansour et al., 1994). Furthermore, PI3K and Akt provide survival signals downstream of activated Ras when epithelial cells are detached from the matrix, and activated forms of PI3K or Akt can rescue MDCK epithelial cells from anoikis (Khwaja et al., 1997). This indicates that both Ras downstream effector pathways, the Raf-1/MAPK and the PI3K pathways are able to prevent anoikis.

1.7 Protection from Apoptosis

Acquired resistance to apoptosis is one of the major hallmarks of cancer. Protection of cells from apoptosis can be achieved by tumour cells through a variety of strategies. Activating mutations in oncogenes such as ras and loss of function of tumour suppressors including p53 are major causes for cellular survival. The tumour suppressor p53 is mutated in about 50% of human cancers (Harris, 1996). In healthy cells p53 can trigger cell cycle arrest enabling DNA repair or elicit apoptosis by promoting the expression of pro-apoptotic proteins Bax, DR5 and
others in response to DNA damage and also in response to hypoxia and overexpression of oncopogenes. In the case of non-functional mutant p53, the induction of apoptosis is abrogated (Levine, 1997). Activation of survival pathways thereby facilitate the accumulation of more mutations that increase cellular malignancy. An important function of Ras is the direct activation of cellular survival pathway via activation of the PI3K/Akt and the Raf/MAPK pathway (Downward, 1998). Akt and Rac possibly facilitate the activation of the NF-κB transcription factor (Irani et al., 1997); (Romashkova and Makarov, 1999), which plays an anti-apoptotic role in Ras function.

1.7.1 Akt and survival

The discovery that c-Akt was the cellular homologue of the retroviral oncogene v-Akt suggested that it has a role in cellular transformation and cancer. Akt was found to be overexpressed in breast, pancreatic and ovarian carcinomas (Staal et al., 1977); (Bellacosa et al., 1991). Expression of activated forms of PI3K or Akt protects cells from apoptosis induced by ultraviolet irradiation, withdrawal of serum factors, anoikis, over-expression of Myc, treatment with anti-Fas antibody and TGFβ.

The serine/threonine kinase Akt mediates cell survival by phosphorylating and inactivating proteins involved in the apoptotic response, such as the pro-apoptotic Bcl-2 family member Bad and forkhead (FH) transcription factors (FKHR, FKHL1 and AFX), which seem to be involved in the transcription of pro-apoptotic proteins (reviewed in (Vanhaesebroeck and Alessi, 2000)). Bad phosphorylation induces its interaction with 14-3-3 proteins in the cytosol and blocks its pro-apoptotic function (Datta et al., 1997). It is thought that phosphorylation of FH transcription factors by Akt promotes their interaction with 14-3-3 proteins in the cytosol. One study showed that Akt can phosphorylate and thereby inhibit human caspase-9 when over-expressed (Cardone et al., 1998), but the physiological relevance is unclear, since the Akt phosphorylation site in caspase-9 is not conserved. Furthermore, over-expression studies with Akt suggested a role for Akt in regulating NF-κB via phosphorylation of IκB kinase (IKK) (reviewed in (Datta et al., 1999)),

1.7.2 Raf and survival

Several studies demonstrated that activation of ERKs can protect against a variety of apoptotic stimuli. An indication that Raf-1 may play a role in preventing anoikis of epithelial cells came
from two studies using an inducible Raf-1 fusion protein (Rytomaa et al., 2000); (Le Gall et al., 2000). Furthermore, the Raf/MAPK pathway was found to protect against TNFα induced apoptosis of L929 cells (Gardner and Johnson, 1996), UV induced apoptosis of human primary neutrophils (Frasch et al., 1998) and against Fas induced apoptosis in fibroblasts (Kazama and Yonehara, 2000).

It appears that inhibition of apoptosis by Raf/MAPK pathway is dependent on cell type and stimuli. In fibroblasts, the ERK pathway did not appear to be involved protection from UV-induced apoptosis (Kulik et al., 1997). In cerebellar neurons, IGF-1 also promotes survival independently of ERK activation, but dependent on PI3K (Dudek et al., 1997). It is suggested that Raf-1 might promote survival independently of the activation of the MAPK pathway. It was found that a GFP-Raf-1 fusion protein can be targeted by Bcl-2 to the mitochondria (Wang et al., 1996). Raf-1 was also shown to phosphorylate Bad via activation of the Raf/MAPK pathway, a pro-apoptotic member of the Bcl-2 family, thereby preventing its translocation to the mitochondria (Scheid et al., 1999); (Bonni et al., 1999). It has been shown that GM-CSF-induced survival from detachment induced apoptosis (anoikis) in hematopoietic cells requires Raf-MAPK activation but is PI3K-independent (Scheid and Duronio, 1998). Furthermore cell survival was also found to be mediated by Raf-1/MAPK via p90Rsk1 activation in neurons (Bonni et al., 1999). In epithelial cells, Raf-transformation led to NF-kB activation, thereby protecting cells from TGFβ induced apoptosis (Arsura et al., 2000). Whereas Akt blocks apoptosis upstream of cytochrome c release, B-Raf was found to confer survival in fibroblasts at the level of cytosolic caspase activation downstream of cytochrome c release (Erhardt et al., 1999).

In some cases, Ras promotes apoptosis via the Raf/MAPK pathway preferentially in fibroblasts or lymphocytes (Downward, 1998). For example cellular stress or TNFα treatment were found to promote apoptosis depending on Raf-1 activation. In c-Myc expressing fibroblasts, activation Ras induced Raf-1 activation or expression of a constitutively active form of Raf-1 (Raf-CAAX) promoted apoptosis in the absence of growth factors (Kauffmann-Zeh et al., 1997). This might involve Raf-1 induced expression of p53 and the induction of p53 dependent apoptosis.

1.7.3 Raf and Akt

Akt regulates many components of the apoptotic cascade and suppresses apoptosis or cell
death. In some cases, PI3K and Akt may contribute to Raf-1 activation as PI3K inhibitors and dominant negative forms of PI3K were found to reduce MAP kinase activity (Sutor et al., 1999); (Wennstrom and Downward, 1999). In contrast, over-expression of both Akt and Raf-1 resulted in phosphorylation of Raf-1 at Ser259 by Akt, leading to inhibition of the Raf-MAPK pathway (Zimmermann and Moelling, 1999). Akt does not inhibit Raf-1 in undifferentiated myoblast precursor cells but does when they are differentiated into skeletal muscle myotubes (Rommel et al., 1996). It seems that the inhibition of the Raf-MAPK pathway by Akt is cell type specific and the biological relevance is not clear at present.

1.8 TGFβ signalling

1.8.1 The TGFβ superfamily

The transforming growth factor β (TGFβ) superfamily consists of more than 30 highly related polypeptide growth factors, including TGFβ isoforms 1-3, activins and bone morphogenetic proteins (BMPs) (reviewed in (Massague, 1998)). These secreted signalling molecules are highly multifunctional and regulate an array of cellular responses, such as proliferation, differentiation, motility and apoptosis, dependent on the cellular context. They have critical roles during early embryogenesis and in maintaining tissue homeostasis during adult life. The TGFβ isoforms play an important role in immunosuppression: they enhance the function of monocytes and neutrophils, suppress proliferation of lymphocytes, regulate differentiation of a large number of immune cell lineages and antagonise the function or expression of several cytokines (reviewed in (Letterio and Roberts, 1998)). The BMPs have key roles in bone morphogenesis, gastrulation and organ development in mice and human as well as mesoderm patterning in *X.laevis*. The activins regulate the secretion of pituitary follicle stimulating hormone (FSH), erythroid cell differentiation and mesoderm induction during embryogenesis in *X.laevis* (reviewed in (Massague, 1998)).

The founding member of the family, TGFβ1, was originally identified to promote in cooperation with TGFα anchorage independent growth of normal kidney fibroblasts in soft agar (Roberts et al., 1980); (Roberts et al., 1981). Moreover, TGFβ was found to be a regulator of mesenchymal growth, but it was anti-mitogenic in epithelial cells (Moses et al., 1990). Probably the most studied TGFβ function is the ability to reversibly inhibit proliferation of many cell types, such as epithelial, endothelial, neuronal, hematopoietic and
lymphoid cells. Recent reports demonstrate that TGFβ is also involved in wound healing and tissue repair in stimulation of extracellular matrix (ECM) formation, cell migration and angiogenesis (reviewed in (O'Kane and Ferguson, 1997)). Knockout studies in mice showed that TGFβ plays many essential roles during embryonal development, which is reflected in the lethal phenotype of TGFβ ligand and TpR-II null mice (Bottinger et al., 1997). Furthermore, defects in TGFβ signalling are found in a number of human diseases including cancer, auto-immune diseases and fibrosis (Border and Noble, 1994); (Massague, 1998).

1.8.2 Mechanisms of TGFβ activation

TGFβ is synthesized as a prohormone that is cleaved in the secretory pathway into an N-terminal propeptide (latent associated protein; LAP) and a C-terminal fragment that constitutes the mature TGFβ. After secretion TGFβ remains non-covalently bound with its propeptide, retaining TGFβ in a latent form on the cell surface, which cannot be recognized by the TGFβ receptor(Gentry et al., 1987). Another component of the latent TGFβ complex is a large secretory glycoprotein termed latent TGFβ-binding protein (LTBP), which is covalently bound to LAP. LTBP is involved in storage of the latent TGFβ complex in the extracellular matrix and in its activation. The physiological multistep activation process of latent TGFβ is currently only partially understood, but it also involves binding of latent TGFβ to the cell surface via the mannose 6-phosphate receptor (Dennis and Rifkin, 1991). Activation of TGFβ was found to be mediated by the protease plasmin or by thrombospondin1 in vitro inducing conformational changes of LAP and binding of TGFβ to its receptor (Lyons et al., 1990); (Crawford et al., 1998), which also seems to be the responsible mechanism for activation of TGFβ in vivo. Other reports suggest that matrix-metalloproteinases, which are implicated in tumour invasiveness, can activate latent TGFβ (Yu and Stamenkovic, 2000).

1.8.3 TGFβ receptor signalling

The TGFβ family signals are transduced into the cell via serine/threonine kinase receptors on the cell surface (reviewed in (Massague, 1998)). These can be divided into two subfamilies: type1 receptors and typeII receptors. The receptor specific for TGFβ ligand is composed of TβRI (originally known as Alk-5) as typeI receptor and TβRII as typeII receptor, which heteromerise upon upon TGFβ binding. Two other cell-surface TGFβ-binding proteins are known, betaglycan (type-III receptor) and endoglin. They modulate cellular responses to TGFβ, but have no kinase activity. Betaglycan and endoglin may function by regulating
TGFβ access to TβRII (Wang et al., 1991); (Lastres et al., 1996). The serine/threonine kinase domains in type I and type II receptors are highly conserved (Mathews and Vale, 1991) as mammalian cell mutants defective in either TβR-I (Laiho et al., 1990) or TβR-II (Boyd and Massague, 1989) lack a wide range of TGFβ response. Activation of the receptor occurs when TGFβ ligand binds to TβRII, which results in autophosphorylation (Lawler et al., 1997). This subsequently leads to recruitment of TβRI, causing the formation of a heteromeric receptor complex. TβRII mediates phosphorylation of TβRI on serine and threonine residues in the highly conserved GS domain which is required for controlling the activity of the preceding catalytic domain (Wieser et al., 1995); (Wrana et al., 1994). A Leu-Pro motif, following the GS domain, can function as a binding site for the immunophilin FKBP12, which may act as a negative regulator of receptor fuction (Charg et al., 1996). Following ligand binding, the heteromeric receptor complex phosphorylates and thus activates intracellular effectors, known as Smad proteins, which to date are the only direct receptor substrates initiating intracellular signalling (Macias-Silva et al., 1996); (Kretzschmar and Massague, 1998).

1.8.4 TGFβ downstream effectors: the Smad proteins

The name Smad is a fusion of two gene names, D.melanogaster mothers against dpp (Mad) (Sekelsky et al., 1995) and C.elegans Sma (Savage et al., 1996), which were the founding members of the Smad family. Multiple studies in D.melanogaster, X.laevis, C.elegans and mammals have defined three major classes of Smads, based on sequence homology: the receptor regulated Smads, (R-Smads), the common Smads (Co-Smads) and the inhibitory Smads (I-Smads) (Figure 1.11) (reviewed in (Massague and Wotton, 2000)).

R-Smads are critical for specifying the biological response, as Smad2 and Smad3 are activated by TGFβ and activin receptors, while Smad1, 5 and 8 are activated by BMP receptors. Once activated, the R-Smads form heteromeric complexes with the second class of Smad proteins, the common mediators or Co-Smads. In mammals a single Co-Smad, Smad4 has been identified, while in X.laevis the Co-Smads include Smad4 and Smad4β (also known as Smad10) (Howell et al., 1999). In addition to these positively acting Smads, the third class of Smads, the inhibitory Smads (I-Smads: Smad6 and Smad7) antagonise
Figure 1.11. The Smad family. Phylogenetic tree of Smads. All vertebrate Smads are human, except for Smad4p, which is X.laevis.

signalling by either targeting the TGFβ receptor complex or possibly by interfering with the R-Smad/Smad4 complex.

The TGFβ/Smad pathway has been conserved through evolution in different species including D.melanogaster, C.elegans and X.laevis. A large body of work exists describing the functions of the Smad proteins as transducers of TGFβ signals into the nucleus, where they control gene expression (reviewed in (Heldin et al., 1997)). Several groups have generated mice that are deficient in Smads. Mice that are deficient for Smad2 and Smad4 are embryonic lethal, indicating critical, non-redundant roles in early embryonic development. Smad4 is required for gastrulation and anterior development of the mouse embryo (Sirard et al., 1998). Smad 2 null embryos fail to induce mesodermal differentiation (Weinstein et al., 1998); (Nomura and Li, 1998) or entirely lack tissues of the embryonic germ layer (Waldrip et al., 1998). Smad3 targeted mice are viable and indicate a role for Smad3 as a major mediator of TGFβ induced growth inhibition (see section 1.8.11).

1.8.5 Activation of Smad proteins

In the absence of a TGFβ signal, it has been suggested that Smad2, Smad3 and Smad4 are
retained in the cytoplasm by binding to microtubules, whereas TGFβ stimulation induces dissociation, allowing translocation of the Smads to the receptor (Figure 1.12) (Dong et al., 2000). Activation of the TβRII/TβRI receptor complex leads to recruitment of Smad2 and Smad3, which is mediated by a protein termed SARA (Smad anchor for receptor activation) (reviewed in (Wrana, 2000)). SARA is a membrane associated protein linking unphosphorylated Smad2 and Smad3 directly to the activated TβRI which phosphorylates these Smads on two Serine residues in a SS[V/M]S motif at their extreme C-terminus.

**Figure 1.12. Model for the regulation of Smad signalling.** K, kinase domain; P, phosphorylation. (See text for details).

The phosphorylated and thus activated R-Smads can form homo-oligomers as well as hetero-oligomers with each other as well as with Smad4. Binding to Smad4 leads to nuclear translocation of the heterotrimeric R-Smad/Smad4 complexes (Kawabata et al., 1998), although R-Smads are able to translocate into the nucleus in the absence of Smad4. This
seems possible, since Smad2 and Smad3 contain nuclear localisation signals (NLS) (Xiao et al., 2000). In addition, Smad4 contains a constitutively active nuclear localisation signal (NLS) beside a nuclear export signal, which allows Smad4 to shuttle continuously between the cytoplasm and the nucleus (Pierreux et al., 2000).

1.8.6 Structure of Smad proteins

The R-Smads and Co-Smads share two highly conserved domains, termed Mad-homology (MH) domains: MH1 at the N-terminus and MH2 at the C-terminus. These two domains are connected by a less well-conserved proline rich linker sequence which differs substantially between the different Smad classes (Figure 1.13) (reviewed in (Massague, 1998)).

![Figure 1.13. Smad domains and their functions.](image)

Receptor regulated Smads interact with activated type I receptors via the MH2 domain and become activated by receptor mediated phosphorylation of two serine residues (*) within the C-terminal SSXS motif. The principal interactions of the Smad domains are listed.

The MH1 and MH2 domains lack intrinsic enzymatic activity and function by controlling protein-protein or protein-DNA interactions. It has also been found that MH1 and MH2 bind to each other and this autoinhibition keeps the R-Smads in an inactive state. The MH1 domain
is responsible for the interaction with transcription factors, such as ATF2 and c-Jun. The MH1 domains of Smad3 and Smad4 are responsible for DNA binding (Shi et al., 1998) whereas Smad2 is unable to bind directly to DNA, since its MH1 domain contains an insert derived from an extra exon (exon3) in the DNA binding region. If this insert is removed, Smad2 acquires DNA binding properties similar to Smad3 (Dennler et al., 1999).

The MH2 domain of Smads is important for their homo- and heteromerisation and for transcriptional activation, but it does not bind DNA. The MH2 domain of R-Smads contains their receptor phosphorylation sites (SSxS) at the very C-terminus (Macias-Silva et al., 1996). TβRI and other proteins, such as transcription factors, interact with R-Smads via its MH2 domain.

Whereas the MH1 and MH2 domains are functionally well characterized, little is known about the role of the linker region. The linker of Smad1 contains four ERK-MAPK consensus phosphorylation motifs (PXS/TP), whereas Smad2 harbors one PXS/TP motif and three serine/proline motifs, which may serve as phosphorylation sites for proline-directed kinases, such as JNK and ERK-MAPK. The linker region of Smad3 contains two PXS/TP motifs and two serine/proline motifs, which might enable regulation of R-Smads by kinases.

1.8.7 Cross-talk between Smad signalling and other kinase-induced signalling pathways

Smad activation might not be restricted to the activation of TGFβ receptors, as recent studies revealed that additional kinase signalling pathways can regulate Smads. It has been shown that activation of the ERK-MAPK pathway by HGF/SF and EGF can phosphorylate four ERK-MAPK consensus phosphorylation sites PX[S/T]P in the linker regions of BMP activated Smad1 and Smad5, which results in inhibition of their nuclear translocation (Kretzschmar et al., 1997). It has been suggested that ERK-MAPK activated by expression of oncogenic Ras in mammary epithelial cells (Ep2Ras) can phosphorylate activated Smad2 and Smad3 in their linker region, which seemed to only partially block their nuclear translocation. This might be due to a different combination of PX[S/T]P and PXS/TP phosphorylation motifs (Kretzschmar et al., 1999). However, in vivo studies have revealed that Ras signalling strongly co-operates with Smads during mesoderm induction in X.laevis, which is dependent on MAPK activation (Cornell and Kimelman, 1994); (LaBonne and Whitman, 1994). During epithelial mesenchymal transition (EMT) in Ep2Ras cells some
TGFβ response is retained which is required for promoting EMT and invasive growth in vivo (Oft et al., 1996); (Oft et al., 1998), indicating that complete blocking of Smad2 and Smad3 translocation would not be consistent with in vivo systems.

It has been suggested that expression of MAPK kinase kinase-1 (MEKK-1) leads to phosphorylation of Smad2, thereby enhancing formation of Smad2/Smad4 complexes (Brown et al., 1999), but the physiological relevance is unclear. Expression of dominant-negative forms of MEKK-1 and JNK1 inhibits TGFβ induced reporter gene expression, which might indicate a role for this pathway in TGFβ signalling (Atfi et al., 1997); (Hocevar et al., 1999), but whether it involves Smad inhibition is not clear at present.

1.8.8 Smad mediated transcription

Smad3/Smad4 complexes have been shown to act as transcription factors through their ability to directly bind DNA and to induce transcription through co-operation with other transcription factors (Figure 1.13) (reviewed in (Derynck et al., 1998). In response to TGFβ the Smad3/Smad4 complex associates with specific DNA Smad binding elements (SBE) containing 5'-AGAC-3' sequences (Yingling et al., 1997); (Dennler et al., 1998). Since SBE sites are found in many promotors, of which some are not even responsive to TGFβ family members, it is difficult to clearly determine Smad target genes. Smad binding sites other than SBE elements have been identified in the goosecoid promotor in X.laevis, in which Smad3 and Smad4 bind to GC rich sequences, implying that the Smad-DNA interaction sites might be more variable in sequence (Labbe et al., 1998). In addition, Smad binding sites in the promotors for many TGFβ-responsive genes have not been identified so far.

So far only few Smad3/Smad4 binding sites have been identified in the promotors of TGFβ responsive genes, such as the plasminogen activator inhibitor (PAI-1) (Dennler et al., 1998); (Song et al., 1998); (Stroschein et al., 1999), typeI collagen (Chen et al., 1999), c-jun (Wong et al., 1999) and junB (Jonk et al., 1998). Studies in Smad3 null fibroblasts have shown that Smad3 seems to be required for the expression of c-jun and PAI-1 (Wong et al., 1999); (Datto et al., 1999). Furthermore, TGFβ can induce transcription of the cell cycle inhibitors p21Cip1 and p15INK4b in a Smad3 dependent manner (Datto et al., 1995); (Moustakas and Kardassis, 1998); (Seoane et al., 2001).

Since the interaction of Smads with DNA is of low affinity, other DNA-binding factors seem to be required for efficient transcription of target genes. This is supported by the finding that
SBE sites of some promotores are located in close proximity to binding sites for other transcription factors. One example is the PAI-1 promoter, which also contains AP-1 and ATF-2 recognition sequences and mutation of each of these sites impairs TGFβ-dependent activation (Hua et al., 1999); (Sano et al., 1999).

Furthermore, it has been shown that Smad3 can interact directly with transcription factors such as AP-1 family members c-Fos, c-Jun (Zhang et al., 1998); (Liberati et al., 1999) and the ATF/CREB family member ATF2 (Sano et al., 1999). In contrast to Smad3, Smad2 does not bind to DNA and therefore requires the binding to transcription factors. It has been shown that Smad2/Smad4 complexes bind to transcription factors of the FAST (forkhead activin signal transducer) family implied in regulating gene transcription during *X.laevis* development (Chen et al., 1997); (Germain et al., 2000). Smad2 and Smad3 have also been found to bind to co-activators such as CBP/p300 (Feng et al., 1998); (Janknecht et al., 1998) but also to co-repressors, such as TGIF (Wotton et al., 1999) and Ski (Luo et al., 1999), indicating that Smads are involved in complex regulatory mechanisms of gene expression. Interactions between transcription factors and co-activators or repressors may allow a higher degree of promoter specificity than would be provided by a single responsive element and a single transcription complex alone.

1.8.9 Switching off the TGFβ signalling pathway

A variety of negative control mechanisms are known to regulate Smad activity, which abrogates TGFβ signal transduction. Interestingly, the inhibitory Smad, Smad7, is transcriptionally upregulated by TGFβ, thus mediating a negative feedback loop to block TGFβ responsive gene expression. Smad7 inhibits phosphorylation of R-Smads by acting as pseudo-substrate for the type-I receptors in response to TGFβ and might also induce its degradation (reviewed in (Heldin et al., 1997)). Expression of Smad7 was also found to be induced by EGF stimulation (Afrahtke et al., 1998), by interferon (IFN) γ and TNFα (Bitzer et al., 2000), but how these factors influence TGFβ-induced transcription of physiological relevant target genes is not clear at present.

Inactivation of Smads can also occur in the nucleus. One mechanism suggested might involve ubiquitin-dependent proteasomal degradation of Smads, since proteasome inhibitors were found to stabilize phosphorylated Smad2 in the nucleus (Lo and Massague, 1999). There is evidence for a recycling mechanism for Smad2 and Smad3, since these Smads contain
nuclear export signals (NES). This enables the dephosphorylated Smads to shuttle back into
the cytoplasm (Pierreux et al., 2000). So far, nothing has been described about the role of
phosphatases to dephosphorylate and therefore abrogate the activity of Smads.

1.8.10 Alternative TGFβ downstream effector pathways

A number of reports have shown that TGFβ signalling can activate different MAPK
pathways, including the SAPK/JNK pathway, the p38 pathway or the ERK-MAPK
pathway, which seems to be dependent on the experimental conditions and the cell line used
(reviewed in (Massague and Chen, 2000)). In a fibrosarcoma cell line TGFβ was found to
rapidly activate JNK via MKK-4, inducing fibronectin expression in a Smad4-independent
way (Hocevar et al., 1999). Another report showed that TGFβ rapidly activates JNK in
epithelial cells, thereby mediating phosphorylation and nuclear translocation of Smad3 (Engel
et al., 1999). Activation of p38 through MKK-3 was observed in response to TGFβ in lung
and kidney epithelial cell lines (Hanafusa et al., 1999); (Sano et al., 1999). The MAPK
kinase kinase family member TAK (TGFβ activated kinase) has been shown to be activated
by TGFβ (Yamaguchi et al., 1995). This can mediate activation of either JNK or p38
(Shibuya et al., 1996), which are both implied in promoting apoptosis at least in response to
cellular stress signals. TAK was also found to mediate transcriptional activation of the TGFβ
responsive PAI-1 reporter, containing three AP-1 sites and transcription is enhanced by
expression of the TAK-activator TAB1 (Shibuya et al., 1996). A downstream
phosphorylation target of TAK1 is the kinase SEK1, which is involved in the SAPK/JNK
pathway, leading to activation of c-Jun (Yamaguchi et al., 1995); (Shirakabe et al., 1997).
TAK1 was also found to function as a negative regulator of the Wnt signalling pathway,
which can regulate proliferation by β-catenin/TCF mediated transcription (Ishitani et al.,
1999). Therefore, it might be speculated that TAK could play a role in the regulation of
inhibitory TGFβ effects, but evidence is still missing. It has been suggested that activated
MEKK-1, an activator of the stress activated JNK, can mediate Smad2 phosphorylation and
enhanced formation of Smads/Smad4 complexes (Brown et al., 1999), but whether this
observation is physiologically relevant is not clear.

1.8.11 TGFβ as a negative regulator of cell cycle

Induction of growth arrest by TGFβ occurs via interference with cell cycle regulators,
arresting the cell cycle in middle to late G1 (Moses et al., 1990). Particularly sensitive to
TGFβ are cultures of epithelial and endothelial cells. Several mechanisms for TGFβ mediated cell cycle arrest have been postulated, depending on the cell type. TGFβ inhibits the proliferation of epithelial cells by inducing expression of the CDK inhibitors p15INK4b and p21Cip1 (Hannon and Beach, 1994); (Reynisdottir et al., 1995). Expression of p15INK4b releases p27KIP1 from cyclinD/CDK4/6 complexes, enabling p27KIP1 to bind to CDK2 and inhibit CDK2/cyclinE activity. This leads to inhibition of pRb phosphorylation and G1-arrest (Polyak et al., 1994); (Reynisdottir and Massague, 1997). TGFβ also suppresses the expression of the c-myc protooncogene in epithelial cells (Alexandrow et al., 1995); (Pietenpol et al., 1990); (Claassen and Hann, 2000). Moreover, TGFβ can induce cell cycle arrest by suppressing the transcription of the positive cell cycle regulatory genes such as the phosphatase Cdc25A (Iavarone and Massague, 1997), cyclinD1 (Ko et al., 1995), cyclinA (Feng et al., 1995) and CDK4/6 (Ewen et al., 1993). TGFβ has also been shown to inhibit the activity of the cyclin-dependent-kinase Cdc2 (Howe et al., 1991) and CDK2 (Koff et al., 1991). In response to TGFβ, the zinc finger transcription factor TIEG (TGFβ-inducible early-response gene) can be activated, leading to inhibition of epithelial cell proliferation (Cook et al., 1998). Alternatively, TGFβ can induce G1 arrest in epithelial cells via inhibition of p70S6kinase independently of Smads. This occurs by association of the TGFβ receptor with the protein phosphatase-2A (PP2A)-alpha which subsequently binds to and phosphorylates p70S6kinase resulting in its inactivation (Petritsch et al., 2000). An example of TGFβ-mediated inhibition of proliferation in vivo was shown by Silberstein and co-workers: Implantation of slow-release polymers impregnated with TGFβ1 in mouse mammary glands inhibited branching morphogenesis by stopping invasion of ductal end buds (Silberstein and Daniel, 1987); (Silberstein et al., 1992). The requirement of Smad4 for TGFβ mediated growth inhibition has been shown in colon carcinoma cells, in which a targeted disruption of Smad4 renders these cells insensitive to TGFβ induced growth inhibition (Zhou et al., 1998). Knockout studies showed that Smad3 is a major mediator of TGFβ-induced growth inhibition. Smad3 targeted mice have been reported independently by three groups. These mice do not display developmental defects: they are viable and survive into adulthood (Zhu et al., 1998); (Datto et al., 1999); (Yang et al., 1999). One report shows that Smad3 deficient mice spontaneously form colorectal adenocarcinomas which are highly invasive, indicating that the inactivation of the TGFβ signalling leads to maintenance of the proliferative state of some epithelial cells (Zhu et al., 1998). Smad3 null MEFs, derived from different Smad3 knockout mice, have lost the response to TGFβ-mediated growth inhibition.
Smad3 null mice generated by Yang et al. showed increased wound healing due to a higher rate of re-epithelialisation, which was a result of the loss of TGFβ mediated cell cycle arrest (Ashcroft et al., 1999). The discrepancy in the phenotypes of the Smad3 deficient mice is not clear at present but it might be suggested that differences in the genetic background of the Smad3 null animals could cause the different phenotypes.

1.8.12 TGFβ as a positive regulator of proliferation

The effects of TGFβ on the growth of mesenchymal cells are bimodal. In fibroblasts and smooth muscle cells TGFβ is growth inhibitory under high mitogen concentration, whereas under low mitogen concentrations TGFβ is growth stimulating. This occurs by induction of an autocrine platelet-derived growth factor (PDGF) signalling loop: the expression of both PDGF ligand as well as PDGF receptor are induced by TGFβ (Leof et al., 1986); (Battegay et al., 1990). In addition, TGFβ can mediate mitogenesis and stromal connective tissue formation by inducing the expression of fibroblast growth factor-2 (FGF-2) (Pertovaara et al., 1993), vascular endothelial growth factor (VEGF) (Pertovaara et al., 1994), and the PDGF-related protein connective tissue growth factor (CTGF) (Igarashi et al., 1993).

1.8.13 TGFβ and apoptosis

Beside the regulation of cell cycle, TGFβ is also a potent inducer of apoptosis in a variety of cell types, including B lymphocytes, Burkitt’s Lymphoma (BL) cell lines, hepatocytes and hepatoma cells and epithelial cell lines (Inman and Allday, 2000). TGFβ-dependent apoptosis is essential for the elimination self reactive B-lymphocytes during development for maintenance of immune-tolerance (Lebman and Edmiston, 1999) and myeloid cell development is controlled by TGFβ induced apoptosis (Selvakumaran et al., 1993).

The mechanism by which TGFβ mediates apoptosis are largely unclear. TGFβ leads to activation of the caspase cascade in human hepatocytes and prostate cancer cell lines and treatment with the broad spectrum caspase inhibitor zVAD-fmk blocked TGFβ induced apoptosis (Chen and Chang, 1997); (Inayat-Hussain et al., 1997); (Choi et al., 1998). Activation of caspase-3 by TGFβ was found in human B-lymphocytes and lymphoma cells (Wang et al., 1996); (Saltzman et al., 1998). Furthermore, TGFβ leads to activation of the initiator caspase-8 in lymphoma cells (Inman and Allday, 2000) and hepatoma cells (Shima et al., 1999). The latter study showed that during TGFβ-induced apoptosis down-regulation of
XIAP and Bcl-X<sub>L</sub> expression can occur and apoptosis can be prevented by EGF (Shima et al., 1999). The involvement of cytochrome c release from the mitochondria and apoptosome formation in response to TGFβ have been described recently (Freathy et al., 2000). TGFβ induced apoptosis but not growth arrest was blocked by IL-6 in a PI3K and Akt-dependent way in hepatoma cells (Chen et al., 1998). Similar results were obtained by Brown and co-workers in B-lymphoma cells, where apoptosis was blocked by the caspase inhibitor BD-fmk, but TGFβ induced growth arrest was not affected (Brown et al., 1998). TGFβ might induce apoptosis via activation of the transcription factor TIEG1 leading to caspase3 activation (Ribeiro et al., 1999). Recently, an apoptosis related protein in the TGFβ signalling pathway, termed ARTS, was found to enhance TGFβ induced apoptosis and activate caspase-3 in prostatic epithelial cells. ARTS is located at the mitochondria and translocated to the nucleus during apoptosis (Larisch et al., 1998; Larisch-Bloch et al., 2000), but the mechanism by which it might promote apoptosis is not clear at present.

Down-regulation of anti-apoptotic Bcl-2 family members during TGFβ induced apoptosis was also observed in a lymphoma cell line (Saltzman et al., 1998) and ectopic expression of Bcl-2 in hepatoma cells (Choi et al., 1998) or hepatocytes (Huang and Chou, 1998) prevented TGFβ induced apoptosis. It seems that c-Myc down-regulation is important for TGFβ to mediate apoptosis, since expression of <i>c-myc</i> in a premature B cell line is able to block apoptosis (Arsura et al., 1996). Another study showed that TGFβ induces Bax and p53 expression and suppressed activation of the NF-κB survival pathway in epithelial RLE cells (Teramoto et al., 1998). NF-κB activity was also found to be repressed in hepatocytes by TGFβ, but expression of NF-κB component RelA promotes survival (Arsura et al., 1997).

Although Smad proteins are key effectors in TGFβ dependent growth inhibition, their role in the induction of apoptosis is still unclear. During TGFβ induced apoptosis in human prostatic carcinoma cells, Smad7 expression was increased, thereby blocking Smad2/Smad4 or Smad3/Smad4 complexes (Landstrom et al., 2000). Recent data indicate that Smad proteins and the AP-1 transcription factor complex can mediate TGFβ induced apoptosis in Hep3 cells: this was blocked by expression of Smad7, dominant negative Smad3 and dominant negative FosB (Yamamura et al., 2000). Similar findings were obtained by Patil and co-workers, showing that dominant negative Smad2 or Smad3 as well as expression of Smad7 prevented apoptosis and growth inhibition in a premature B cell line. The TGFβ superfamily member BMP2 was found to induce apoptosis in mouse hybridoma cells via activation of 55
TAK1, a MAPKKK, which results in activation of the p38 stress activated MAPK. This was prevented by expression of inhibitory Smad6 (Kimura et al., 2000). This indicated that the role for Smad2, Smad3 and Smad4 in promoting apoptosis is not clear to date, since the inhibitory Smads might be able to interact directly with the TGFβ receptor, thereby blocking all downstream signalling pathways.

1.8.14 TGFβ as a mediator of synthesis and degradation of the extracellular matrix

TGFβ has a major role in the regulation of extracellular matrix (ECM) synthesis, degradation and remodelling: processes that are important during development and tumour invasion. TGFβ stimulates the synthesis of multiple ECM components, including elastin (Marigo et al., 1994), collagens (Ritzenthaler et al., 1993) and plasminogen activator inhibitor (PAI-1) (Dennler et al., 1998); (Song et al., 1998), whose responsive regions in the promoters contain Sp1 sites or CTF/NF-1 sites. Furthermore, TGFβ was found to affect the alternative splice pattern of fibronectin (Borsì et al., 1990) and the glycosylation pattern of proteoglycans (Bassols and Massague, 1988). TGFβ stimulates the secretion of "embryonic" or early matrix proteins such as tenascin, thrombospondin-1 and fibronectin, whereas its effects on the expression levels of the proteins present in mature connective tissue, such as elastin and collagenI are more modest, implying a role for TGFβ in ECM remodelling. Regulation of invasion and proteolytic activity by TGFβ can be altered in cancer cells. For example in a lung adenocarcinoma cell line, TGFβ enhances the expression of uPa (Keski-Oja et al., 1988) and its receptor (Lund et al., 1991), leading to an increase in plasminogen activation. Cleavage of plasminogen to plasmin can mediate release of TGFβ from its latent form (Taipale et al., 1994), leading to a positive TGFβ feedback loop. In addition, thrombospondin1 seems to mediate a positive feedback loop, since it can activate TGFβ but is also transcriptionally regulated (Schultz-Cherry and Murphy-Ullrich, 1993); (Ruoslahti, 1999). Depending on the cellular environment TGFβ may thus act as feedback regulator for remodelling of the extracellular matrix.

1.8.15 TGFβ and cancer

TGFβ can have positive or negative effects on tumourigenesis, depending on the differentiation state of the tumour cells. During early tumourigenesis, it can act as a tumour suppressor (Cui et al., 1996); (Glick et al., 1996); (Pierce et al., 1995), but at later stages it can act as a stimulator of tumour progression leading to invasiveness and metastasis,
which is characterised by loss of responsiveness to anti-proliferative TGFβ effects (reviewed in (Akhurst and Balmain, 1999)). In a number of human tumours, TGFβ is overexpressed (Derynck et al., 1985); (Krieg et al., 1991) and it has also been found to be produced in the tumour stroma and micro-environment (Sieweke and Bissell, 1994), especially in the case of bone metastasis (Guise and Mundy, 1998). Since TGFβ can promote angiogenesis (Roberts et al., 1986), wound healing (Ashcroft and Roberts, 2000) and immunosuppression (Torre-Amione et al., 1990), at least some of its positive effects on tumour progression may be through action on cells other than those of the tumour itself.

The tumour suppressing functions of TGFβ during early tumour stages have been demonstrated in vivo in a mouse skin model of chemical carcinogenesis and treatment with DMBA induced activating H-Ras mutations initiating formation of benign papillomas. Keratinocyte targeted expression of dominant negative TGFβ receptor type-II (TβRII) increased the incidence of tumours, whereas mice expressing TGFβ1 had much reduced papilloma formation (Cui et al., 1996). In many cases tumour cells remain growth responsive to TGFβ relatively late in tumourigenesis and loss of growth responsiveness correlates with malignant tumour progression (Haddow et al., 1991); (Hebert and Birnbaum, 1989).

Some human cancer types including squamous cell carcinomas (Shipley et al., 1986); (Reiss et al., 1993), and retinoblastoma cells (Kimchi et al., 1988) are refractory to TGFβ induced growth arrest. As TGFβ is also growth inhibitory in endothelial and haematopoietic cells, loss of sensitivity to TGFβ can also promote tumourigenesis in these cell types. Advanced tumour cells, such as sarcoma cells and carcinoma cells which have lost the growth inhibitory response to TGFβ, become more invasive and tumourigenic after TGFβ1 transgene expression (Samuel et al., 1992); (Chang et al., 1993); (Cui et al., 1996).

Multiple mechanisms can account for the loss of responsiveness to TGFβ. These include down-regulation of the TGFβ receptor expression (Zhao and Buick, 1995), mutation of the TβRI or TβRII and mutations in Smad4 or Smad2. Overall, the frequency of TGFβ receptor mutation in human tumours is quite low. TβRII is mutated in human colon cancer cell lines from families with genetic defects in DNA repair (RER) (Markowitz et al., 1995) gastric cancer from families with RER (Shinmura et al., 1998) and in cervical cancer (Chen et al., 1999) and in colon carcinoma (Grady et al., 1999), and TβRI in some T cell lymphomas (Schiemann et al., 1999). The other major mechanism for inactivation of TGFβ signalling in tumours is through loss of function of Smad4 (Schutte, 1999). Smad4, which was identified as a tumour suppressor protein (Hahn et al., 1996) is inactivated in about 30% of
pancreatic carcinomas. In colon carcinomas, inactivating mutations in Smad4 and Smad2 are also found in colon carcinomas (reviewed in (Riggins et al., 1997)). Mutations in Smad3, however, have not been found in human cancers so far. Mutations in TGFβ signalling components seem to allow tumour cells to evade TGFβ-induced growth arrest, but some TGFβ responsiveness seems to be retained, since studies in mouse models demonstrated that TGFβ signalling is still required for promoting invasiveness and metastasis. There is evidence that for example tumour cells carrying mutations in Smad4 are able to partially respond to TGFβ (Hocevar et al., 1999). This might be due to signalling through Smad-independent pathways. Alternatively, other functional Smad4 homologues might exist, which has been described for X.laevis (Howell et al., 1999); (Newman and Krieg, 1999). However, most advanced tumours do not show mutations of the receptor or Smads, leaving the integrity of the TGFβ signalling pathway intact (reviewed in (Akhurst and Balmain, 1999)).

Insensitivity towards TGFβ growth inhibition can be caused by expression of oncogene products. Expression of H-ras oncogene induces TGFβ resistance in a variety of epithelial cell lines (Houck et al., 1989); (Kerr et al., 1991); (Fiml us et al., 1992). Strong activation of Ras can down-regulate the expression of TβRII leading to reduced TGFβ sensitivity (Zhao and Buick, 1995). Many studies showed that variations in TβRII, rather than TβRI, determine the growth responsiveness to TGFβ in vivo and in vitro (Cui et al., 1995); (Geiser et al., 1992); (Portella et al., 1998)c. TGFβ can also promote an epithelial-to-mesenchymal transition (EMT) in tumour cells which express activating mutations of H-Ras oncogene. This dedifferentiation process leads to the progression from squamous cell to spindle cell carcinoma in a mouse model of skin carcinogenesis (Miettinen et al., 1994); (Caulin et al., 1995) (reviewed in (Akhurst and Balmain, 1999)). Furthermore, mink lung epithelial cell lines transformed by the c-myc oncogene (Kerr et al., 1991) or expressing mutant tumour suppressor p53 (Ewen et al., 1995) are refractory to TGFβ growth inhibition. Furthermore, loss tumour suppressors such as p16INK4a have been observed to contribute to the lack of insensitivity to anti-proliferative TGFβ effects in spindle cell carcinomas in vivo (Linardopoulos et al., 1995). Since the CDK inhibitor p15INK4B is known to be induced by TGFβ in epithelial cells (Hannon and Beach, 1994), loss of the INK4 locus would prevent this growth-inhibitory mechanism. In addition, cells transformed by viral proteins from tumour viruses, including SV40 largeT antigen and the adenoviral protein E1A, lose sensitivity to TGFβ-mediated growth inhibition (Pietenpol et al., 1990); (Laiho et al., 1991);
(Kumar et al., 1995). These viral products are able to inactivate essential cell cycle regulators, such as members of the pRb family.

It seems, therefore, that loss of sensitivity to anti-proliferative TGFβ effects allows TGFβ to promote its pro-malignant features, promoting cell invasiveness and metastasis.

1.9 The epithelial phenotype

1.9.1 Molecular architecture of the epithelial phenotype

The epithelium is one of the principal tissue types of the body, characterized by an apical-basal polarity of the cell. The apical surface, facing the external environment (growth medium), is often rich in microvilli or cilia, whereas the basal surface is attached to the basement membrane, where transmembrane adhesion receptors (integrins) interact with components of the extracellular matrix (ECM), including collagen, laminin, vitronectin and fibronectin (reviewed in (Gumbiner, 1992)). The lateral surfaces adhere to those of neighbouring cells via specialised junctions, including tight junctions, adherens junctions, desmosomes and gap junctions (reviewed (Gumbiner, 1996)). There is a large body of evidence that cell adhesion proteins not only function to form the stable cellular structure, but are also part of dynamic signalling events transducing signals into the cell and also responding to internal signals. Therefore proper formation of intercellular junctions and interactions with the ECM are critical for the maintenance of the epithelial phenotype. For example, the destabilisation of junctions allows invasiveness of epithelial cells and the progression of carcinomas (reviewed in (Birchmeier and Behrens, 1994)).

1.9.2 Cell-Cell adhesion

1.9.2.1 Tight junctions

Tight junctions regulate paracellular diffusion across epithelial monolayers and the maintenance of the assymetric distribution of proteins and lipids to the apical and basolateral plasma membrane domain of epithelial cells (reviewed in (Mitic and Anderson, 1998)). The backbone of tight junctions is composed of transmembrane proteins, such as occludin. At the inner membrane surface, occludin is associated with zonula occludens (ZO) proteins ZO-1, ZO-2 and ZO-3, which are members of the membrane-associated guanylate kinase
(MAGUK) protein family. They link occludin to the actin cytoskeleton of the cell or to intracellular signalling proteins (Tsukita et al., 1999). These include some PKC isoforms and the Ras effector AF-6, which binds to ZO-1 (Yamamoto et al., 1997). Disruption of tight junctions has been associated with oncogenic activation. For example, the expression of oncogenic Raf-1 can reduce occludin expression, thereby disrupting tight junctions and ZO-1 distribution, while over-expression of occludin in these Raf-1 activated cells prevented anchorage-independent growth (Li and Mrsny, 2000). Furthermore, inhibition of the MAP kinase signalling pathway in Ras transformed cells was able to restore the formation of tight junctions (Chen et al., 2000).

1.9.2.2 Adherens junctions

Adherens junctions are located below tight junctions on the lateral membrane of epithelial cells. Intercellular contacts are formed by members of a family of calcium-dependent adhesion molecules, called cadherins. These are important for cell recognition and cell targeting during development (Takeichi, 1991). Several tissue specific cadherins have been identified, including epithelial (E)-cadherin, neuronal (N)-cadherin and vascular endothelial (VE)-cadherin. Cadherins are homophilic transmembrane glycoproteins (reviewed in (Steinberg and McNutt, 1999)) and the extracellular domain is composed of five repeating regions (C1-C5) that interact with the extracellular domains of cadherins in adjacent cells. The cytoplasmic domain can bind to plaque proteins β-catenin or γ-catenin (plakoglobin), which are members of the Armadillo family of proteins (Hulsken et al., 1994). Another catenin, termed α-catenin, interacts with β-catenin or γ-catenin and links the cadherin complex to the actin cytoskeleton (Rimm et al., 1995) either directly or indirectly via actin binding proteins such as α-actinin, vinculin and ZO-1 (reviewed in (Steinberg and McNutt, 1999)).

It seems that β-catenin and γ-catenin may be involved in the regulation of cell-cell adhesion, since they can be phosphorylated at tyrosine residues, which results in their dissociation from the cadherin/catenin complex (reviewed in (Provost and Rimm, 1999)). This can occur directly by activation of the Src kinase, but also indirectly in response to growth factors including EGF, TGFβ, PDGF and HGF/SF (reviewed in (Daniel and Reynolds, 1997)) and thus contribute to invasive properties of tumour cells (Behrens et al., 1993). This seems to be regulated by activation of Ras, which induces the destabilisation of E-cadherin/ β-catenin complexes in MDCK cells by mechanisms involving both the MAP kinase pathway and the PI3K pathway (Potempa and Ridley, 1998). Members of the Rho family of GTPases, which
regulate the actin cytoskeleton, also modulate cadherin mediated cell adhesion. For example, microinjection of dominant negative Rac1 or treatment with the Rho inhibitor botulinum toxin C3 reduces the accumulation of E-cadherin at cell-cell contacts in keratinocytes (Braga et al., 1997) and in MDCK cells (Takaishi et al., 1997).

In addition to its role in E-cadherin based cell-adhesion, β-catenin has been identified as a direct signalling component of the wnt/wingless (wnt/wg) signalling cascade (reviewed in (Willert and Nusse, 1998)). This pathway is highly conserved from D.melanogaster to mammals and plays a central role during embryonal development but also during tumourigenesis. In the absence of wnt/wg signalling, cytosolic β-catenin was found in complex with the tumour suppressor APC (adenomatous poliposis coli), GSK3β and axin, or the related protein conductin. In this complex, GSK3β phosphorylates β-catenin (Easwaran et al., 1999) (Yosh 1996), which targets it for ubiquitination and subsequent proteosomal degradation.

Upon wnt/wg signalling GSK3β is inhibited, which leads to stabilisation and accumulation of β-catenin in the cytoplasm, enabling β-catenin to translocate to the nucleus where it forms a complex with transcription factors of the LEF-1/TCF family and regulates transcription of several target genes (reviewed in (Behrens, 1999)). Interestingly, the signalling function of β-catenin is activated in many human tumours increasing β-catenin mediated transcription (Barth et al., 1997); (Gumbiner, 1997). This can occur due to mutations in the APC tumour suppressor, which is observed in about 80% of familial adenomatous polyposis (FAP) colon cancer (Kinzler and Vogelstein, 1996), thereby losing its ability to bind and target β-catenin for degradation. Alternatively, activating mutations in the β-catenin gene have been observed in various colon and melanoma cancer cell lines and in hepatocellular carcinomas (Morin et al., 1997); (Rubinfeld et al., 1997); (Ilyas et al., 1997).

Although there are a several known transcriptional targets for the wnt/wg pathway during development, there is limited knowledge of targets relevant to human tumourigenesis. Recently, c-Myc and cyclin D1 have been identified as TCF/β-catenin transcriptional targets in colon cancer (He et al., 1998); (Tetsu and McCormick, 1999). Both the c-myc and cyclinD1 promotor contain LEF-1/TCF binding sites and appear to be constitutively active in several colon cancer cell lines (Mann et al., 1999). Furthermore, activation of the β-catenin/TCF complex leads to expression of the matrixmetalloproteinase matrilysin (Crawford et al., 1999), and enhances expression of the urokinase-plasminogen activator receptor (Mann et
1.9.2.3 Desmosomes

Desmosomes are intercellular junctions connected with the intermediate filament cytoskeleton network, which is formed by cytokeratins in epithelial cells: thereby defining them as important regulators of structural integrity of tissue. Desmosomes contain two transmembrane proteins of the cadherin family, the desmogleins and desmocollins (Garrod, 1993). Desmosomal cadherins bind to a number of cytoplasmic plaque proteins, which belong to the Armadillo family of proteins, and desmoplakin. Desmoplakin and the plakophilins bind directly to cytokeratin filaments.

Plakophilins are also found in the nucleus, suggesting that these proteins may be involved in signalling pathways (Bonne et al., 1999). There is evidence to suggest that the assembly of desmosomes is independent of adherens junctions. For example, one study showed that mouse blastocysts lacking E-cadherin were able to form desmosomal junctions (Riethmacher et al., 1995). Another report suggested that desmosomes are regulated separately from adherens junctions, because, unlike adherens junctions, desmosomes do not seem to be disrupted by the expression of constitutively active Ras (Potempa and Ridley, 1998).

1.9.2.4 Gap junctions

Gap junctions are plasma membrane channels that directly connect neighbouring cells, thereby providing a pathway for the diffusion of small molecules including ions, amino acids, nucleotides and second messengers (calcium, cAMP, cGMP and IP₃). Gap junction channels are composed of connexins consisting of hexamers, also termed connexons (reviewed in (Paul, 1995)). The physiological regulation of gap junctions is thought to represent a cell survival mechanism: closure of the channels in response to cell injury or apoptotic stimuli effectively isolates the cell and therefore prevents damage from spreading to adjacent cells.

1.9.3 Cell-matrix adhesion

The attachment of epithelial cells to the extracellular matrix is crucial for the maintenance of tissue integrity. A number of different adhesion receptors are known, which function as a link between ECM and the cytoskeleton, including integrins and syndecans. Integrins are members of a large family of heteromeric transmembrane proteins with different α and β
subunits. In many epithelia there are specific points of connections linking cytokeratin intermediate filaments to the ECM (reviewed in (Jones et al., 1998)). The main adhesion receptor is the integrin heterodimer $\alpha_\beta_4$, which binds to laminin isoforms, and is crucial for the organisation and maintenance of the epithelial morphology. In addition, the $\alpha_\beta_4$ integrin is also involved in the transduction of signals induced by the extracellular matrix. These signalling pathways modulate a diverse range of cellular events including cell proliferation, apoptosis and migration (Mainiero et al., 1997). Upon ligand binding, $\beta_4$ integrin is tyrosine phosphorylated and can lead via activation of Ras to activation of the ERK-MAPK pathways, thereby controlling keratinocyte proliferation (Mainiero et al., 1997).

Other integrin receptors interact with extracellular matrix proteins, such as fibronectin, collagen and vitronectin thereby forming large protein aggregates termed focal adhesions, which are involved in mediating cell migration and survival. Focal adhesions are composed of integrin receptors, multiple cytoplasmic proteins that link the integrins to the actin cytoskeleton signal transduction proteins, such as focal adhesion kinase (FAK) (Critchley, 2000). Signalling from FAK can directly activate PI3K (Chen et al., 1996) and the Raf/ERK MAPK pathway in a Ras-dependent manner (Schlaepfer et al., 1994). Beside signalling from the ECM to the cell, the transduction of cellular signals via integrins to the outside also plays a crucial role in carcinoma migration and invasion (Rabinovitz et al., 1999).

1.10 Epithelial mesenchymal transition (EMT)

1.10.1 Regulation of EMT

Epithelial to mesenchymal transition (EMT) is an essential morphogenetic process during embryonal development but significantly it is also found to be involved in tumour progression (reviewed in (Birchmeier et al., 1993)). EMT is also seen as part of normal response to tissue damage, for example in the kidney, where stromal cells directly derived from tubular epithelial cells invade an injured site (Strutz et al., 1995).

During EMT, dedifferentiation of epithelial cells occurs, which then acquire a mesenchymal phenotype accompanied by increased cell migration. Loss of the polarised epithelial phenotype is characterised by repression of the junctional marker proteins E-cadherin and ZO-1, and cytokeratin. The transepithelial resistance is reduced due to loss of tight junction integrity (reviewed in (Hay, 1995)). A mesenchymal phenotype is characterised by
expression of the intermediate filament vimentin and the ECM component fibronectin. It was also found that the expression of N-cadherin is upregulated during EMT (Islam et al., 2000). Mesenchymal cells are generally loosely associated and non-polarised. The result of EMT is the production of migrating cells with the capacity to invade ECM. To maintain vimentin filament integrity, activation of phosphatases such as PP2A might be required, since treatment with phosphatase inhibitors resulted in filament disassembly (Cheng et al., 2000).

In vitro, signalling via tyrosine kinases and oncogene expression, which interferes with the integrity of cell-cell junctions, can induce EMT. For example, expression of a truncated ZO-1 protein, which no longer localises to the membrane, induces EMT in MDCK cells, thereby mediating loss of E-cadherin and activating the β-catenin signalling pathway. Activation of β-catenin appears to play a key role during EMT, since expression of APC was able to reverse the transformed fibroblastoid phenotype (Reichert et al., 2000). The importance of β-catenin signalling was also demonstrated in mouse mammary epithelial cells (EpH4), expressing an inducible c-Fos protein (Fos-ER). Sustained activation of Fos-ER induces EMT (Reichmann et al., 1992) and the mesenchymal cells show increased β-catenin dependent reporter activity and colocalised with the transcription factor LEF-1 in the nucleus (Eger et al., 2000). On the other hand, long-term activation of an inducible c-Jun protein (Jun-ER) was able to delocalise E-cadherin, but was not sufficient to induce EMT (Fialka et al., 1996).

A number of reports have shown that FGF-1, TGFα, EGF or HGF/SF stimulation can lead to the induction of EMT in a rat bladder carcinoma cell line NBT-II (Boyer et al., 1989); (Savagner et al., 1997). FGF-1 treatment led to dissociation of desmosomes before adherens junctions were affected (Boyer et al., 1992). In addition, EMT specific activation of c-Src occurred and expression of c-Src in NBT-II cells sensitised them to induction of EMT by FGF-1. In the same study, it was suggested that c-Src is more involved in cytoskeleton remodeling, whereas Ras signalling controls gene expression required for EMT. Another report showed that expression of v-Src promoted phosphorylation of E-cadherin and β-catenin, causing loss of adherens junctions and EMT (Behrens et al., 1993). Furthermore, inducible activation of the matrix metalloproteinase (MMP) stromelysin-1 (SL-1) resulted in cleavage of E-cadherin which eventually led to EMT in a mouse mammary cell line (Lochter et al., 1997). During EMT of lens epithelia in collagen matrices, α5β1 integrin is upregulated and antibodies to β1 integrin block EMT, suggesting that integrin/ECM interactions are also important for the transformation process (Zuk et al., 1994).
1.10.2 EMT in development

EMT occurs at several critical stages during development, such as gastrulation, neural crest migration and organogenesis (reviewed in (Hay, 1995)). At the blastula stage of the early embryo, only one single cell type exists, which is of epithelial nature. During gastrulation the three germ layers are formed from the blastocyst. Whereas ectoderm and endoderm remain largely epithelial, the mesoderm is formed by a conversion of epithelial cells to mesenchyme (reviewed in (Viebahn, 1995)). Another example of EMT is early development of the heart (Markwald et al., 1996). Later on in embryogenesis unwanted epithelia, such as that of the palate medial edges is remodelled by mechanisms involving EMT (Trelstad et al., 1982); (Fitchett and Hay, 1989); (Griffith and Hay, 1992). A number of growth factors from the TGFβ and FGF family have been found to play a role in these embryonic processes. For example, TGFβ and other growth factors play a role in EMT during neural crest emigration (McCarthy and Hay, 1991); (Duband et al., 1995). This appears to involve activation of the transcription factor Slug as treatment with Slug antisense oligonucleotides prevented EMT in chicken neural crest cells (Nieto et al., 1994). Also acidic FGF and TGFα promote EMT during development (Boyer and Thiery, 1993). Furthermore, oncogenes such as c-fos (Reichmann et al., 1992), v-mos and v-ras (Behrens et al., 1989) were found to promote the transition during development.

1.10.3 EMT and cancer

Many carcinomas with activated ras oncogenes have undergone EMT. While the exact mechanisms by which Ras promotes EMT are not fully understood, EMT is likely to be important in the behaviour of a number of tumour types, in particular in the establishment of invasiveness and formation of metastasis. Balmain and co-workers have established a mouse skin model of tumour progression, demonstrating the development of highly invasive spindle cell carcinomas following EMT (Stoler et al., 1993); (Cui et al., 1996). In this mouse skin model, treatment with a chemical carcinogen such as dimethylbenzantrazene (DMBA) induces activating G->A mutation at codon12 of the h-ras gene. This leads to the formation of benign papillomas in a subset of keratinocytes. Due to further genetic changes including the inactivation of the p53 gene and loss of the INK4 locus, some cells develop into squamous carcinomas, which still exhibit an epithelial phenotype. In a subset of these cells, dedifferentiation from an epithelial to a mesenchymal phenotype (EMT) occurs due to genetic and epigenetic alterations, leading to a highly malignant spindle cell carcinoma. These spindle
cells express vimentin, but no cytokeratins and E-cadherin, and are highly invasive and metastatic (Klein-Szanto et al., 1989); (Navarro et al., 1991); (Stoler et al., 1993); (Cano et al., 1996). Similar spindle cell tumours have been described in brain, skin, colon and breast of both humans and mice, and it seems that these tumours are of epithelial origin (Sonnenberg et al., 1987); (Buchmann et al., 1991).

The dependence of EMT on co-operating signalling pathways was also demonstrated in mouse mammary epithelial cells expressing activated \( h\)-ras (EpRas) which underwent EMT in response to TGFβ and formed tumours in mice (Oft et al., 1996). TGFβ has also been shown to mediate epithelial to mesenchymal conversion of a malignant metastatic keratinocyte cell line expressing activated H-Ras (Portella et al., 1998). However, expression of dominant negative TGFβ receptor type II blocks EMT and thus the formation of invasive spindle tumours \textit{in vivo}, which is also observed in other tumour cell lines (Miettinen et al., 1994); (Caulin et al., 1995); (Oft et al., 1998).

1.10.4 Invasiveness

Progression of epithelial tumours to the invasive stage often require EMT, which results in abrogation of cell-cell contacts, remodeling of the actin cytoskeleton, increased motility and proteolytic processing and secretion of ECM (Frixen et al., 1991); (Navarro et al., 1991); (Blasi, 1993); (Cano et al., 1996). A large body of evidence shows that there is a correlation between mutational inactivation of the E-cadherin gene and invasiveness (reviewed in (Birchmeier and Behrens, 1994); (Christofori and Semb, 1999)). Some human cell lines derived from bladder, breast, lung and pancreas carcinomas showed an epithelial cell morphology, expressed E-cadherin and were non-invasive, whereas cell lines with a fibroblastoid morphology were invasive and had often lost E-cadherin expression (Frixen et al., 1991). Restoration of E-cadherin function by expression of E-cadherin in cultured cancer cells or carcinogenesis mouse models impairs the invasive and metastatic features and following addition of E-cadherin directed antibodies re-established the invasive mesenchymal phenotype (Behrens et al., 1989).

Cell motility is another defining characteristic of invasive tumours, enabling cells to migrate into adjacent tissues or to transmigrate through the basement membrane and extracellular matrices. Many growth factors such as PDGF, FGF, EGF, HGF/SF, IGF-1 and TGFα and the activation of oncogenes are known to induce cell motility (Stoker and Gherardi, 1991).
This involves activation of the ERK-MAPK pathway, which results in phosphorylation of myosin light chain kinase (MLCK) required for contractility and cell migration (Klemke et al., 1997), but also activation of Rho GTPases, including Rac, Cdc42 and RhoC, which control the remodelling of the cytoskeleton and affect cell-cell adhesion, thereby promoting motility. The RhoGTPases can also be activated by integrins, involving kinases such as FAK and src and for example expression of dominant negative Rac (RacN17) abolishes migration (Klemke et al., 1998). The integrins β1, β2 or β3 can associate with uPAR (uPA-receptor) and activate the ERK-MAPK pathway either directly or indirectly via FAK or Ras, which can result in phosphorylation of the MLCK (Nguyen et al., 1999). Activation of the ERK-MAPK pathway also leads to transcriptional activation of components required for remodelling of the ECM, including uPA, uPAR and matrixmetalloproteases (MMPs) (reviewed in (Chambers and Matrisian, 1997)). Several human tumours including colorectal, mammary, lung and kidney tumours, were found to overexpress uPA, which is directly associated with the invasive capacity of these tumours (Ossowski and Reich, 1983); (Markus, 1988).
Chapter 2

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

PD98059 and EGF were purchased from Calbiochem. LY294002 were from Biomol. Hoechst33258 was from Molecular Probes. zVAD-fmk was purchased from Bachem. Propidium iodide came from Becton Dickinson. Recombinant human TGFβ, was from R&D Systems. Recombinant human TNFα was from Boehringer Mannheim. Foetal bovine serum (FBS) was from Gibco BRL. Insulin, HGF/SF, 4-Hydroxy-tamoxifen, Staurosporine, Cycloheximide and Etoposide were obtained from Sigma. [γ-32P] rUTP (400 Ci/mmol), [α-32P] dCTP (3000 Ci/mmol), [α-32P] dATP (3000 Ci/mmol) were purchased from Amersham and dNTPs came from Pharmacia. Z-EK(bio)D-aomk peptide was purchased from Peptide Institute, Osaka. Unless otherwise stated, all other reagents were obtained from Sigma.

2.1.2 Antibodies

Primary antibodies

anti-Bcl-XL rabbit Transduction Laboratories
anti-β-catenin mouse Transduction Laboratories
anti-caspase-8 (C20) goat Santa Cruz
anti-cyclinA (H4320) rabbit Santa Cruz
anti-cyclinD1 (DCS6) mouse Neomarkers
anti-cytochrome c mouse PharMingen
anti-cytokeratin 8, 18 rabbit a gift from H.Beug
anti-E-cadherin mouse Transduction Laboratories
anti-estrogen-receptor rabbit Santa Cruz
anti-Grb2 mouse Transduction Laboratories
anti-p21Cip1 goat Santa Cruz
anti-panERK mouse Transduction Laboratories
anti-PCNA (PC10) mouse ICRF
anti-phospho-ERK1/ERK2 mouse New England Biolabs
anti-Smad2 mouse Transduction Laboratories
anti-Smad2 (SED) rabbit a gift from P. ten Dijke
anti-Smad4 (B8) mouse Santa Cruz
anti-TGFβ1,2,3 mouse R&D systems
anti-TGFβ1 chicken R&D systems
anti-uPA mouse Neo Markers
anti-vimentin (V3B) mouse Boehringer Mannheim
anti-ZO-1 rabbit Zymed

Secondary antibodies
HRP-anti-rabbit IgG donkey Amersham
HRP-anti-mouse IgG sheep Amersham
HRP-anti-goat IgG rabbit Pierce
HRP-anti-chicken IgG rabbit Pierce

Cy3-labeled goat anti-rabbit IgG (1:1000) Amersham
Cy5-labeled donkey anti-mouse IgG H+L (1:100) Jackson
FITC-labeled donkey anti-mouse IgG (1:200) Jackson
The GFP tagged ΔRaf-1:hbER [DD] cDNA (Woods et al., 1997) in pBabe-puro was provided by M.McMahon (DNAX Research Institute, Palo Alto, CA). *X.laevis* Fast1 in pEF-Flag, *X.laevis* Smad2 in pEF-myc and pFTX5 (Howell and Hill, 1997), human Smad3 (hSmad3) in pcDNA3 (a gift from P. ten Dijke) was subcloned into pFTX5, pEF-lacZ and the ARE-Luc construct comprising three copies of the Mix.ARE promoter in pGL3 were kindly provided by C.S.Hill (ICRF, London). Full length human Smad3 was digested out of pFTX5 as a EcoRI-Xhol insert and subcloned into the EcoRI-Sall sites of pBabe-hygro and pBabe-bleo. pWZL-Neo-EcoR encoding for the retroviral ecotropic receptor was a gift from M. Serrano (CNB, Madrid). The pGEM-T vector came from Promega. The humanGAPDH cDNA (51-231) in pGEM-T was a gift from R.Treisman (ICRF, London). The human Smad2 cDNA (26-261), human Smad3 cDNA (321-626), human Smad4 cDNA (281-684), human p21CIP cDNA (76-570) and human γ-actin cDNA (BamHI-Hindlll fragment) cloned into pGEM-T were kindly provided by C.S.Hill (ICRF, London). The pGEM-T constructs (Sp6 promoter) were linearised using *NcoI* restriction enzyme, for linearisation of pGEM-T containing Smad2 cDNA (T7 promoter) the restriction enzyme *SpeI* was used.

2.1.3 Primers and oligonucleotides

T7 primer: 5' TAATACGACTCACTATAGGG 3'

Sp6 primer: 5' GATTTAGGTGACACTCATA 3'

E-cadherin primer for generation of E-cadherin template

forward 5' TGACAGAGCCTCTGGATAGAG 3'

reverse 5' CTCGTTCCTCACCCACCTGAC 3'

c-jun oligonucleotides for 32P labeled EMSA probe (Smad3/4 binding site in bold) were a gift from C.S.Hill (ICRF):

CH338: 5' GGAGGTGCGCGGAGTCAGGCAGACAGACAGACACAGC 3'

(c-jun GACA sense)

CH339: 5' TGCCGACCTGGCTGGCTGGCTTGTGCTGCTGCTGCTGCTGCTGCTG 3'

(c-jun CTGT antisense)
2.2 Methods

2.2.1 Tissue culture

2.2.1.1 Cell lines

MDCK cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS). MDCK Raf:ER cells which have undergone EMT (MDCK RafT) were cultured in phenolred-free DMEM supplemented with 10% charcoal treated FCS (incubation of 500 ml FCS with 0.05 g activated charcoal and 0.5 g dextran T-70 (Pharmacia) for 2 h at 37°C, followed by sterile filtration) and 50 μM 4HT. MCF-10A cells were grown in Ham's nutrient mixture F12/DMEM (1:1) containing 5% horse serum (Gibco BRL) and 10 μg/ml insulin, 20 ng/ml EGF 5 μg/ml hydrocortisone and 100 ng/ml cholera toxin.

To generate MDCK and MCF-10A cell lines stably expressing ΔRaf-1:hbER [DD] and control MDCK and MCF-10A cell lines carrying empty vector, wild type cells were first infected with an amphotropic retrovirus encoding for the ecotropic receptor (pWZL-Neo-EcoR) to allow the subsequent infection with ecotropic viruses. Retrovirus stock was obtained from the medium of NIH3T3 GP+E packaging cells that had been transfected using Lipofectamine (Gico BRL) with either ΔRaf-1:hbER-pBabe-puro or empty vector and then selected with 0.5 mg/ml G418 for 10 days. For the following infection MDCK and MCF-10A cells, which stably express the ecotropic receptor, were infected with retrovirus containing supernatants and then cultured in the appropriate medium containing 2.5 μg/ml of puromycin to select for virus infected cells. The puromycin resistant cells were pooled and sorted twice by fluorescence activated cell sorting (FACS) for GFP expression. MDCK and MCF-10A cells expressing ΔRaf-1:hbER [DD] and control cells were cultured in phenol-red free DMEM supplemented with 10% charcoal treated FCS.

MDCK RafT cells and MDCK Raf:ER cells stably expressing human Smad3 were generated by retroviral infection. Therefore, GP+E packaging cells were transfected using Lipofectamine (Gico BRL) with either Smad3-pBabe-bleo, Smad3-pBabe-hygro or empty vector and then selected with 0.5 mg/ml G418 for 8 days. For the following infection MDCK RafT cells were incubated with retrovirus containing supernatants for 7 hours (pBabe-hygro) or overnight (pBabe-bleo) and then cultured in medium containing the appropriate antibiotic.
(100 μg/ml hygromycin, 25 μg/ml bleocin) to select for virus infected cells.

The generation of MDCK cell lines stably expressing Raf-CAAX, V12 H-Ras and activated p110 have been previously described (Khwaja et al., 1997). MDCK cell lines expressing dominant negative FADD (dnFADD), Bcl-2 and Bcl-X\textsubscript{L} have been described previously (Rytömaa et al., 1999).

2.2.1.2 Concentration of cell culture supernatant

Conditioned medium was generated from Raf-1 transformed MDCK ΔRaf-1:hbER [DD] cells and wild type MDCK cells cultivated in DMEM supplemented with 2% FCS. Cell culture supernatant was concentrated three times by ultrafiltration (Biomax-10K, Millipore).

2.2.1.3 Transient transfections

MDCK Raf:ER cells were transiently transfected using Lipofectamine (Gibco BRL) with the following amounts of vectors per 3.5 cm dish: 1 μg ARE-Luc reporter, 0.05 μg XFast1 expression vector, 0.25 μg lacZ as an internal control for transfection efficiency and 0.2 μg pEF empty vector. Before measuring Luciferase activity, cells were stimulated with 2 ng/ml TGFB for 6 h following 4HT treatment as indicated. Luciferase assays were performed according to the procedures recommended by the supplier (Promega). The β-galactosidase assays were performed using chlorophenolred-β-D-galactopyranoside (Calbiochem) as a substrate and quantified photometrically at 595 nm. All transfections were normalised to β-galactosidase activity.

2.2.1.4 Collagen gel culture

Collagen gel culture was performed by A. Khwaja as follows: Type I Collagen (Vitrogen100, Collagen Corporation) solution was mixed with 10x DMEM and water to a final concentration of 2 mg/ml and neutralised with 0.1 M NaOH on ice. 700 μl per 3.5 cm dish were allowed to gel. MDCK wild type cells and MDCK cells stably expressing Raf-CAAX, V12Ras and p110 were detached by incubation with trypsin and 5 x 10\textsuperscript{3} cells were resuspended in 1 ml of the collagen mixture and plated on top of the previously gelled collagen layer. After gelation, 1 ml of DMEM supplemented with 10% FBS was added and subsequently changed every third day. Structures were photographed at 20x magnification after 8 days.

Alternatively, collagen gel culture was performed as follows: MDCK wild type cells and
MDCK Raf:ER cells were detached by incubation with trypsin and $3 \times 10^5$ cells were suspended in 100 µl of rat collagen type I (Vitrogen100, Collagen Corporation) at 4°C and plated on cell culture dishes. After gelation for 45 min, the collagen gels were overlayed with DMEM medium containing 10% charcoal treated FCS, 5 ng/ml TGFα (R&D systems) and 0.04 IE/ml Insulin (Novo Nordisk). After six days 2 ng/ml TGFβ or neutralising TGFβ antibodies (100 ng/ml chicken anti-TGFβ1 and 20 µg/ml mouse anti-TGFβ1,2,3) were added for a further 7 days. The medium was changed every second day and the TGFβ and neutralising antibodies were re-added. Structures were photographed at 20x magnification 14 days after plating.

2.2.1.5 Growth in suspension

MCF-10A cells and MDCK cells were detached by incubation with trypsin and plated at a density of $1 \times 10^5$ cells/1.5 ml medium onto normal or poly(2-hydroxyethylmethacrylate) (poly-HEMA) coated 3.5 cm plates. MCF-10A cells were cultivated for 24 h in Ham's nutrient mixture F12/DMEM (1:1) containing only 5% horse-serum. MDCK were cutivated for 8 h in DMEM supplemented with 10% FBS. Coating was carried out with 2x 1 ml of 10 mg/ml poly-HEMA in ethanol for a 3.5 cm plate allowing the ethanol to evaporate between additions. The plates were subsequently washed with PBS.

2.2.1.6 Growth in soft agar

$5 \times 10^3$ cells were mixed with 4 ml of 0.5% low melting point agarose (FMC) and overlayed onto a layer of 0.9% agarose in 6 cm dishes and allowed to set for 30 min at room temperature. 3ml of DMEM supplemented with 10% FBS was added and the cells were cutivated for 3 weeks. The medium was changed every third day. Cells were stained by adding 1 ml of 0.2% neutral red in PBS for 4 h at 37°C. Gels were dried onto Whatman paper and colonies were counted.

2.2.1.7 Hoechst staining

MDCK cells were detached by incubation in trypsin, washed in PBS and fixed in 3.7% formaldehyde in PBS for 30 min at room temperature. Cells were stained for 10 min with 1 µg/ml Hoechst33258 (Molecular Probes) and examined by fluorescence microscopy following UV illumination. A minimum of 500 cells/ sample was scored for apoptotic phenotype.
2.2.2 Bacterial culture

2.2.2.1 Preparation of competent E.coli

Competent DH5α E.coli bacteria were generated by inoculating a single bacterial colony into 50 ml LB medium (10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl) and incubating overnight in a 37°C shaker. 25 ml of the overnight culture were inoculated into 475 ml LB medium containing 16 mM MgSO₄, 10 mM KCl and 1 mM KOH. The culture was grown in a 37°C shaker to an OD₆₀₀nm of 0.4-0.5, chilled on ice for 5 min and sedimented by centrifugation at 2300 x g for 15 min at 4°C. The pellet was resuspended in 150 ml ice-cold TFBI buffer (30 mM KOAc, 50 mM MnCl₂, 100 mM RbCl and 19% (w/v) glycerol, pH 5.8). After a 10 min incubation on ice, the bacteria were sedimented by centrifugation at 2300 x g for 15 min at 4°C and resuspended in 20 ml TFBII buffer (10 mM MOPS, 55 mM CaCl₂, 10 mM RbCl and 19% (w/v) glycerol, pH 7.0). 50 μl aliquots were frozen in liquid nitrogen and stored at -80°C.

2.2.2.2 Transformation of E.coli by heatshock

50 μl competent DH5α E.coli were thawed on ice, combined with 1 μl of plasmid DNA (~20 ng) and incubated on ice for 30 min. The bacteria were incubated for 75 sec at 42°C, placed on ice for 2 min and mixed with 1 ml LB medium. The bacteria were incubated at 37°C for 1 h and 5, 10 and 50 μl were spread on LB plates (LB medium containing 15 g/l agar) containing appropriate selective antibiotics (ampicillin 100 μg/ml). The plates were incubated overnight at 37°C.

2.2.3 DNA techniques

2.2.3.1 Purification of plasmid DNA

A single bacterial colony was inoculated into 3 ml of LB medium and incubated overnight in a 37°C shaker. Plasmid DNA was purified using QIAGEN plasmid kits, according to the manufacturer’s protocol. For large scale plasmid DNA purification, 1.5 ml of the overnight culture was inoculated into 300 ml of LB medium and incubated overnight in a 37°C shaker. Plasmid DNA was purified using QIAGEN plasmid kits. The DNA was dissolved in water and stored at -20°C.
2.2.3.2 Quantitation of DNA

Plasmid DNA concentration was determined measuring the $A_{260\text{nm}}$ and $A_{280\text{nm}}$ in a spectrophotometer. An $A_{260\text{nm}}$ of 1 corresponds to 50 µg/ml double stranded DNA and 33 µg/ml single stranded DNA. The ratio $A_{260}/A_{280}$ provides an estimate of the purity of DNA/RNA solutions and should be ~1.7 for DNA preparations.

2.2.3.3 DNA agarose gel electrophoresis

Agarose gels were prepared by dissolving 1% agarose in TAE buffer (40 mM Tris base, pH 8.0, 1 mM EDTA). Ethidium bromide was added to a final concentration of 0.5 µg/ml. DNA samples were mixed with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll (Typ 400)) and electrophoresed at 5-20 V/cm in TAE buffer.

2.2.3.4 Restriction enzyme digestion

Restriction enzyme digestions were performed according to the manufacturer’s protocol (New England Biolabs) and DNA fragments were excised from agarose gels using the QIAquick Gel Extraction kit (QIAGEN).

2.2.3.5 Ligation

Plasmid DNA ligation of were carried out overnight at 1°C in a total reaction volume of 20 µl using T4 DNA Ligase, according to the manufacturer’s protocol (New England Biolabs).

2.2.3.6 Nucleotide sequencing

Fluorescent cycle sequencing was performed using gene-specific primers and the ABI dye termination kit (Perkin Elmer) according to the manufacturer’s protocol. Reactions were carried out in a total reaction volume of 20 µl containing 1-3 µl double stranded DNA (0.2 µg/µl), 3.2 pmol primer and 8 µl reaction mix using a thermal cycler. PCR conditions were as follows: denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, extension at 60°C for 4 min (25 cycles). Samples were ethanol precipitated, electrophoresed and visualised. Electrophoresis and visualisation of the samples was performed by the ICRF Sequencing Service.

2.2.3.7 In vitro translation assay

*In vitro* coupled transcription/translation of hSmad3 or XSmad2 in pFTX vector was
performed following the protocol provided by the supplier (TNT T7 Coupled Reticulocyte Lysate System, Promega). Reactions were carried out in a total reaction volume of 20 μl containing 500 ng plasmid DNA. 5 μl of lysates were analysed by a 15% low-bis SDS-PAGE and Western blotting with anti-Smad2 antibody (Transduction Laboratories).

2.2.3.8 Preparation of ^32P-labelled c-jun probe

The ^32P-labelled c-jun oligonucleotide probe was generated by annealing and filling in the overlapping c-jun oligonucleotides by PCR in the presence of [α-^32P]dCTP and [α-^32P]dATP. Reactions were carried out in a total reaction volume of 10 μl containing 50 ng sense and 50 ng antisense oligonucleotide, 1 μl of PCR buffer (NEB) 0.5 mM dGTP and dTTP, 0.05 mM dCTP and dATP, 1 μl [α-^32P]dCTP (10 μCi/μl), 1μl [α-^32P]dATP (10 μCi/μl) and 0.5 μl Taq Polymerase (NEB). PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72 °C for 1 min (30 cycles). The probe was gel-purified via a native 5% (40:1) acrylamide gel (6,25 ml 40% acrylamide, 3.125 % glycerol in a total volume of 50 ml). Electrophoresis was carried out in 0.5x TBE at 150 V for 1.5 h. The labelled probe was excised from the gel and eluted in elution buffer (33 mM NaOAc, pH 5.2, 0.1% SDS, 1 mM EDTA) overnight at 37°C. The probe was ethanol precipitated in the presence of tRNA (Boehringer Mannheim) and resuspended in 20 μl water.

2.2.3.9 Electrophoretic mobility shift assay (EMSA)

The EMSA reaction was performed as described previously (Germain et al., 2000) at room temperature containing 10 μg of nuclear protein extracts in 10 μl of nuclear extraction buffer (20mM HEPES, pH7.6, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM DTT, 1 mM Na₃VO₄, 10 μg/ml leupeptin and aprotinin, 2 mM benzamidine) and 10 μl of binding buffer (10% glycerol, 10 mM KCl, 5.5 mM MgCl₂, 2 μg poly(dI.dC) and 1x10⁴ cpm of ^32P-labelled c-jun probe. After incubation for 15 min the samples were loaded onto a native 5% (40:1) acrylamide gel (6,25 ml 40% acrylamide, 3.125 ml 2% bis-acrylamide in a total volume of 50 ml) containing 2.5% glycerol. Samples were resolved by electrophoresis in 0.5 x TBE (50 mM Tris base, 50 mM H₂BO₄, 1 mM EDTA, pH 8.3) at 150 V for 3.2 h and visualised by autoradiography. For supershift experiments the following antibodies and competing peptide were added to the nuclear extracts prior to probe addition and incubated at room temperature
for 5 min: 1 µl anti-Smad4 (B8), 1 µl anti-Smad3 without or with 0.5 µl competing peptide (10µg/ml) (a gift from P. ten Dijke), 0.5 µl anti-Smad2 (Transduction Laboratories) and 1 µl of anti-Smad2 (SED).

2.2.4 RNA techniques

2.2.4.1 Isolation of RNA

Total RNA from MDCK cells was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Briefly, 600 µl RLT buffer containing guanidine isothiocyanate was added to 80% confluent cells on a 10 cm dish, which were then scraped and homogenised using a QIA shredder (QIAGEN) to sheer the DNA. RNA was isolated by using RNeasy spin columns and eluted in 90 µl RNase free water.

2.2.4.2 Quantitation of RNA

RNA concentration was determined by measuring the absorbance at A_{260nm} and A_{280nm} in a spectrophotometer. An A_{260nm} of 1 corresponds to 40 µg/ml RNA. The ratio A_{260/280nm} provides an estimate of the purity of DNA/RNA solutions and should be 1.8 – 2.0 for RNA preparations.

2.2.4.3 RNA agarose gel electrophoresis

The quality of total RNA was analysed on a 1% agarose gel in TBE buffer (50 mM Tris base, 50 mM H_{2}BO_{4}, 1 mM EDTA, pH 8.3). Ethidium bromide was added to a final concentration of 0.5 µg/ml. 2 µg of RNA from each sample were mixed with an equal volume of loading buffer (0.025% SDS, 18 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol-blue, 95% formamide), incubated at 65°C for 10 min and electrophoresed at 2-5 V/cm in TBE buffer. The 28S ribosomal RNA bands should be present at approximately twice the amounts of the 18S RNA.

2.2.4.4 RT-PCR for generation of E-cadherin cDNA probe

Complementary DNA (cDNA) was synthesised by a one-step reverse transcription polymerase chain reaction kit (QIAGEN) according to the manufacturer’s protocol. Briefly, the reactions were carried out in a total reaction volume of 50 µl containing 2 µg total RNA and 0.6 µM of each gene-specific primer (forward and reverse). PCR conditions were as
follows: reverse transcription at 50°C for 30 min, initial polymerase activation at 95°C for 15 min, denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1 min, final extension at 72°C for 10 min. The three step cycling was carried out in 30 cycles. The amplified E-cadherin cDNA fragment was cloned into pGEM-T vector (Promega) after poly-A tail generation with 2 units Taq-polymerase (NEB), 300 ng cDNA fragment, 0.02 mM dNTPs (Pharmacia) and the appropriate buffer (NEB) in a total reaction volume of 50 μl. Reactions were carried out for 15 min at 72 °C. E-cadherin template is under the control of the Sp6-promotor. Linearisation was carried out by NcoI restriction enzyme digestion.

2.2.4.5 Probe preparation for RNase protection assays

RNA probes were synthesised using the method of (Melton et al., 1984). The RNA antisense probes were generated by using template cDNA in pGEM-T, which was linearised with the appropriate restriction enzyme. The digest was checked on an agarose gel, purified by phenol/chloroform extraction, ethanol precipitated and resuspended in water.

**Probe labelling:**

The labelling reactions contained 0.5 μl linearised template cDNA(1mg/ml), 0.5 μl of the appropriate RNA-polymerase (T7, New England Biolabs; Sp6, Promega), 2.5 μl [γ-32P] rUTP, 0.5 μl 10x transcription buffer (New England Biolabs), 0.25 μl of rATP (10 mM), rGTP (10 mM) and rCTP (10 mM), 0.25 μl RNAase-inhibitor (Amersham) and 0.25 μl DTT (200 mM). The reactions were incubated at 37°C for 1 h. DNase treatment was then carried out by addition of 95 μl 50x TE buffer (1x TE: 10 mM Tris HCl, 1 mM EDTA, pH 8.0), 0.5 μl RNAase-inhibitor and 1.25 μl of bufferD (400 mM MgCl2, 200 mM CaCl2, 80 mM DTT) and 0.75 μl DNase1 (2.7 U/μl; Worthington Biochemicals) at 37°C for 30 min, followed by a phenol extraction and ethanol precipitation of the generated cRNA in the presence of 10 μg carrier tRNA (Boehringer Mannheim). The dried pellet was dissolved in 1 μl water and 5 μl formamide loading buffer (0.25% xylene-cyanol, 0.25% bromphenol-blue, 20 mM EDTA, pH 8.0 in formamide (Fluka)) and denatured at 95°C for 3 min. The probes were gel-purified using a 6% denaturing acrylamide gel (6% 40:1 acrylamide:bis-acrylamide, 7M Urea in 0.5 x TBE). The gel run was carried out in 0.5x TBE at 11 W for 1.5 h. The labelled probes were excised from the gel and eluted in elution buffer (0.5 M NH4OAc, 0.1% SDS, 1mM EDTA) for 2 h at 55°C. After phenol extraction, the probes were ethanol precipitated in the presence of carrier tRNA, the pellet was dissolved in 20 μl water and counted in a scintillation counter.
2.2.4.6 RNase protection assay

For probe hybridisation the reactions were carried out in a total volume of 30μl containing 15 μg total RNA in 30 μl hybridisation buffer (400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 80% formamide) and 1 μl labelled probe (2 x 10^5 cpm/sample). Samples were incubated at 85°C for 5 min to linearise the RNA and hybridised at 45°C overnight. Digestion of the un-hybridised single stranded RNA was carried out by RNase treatment in 350 μl RNase buffer (10 mM Tris base, pH 7.5, 5 mM EDTA, 300 mM NaCl) with 0.4 μl RNaseT1 (20 U/μl; Calbiochem) at 37°C for 30 min. The RNase was then digested by addition of 5 μl ProteinaseK (10 mg/ml, Sigma) and 0.25% SDS at 37°C for 30 min. After phenol/chloroform (1:1) extraction, the double-stranded RNA was precipitated using 1 volume isopropanol and dissolved in 1 μl water and 5 μl formamide loading buffer. The reactions were electrophoresed (6% denaturing acrylamide gel) at 11 W for 1 h. The gel was fixed (10% methanol, 10% acetic acid), dried and visualised by autoradiography.

2.2.5 Protein techniques

2.2.5.1 Protein quantitation

Protein concentrations were determined using the Bio-Rad protein Assay reagent based on the method of (Bradford, 1976). Sample dilutions and BSA standards were made in 0.8 ml water, incubated with 0.2 ml Bio-Rad Protein Assay for 5 min and the absorption was measured at 595 nm in a spectrophotometer. Protein concentration was calculated from the BSA standard curve.

2.2.5.2 Total protein lysates

Cells were washed twice with ice-cold PBS. After addition of ice-cold Triton lysis buffer (25 mM Tris-HCl, pH 7.6,150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 mM Na_2VO_4, 10 μg/ml aprotinin and leupeptin, 2 mM benzamidine) cells were incubated on ice for 5 min and the lysate was collected by scraping. The lysates were clarified by centrifugation at 12000 x g for 10 min at 4°C. Lysates were frozen in liquid nitrogen and stored at -80°C.

2.2.5.3 Lysis in SDS-sample buffer

Cells were washed twice with ice-cold PBS and lysed with SDS-sample buffer (50 mM Tris
HCl, pH 6.8, 2.5% β-Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). After scraping the cells, the samples were boiled for 5 min. Lysates were stored at -80°C.

### 2.2.5.4 Nuclear extracts

Nuclear extracts were prepared as previously described (Wong et al., 1999). Briefly, cells were washed twice with ice-cold PBS. After addition of ice-cold hypotonic buffer (20mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM DTT, 1 mM Na₃VO₄, 10 μg/ml leupeptin and aprotinin, 2 mM benzamidine) the cells were allowed to swell on ice for 5 min before scraping them. The nuclei were pelleted by centrifugation at 400 rpm for 5 min and resuspended in nuclear extraction buffer (hypotonic buffer + 500 mM NaCl). After rocking for 20 min at 4°C, the lysates were clarified by centrifugation at 12000 x g for 10 min and the nuclear extracts were shock frozen in liquid nitrogen and stored at -80°C.

### 2.2.5.5 Lysis in digitonin buffer

Cell lysis in digitonin buffer was carried out according to (Samali et al., 1999). Cells (6 x 10⁶) were detached by incubation with trypsin, washed once in mitochondrial buffer (70 mM Tris base, 0.25 M sucrose, 1 mM EDTA, pH 7.4) and resuspended in 100 μl of mitochondrial buffer. An equal volume of digitonin (0.2 mg/ml dissolved in MES-buffer: 20 mM EGTA, 20 mM EDTA, 0.25 M D-mannitol, 20 mM MES, pH 7.4) was added to the sample and incubated on ice for 5 min. After clarification by centrifugation at 900 x g for 2 min, the supernatant was clarified by centrifugation at 20000 x g for 5 min to obtain the cytosolic fraction. Samples were frozen in liquid nitrogen and stored at -80°C.

### 2.2.5.6 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels were prepared as described in (Laemmli, 1970). The separating gel was prepared by mixing 2.6 ml bufferA (1.5 M Tris-base, pH 8.8 and 0.1% SDS), acrylamide/bis-acrylamide solution and water. The composition of acrylamide/ bis-acrylamide and water is shown in the following table:
Final acrylamide concentration

<table>
<thead>
<tr>
<th></th>
<th>10%</th>
<th>12%</th>
<th>16%</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>4 ml</td>
<td>3.3 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>30% acrylamide/0.8% bis-acrylamide mix</td>
<td>3.3 ml</td>
<td>4 ml</td>
<td>5.5 ml</td>
</tr>
</tbody>
</table>

The separating gel for a 15% low-bis acrylamide gel was composed as follows: 2.6 ml buffer A (1.5M Tris-base, pH 8.8 and 0.1% SDS), 5 ml 30% acrylamide and 375 μl 2% bis-acrylamide, 1.85 ml water. The 5% stacking gel was made by mixing 1.35 ml bufferB (1 M Tris-base, pH 6.8, 0.1% SDS), 1.7 ml 30% acrylamide/0.8% bisacrylamide mix and 6.8 ml water. For the polymerisation reaction 100 μl 10% APS and 10 μl TEMED were added. Protein samples were mixed with SDS-sample buffer (50 mM Tris HCl, pH 6.8, 2.5% β-Mercaptoethanol, 2% SDS, 0.1% bromophenol-blue, 10% glycerol) and boiled for 5 min. Proteins were separated alongside molecular weight markers (Rainbow Markers, Amersham; MultiMark, NOVEX) in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS) at 100-200V.

2.2.5.7 Western Blotting

Proteins separated by SDS-PAGE were transferred onto polyvinylfluoridine (PVDF) membranes (Millipore) in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.05% SDS) using a semi-dry electroblotter at 12V for 75 min. Membranes were blocked in blocking buffer (PBS containing 5% milkpowder, 0.05% Tween-20) for 1h and incubated with an appropriate primary antibody diluted in blocking buffer. Membranes were washed in blocking buffer three times for 15 min and incubated with blocking buffer containing a HRP-conjugated secondary antibody (1:5000) for 1h and washed with blocking buffer three times for 15 min. Immunoreactive proteins were visualised using enhanced chemiluminescence according to manufacturer’s protocol (ECL, Amersham).

2.2.5.8 Affinity labelling of activated caspases

For affinity labelling of active caspases, standard protein lysates were prepared as described above. Aliquots of 120 μg protein in a volume of 50 μl were incubated for 5 min at 37°C with 0.5 μM Z-EK(bio)D-aomk peptide (Peptide Institute, Osaka), then diluted with 1/3 volume of four times concentrated SDS sample buffer and boiled for 3 min. Equal aliquots were
subjected to 16% SDS-PAGE followed by Western blotting and detection by using HRP-coupled streptavidin (Amersham) and ECL.

2.2.5.9 Immunostaining and confocal microscopy

Cells were grown on glass coverslips and fixed with 3.7% formaldehyde in PBS for 30 min at room temperature. After three washes in PBS, the cells were permeabilised in PBS containing 1.5% BSA and 0.2% Triton X-100 for 10 min followed by three further washes in PBS containing 1.5% BSA. The β-catenin antibody, diluted in PBS containing 1.5% BSA was then applied to the coverslips and incubated for 20 min followed by one wash for 30 min and two washes for 5 min with 1.5% BSA in PBS. The secondary antibody (FITC-anti mouse) was applied in PBS containing 1.5% BSA for 20 min. This was followed by three washes in 1.5% BSA in PBS. Coverslips were mounted in Gelvatol (Monsanto Chemicals) and examined with a BioRAD MRC1000 confocal microscope using a 63 x oil immersion lens.

Alternatively, cells were grown on 24 mm Falcon cell culture inserts (0.4 μm pore size, Becton Dickinson) and then fixed in methanol/acetone (1:1) for 5 min at -20°C, washed three times with PBS and blocked with 0.2% gelatine in PBS for 30 min. After incubation with the primary antibody, diluted in PBS/ 0.2% gelatine for 20 min at room temperature the cells were washed three times with PBS/ 0.2% gelatine and incubated for 20 min with an appropriate secondary antibody diluted in PBS/ 0.2% gelatine. Samples were mounted in Gelvatol and examined with a Zeiss LSM510 Axioplan2 confocal microscope using a 63x NA 1.4 oil immersion lens.

2.2.5.10 TGFβ1 ELISA

To determine the amount of TGFβ1 secretion into cell culture supernatants, cells were cultivated until they had reached 80% confluency in a 10 cm dish. The cells were grown for the last 24 h in DMEM supplemented with 0.5% BSA. The cell culture supernatants were collected and TGFβ1 levels were measured by ELISA (Promega).

2.2.5.11 Cell death ELISA

Cells were harvested and DNA fragmentation ELISA was carried out using the Cell Death Detection ELISA kit (Roche Pharmaceuticals) according to the manufacturer’s instructions.
2.2.6 Cell-cycle analysis

To examine cell cycle distribution, cells were detached by incubation with trypsine, washed in PBS and fixed in 70% ice-cold ethanol. After RNase (100 μg/ml) treatment for 5 min at room temperature cells were stained with 50 μg/ml propidium-iodide (Becton Dickinson) for 5 min. Stained cells were analysed by flow cytometry using 488 nm excitation, carried out by the ICRF FACS Service. The data profiles were analysed by the Dean-Jett-Fox integration method.
Chapter 3

Initial observations:

3 The effects of the Raf-MAPK pathway on the regulation of cell scattering and tubulogenesis.

3.1 Introduction

Hepatocyte growth factor, also termed scatter factor (HGF/SF) is a multifunctional cytokine which is secreted by mesenchymal cells. It functions as a motogenic and mitogenic factor in epithelial cells. HGF/SF causes disruption of cell-cell junctions and migration of epithelial cells grown in a monolayer (Stoker et al., 1987); (Gherardi et al., 1989). *In vitro* HGF/SF promotes the formation of branching tubular structures (tubulogenesis) in epithelial cells cultured in three-dimensional collagen matrices (Montesano et al., 1991). In vivo, HGF/SF induces tubulogenesis plays an important role during kidney organogenesis and mammary gland development (Woolf et al., 1995); (Yang et al., 1995). The formation of branching tubular structures in response to HGF/SF is a complex process involving proliferation, cell migration and remodelling of the extracellular matrix (ECM) by activation of proteases (Pepper et al., 1992). HGF/SF was also found to promote tumourigenesis and cell invasion of some human carcinomas and tumour cell lines (Rong et al., 1994); (Rosen et al., 1994).

The effects of HGF/SF are mediated through binding to its cell surface receptor c-Met (Naldini et al., 1991); (Bottaro et al., 1991) which activates a number of downstream effectors such as the small GTPase Ras and thereby activating PI3K and Raf-1 (Graziani et al., 1993); (Ponzetto et al., 1994). Alternatively, PI3K activation can occur independently of Ras either through direct binding of PI3K to the receptor or via binding of PI3K to the adapter protein Gab1 (Schaeper et al., 2000). Importantly, it has been demonstrated that Ras signalling is required for HGF/SF induced scattering of MDCK cells and expression of constitutively activated Ras in MDCK can induce the scattered phenotype (Schoenenberger et al., 1991); (Hartmann et al., 1994); (Ridley et al., 1995). Furthermore, HGF/SF and Ras induces loss of the adherens junction proteins E-cadherin and β-catenin from intercellular junctions during scattering (Potempa and Ridley, 1998). Beside the role of β-catenin in cell-
cell adhesion, cytoplasmic β-catenin can function as a signalling component, thereby translocating into the nucleus, where it can promote transcription of genes involved in proliferation (reviewed (Behrens, 1999)).

The aim of this study was to characterise the role of Ras downstream effector pathways on scattering and tubulogenesis. As a model system the untransformed immortalised dog kidney epithelial cell line MDCK (Madin-Darby canine kidney) was used, which displays many features of \textit{in vivo} epithelia (Barker and Simmons, 1981). When grown in monolayer in the presence of serum, MDCK cells form compact sheets of polarised cells (Wang et al., 1990). Activated forms of Ras, PI3K and Raf-1 were stably expressed in MDCK cells, which allows the selective study of their effects on MDCK cell morphology. In addition, to investigate the effects of the activated signalling proteins on cell-cell contacts, the abundance of the adherens junction component β-catenin was examined by immunostaining and confocal microscopy.

3.2 Results

3.2.1 Morphological appearance of MDCK clones stably expressing activated signalling proteins

To investigate which Ras effector pathway might be involved in MDCK cell scattering MDCK cells were generated stably expressing activated forms of Ras, Raf-1 and PI3K (Khwaja et al., 1998). V12Ras was derived from H-Ras by introducing a point mutation at codon 12 which renders the protein constitutively active (Rodriguez-Viciana et al., 1997). Raf-CAAX, is a membrane-targeted form of Raf-1 generated by fusion of the Ras C-terminal membrane targeting sequence CAAX to full length Raf-1. This enables Raf-1 signalling in a Ras-independent manner (Leevers et al., 1994); (Stokoe et al., 1994). The catalytic subunit of PI3K, p110, was also membrane targeted by N-terminal fusion of the CAAX motif which leads to constitutive activation (p110-CAAX) (Wennstrom and Downward, 1999). All assays were carried out in the presence of 10% serum, thereby allowing a constitutive basal level of Raf/ERK-MAPK and PI3K activity.

Figure 3.1 shows phase-contrast images of the phenotype of these MDCK cell lines. MDCK cells stably expressing V12Ras and p110-CAAX exhibit a scattered and fibroblastoid
Figure 3.1. Morphological appearance of MDCK cells stably expressing activated signalling proteins. MDCK wild type cells were transfected with cDNAs for V12Ras, Raf-CAAX and PI3-kinase (p110-CAAX). MDCK wild type cells untreated or treated with HGF/SF (10 ng/ml) for 14 h and MDCK clones stably expressing the indicated activated signalling proteins were examined by phase contrast microscopy. Shown are representative phase contrast images. (Figure kindly provided by A.Khwaja).
phenotype similar to the one observed by treatment with HGF/SF. In contrast, MDCK cells expressing Raf-CAAX grow in loosely associated cells colonies with reduced cell-cell contacts, but the cells are not dispersed. This indicates that the MAPK pathway leads to disruption of intercellular contacts whereas the PI3K pathway is sufficient to induce the motile and dispersed phenotype of MDCK cells similar to that observed in response to HGF/SF.

To explore the effects of V12Ras, p110-CAAX and Raf-CAAX on cell-cell adhesion, a component of the cell adhesion complex was monitored: localisation of the adherens junction protein β-catenin was examined by immunostaining in the respective MDCK cell lines. Furthermore, the effects of inhibition of the ERK-MAPK pathway or the PI3K pathway on the localisation of β-catenin and on the morphological appearance of the cells was analysed. Therefore, the different MDCK cell lines were treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY294002 for 8h prior to immunostaining with β-catenin. The immunostained cell samples were analysed using confocal laser scanning microscopy (Figure 3.2). Optical sections were obtained at 0.5 μm intervals and three-dimensional stacks of images were generated.

V12Ras and p110-CAAX expressing cells show little β-catenin staining at the intercellular contacts compared to wild type MDCK cells. In Raf-CAAX expressing cells, β-catenin staining is also reduced at the sites of cell-cell contacts compared to the control cells. Blocking the activation of the ERK-MAPK by treatment with the MEK inhibitor PD98059 restores β-catenin localisation to the sites of cell-cell contacts in all three cell lines, indicating that activation of the Raf-MAPK pathway is required to reduce the tightness of intercellular contacts. Treatment with the PI3K inhibitor LY294002 also restored the β-catenin staining and reverts the dispersed phenotype in MDCK cells expressing V12Ras and p110-CAAX. In the case of Raf-CAAX, inhibition of basal PI3K activity with LY294002 did not restore β-catenin staining and cells were still spread. Although it was reported that increased cytoplasmic levels of β-catenin can translocate to the nucleus forming aggregates with transcription factors (Simcha et al., 1998) and that activated Ras can increase the cytoplasmic pool of β-catenin by reducing E-cadherin/β-catenin complexes (Kinch et al., 1995), specific translocation of β-catenin to the nucleus in the presence of V12Ras in MDCK cells could not be observed under the conditions used here. This might be explained by the finding that nuclear translocation of β-catenin was demonstrated only after ectopic expression of β-catenin (Behrens et al., 1998); (Ikeda et al., 1998); (Simcha et al., 1998) or in melanoma cell
Figure 3.2. Effects of stable expression of V12Ras, Raf-CAAX and p110-CAAX on cell-cell adhesion in MDCK cells. MDCK clones stably expressing V12Ras, Raf-CAAX and PI3-kinase (p110-CAAX) were treated with the MEK inhibitor PD98059 (30 μM), the PI3-kinase inhibitor LY294002 (20 μM) or diluent control for 12 h. Clones were immunostained with an antibody recognising β-catenin and analysed by confocal laser scanning microscopy. Confocal images show three dimensional stacks of horizontal sections. These images represent three independent experiments using three independent clones for each construct.
lines with abnormal high β-catenin expression levels (Rubinfeld et al., 1997). However, it is not yet clear whether breakdown of cell-cell contacts is directly linked to β-catenin/LEF-1 mediated gene expression.

3.2.2 Effects of expression of activated signalling proteins on tubule formation

Since activation of Raf-1 in MDCK cells seems to reduce cell-cell adhesion and PI3K seems to regulate motility, it was examined which of the Ras effector pathways may contribute to tubular morphogenesis in MDCK cells. Therefore, wild type MDCK cells and MDCK cells expressing V12Ras, Raf-CAAX and p110-CAAX were grown in collagen gels and analysed by phase-contrast microscopy after 8 days in culture (Figure 3.3). Whereas wild type MDCK cells grow as hollow cystic structures, treatment with HGF/SF induced the formation of branched tubular structures. MDCK cells expressing p110-CAAX mimick this phenotype, indicating that activation of the PI3K pathway is required and sufficient to induce branched tubular structures. In contrast, both V12Ras and Raf-CAAX expressing MDCK cells did not grow in cystic or tubular structures, but grew in small cell clusters. This indicates that the cells lack the ability to form tight cellular junctions in the presence of activated Ras or Raf. Interestingly, activation of the PI3K pathway through V12Ras was not capable of inducing tubulogenesis.

3.2.3 Inhibition of MAPK activity by PD98059 induces tubule formation of V12Ras expressing MDCK cells grown in collagen gels

While expression of activated Ras and Raf-1 in MDCK cells caused dispersed growth of small cell clusters in collagen gels with little cell-cell interaction, expression of activated PI3K in MDCK cells caused tubulogenesis. Since Ras induces activation of both signalling pathways, it was asked whether V12Ras does not sufficiently activate PI3K to induce tubulogenesis.

In order to address this question, MDCK cells expressing V12Ras were treated with different concentrations of the MEK inhibitor PD98059 in collagen gels to modulate the levels of ERK-MAPK activation (Figure 3.4). Moderate inhibition of MAPK activity by treatment with 6 μM PD98059 changed the phenotype of V12Ras expressing MDCK cells and led to the formation of branched tubules in V12Ras expressing MDCK cells. High levels of PD98059 (30μM), resulting in strong inhibition of ERK-MAPK activity, led to formation of
**Figure 3.3.** Effect of expression of activated signalling proteins on tubule formation in collagen gels. MDCK wild type cells cultured in the presence or absence of HGF/SF (10 ng/ml) and MDCK clones stably expressing activated forms of V12Ras, Raf-CAAX and PI3-kinase (p110-CAAX) were grown in type I collagen matrices and phase contrast Images were taken after 8 days in culture. (Figure kindly provided by A.Khwaja.)
Figure 3.4. Tubule formation of MDCK V12Ras cells grown in collagen gels is induced by treatment with low dose of the MEK inhibitor PD98059. MDCK cells stably expressing V12Ras grown in collagen type I matrices were treated with the indicated concentrations of the MEK inhibitor PD98059 (30 μM) for 6 days. Phase contrast images were taken after 6 days in culture. (Figure kindly provided by B. Marte).
small cystic structures, similar to the phenotype observed in MDCK wild type cells. The reduced size of cell cysts might be due to inhibition of cell proliferation by suppressing ERK-MAPK activation. This experiment shows that strong activation of the Raf/MAPK pathway downstream of activated Ras prevents tubulogenesis, possibly due to disruption of intercellular contacts. From this it can be concluded that in MDCK cells expressing V12Ras, the Raf/MAPK effect can override PI3K induced tubulogenesis.

3.3 Discussion

Here it was demonstrated that constitutive activation of PI3K is sufficient to cause scattering of MDCK cells. In contrast, expression of activated Raf-1 (Raf-CAAX) in MDCK cells, which activates the MAPK pathway, fails to cause scattering but induces reduced cell-cell contacts (Khwaja et al., 1997). Although basal MAPK activation is required, it is not sufficient for MDCK cell scattering. Furthermore, expression of activated Ras, Raf-1 and PI3K show reduced β-catenin localisation to cell-cell junctions. Whereas inhibition of MAPK activity by PD98059 led to restoration of β-catenin to the sites of cell-cell contacts in all three cell lines, LY294002 did not induce relocalisation of β-catenin to the cell junctions when activated Raf-1 was expressed in the cells. The results from this study led to the conclusion that the primary role of the Raf/ERK-MAPK pathway is to reduce cell-cell contacts, whereas the PI3K pathway is required for the induction of motility, provided that basal MAPK activity is not inhibited.

Activation of both the Raf-1/MAPK pathway and PI3K are required for scattering and both can be induced by HGF/SF treatment (Ponzetto et al., 1994). There is evidence that activation of the ERK-MAPK pathway mediates the breakdown of intercellular junctions, since expression of a dominant negative version of MEK1 or treatment with PD98059 blocked the loss of adherens junctions in MDCK cells in response to HGF/SF (Potempa and Ridley, 1998). Furthermore, activation of c-Fos induced rapid dissociation of adherens junctions (Reichmann et al., 1992). Another report demonstrated that ERK-MAPK can induce cell contractility required for migration by phosphorylation of myosin light chain kinase (MLCK) (Klemke et al., 1997).

HGF/SF can induce branching tubular structures of MDCK cells grown in collagen gels, which can be prevented by inhibition of MAPK activity by PD98059 (Khwaja et al., 1998). Activation of MAPK during HGF/SF induced tubulogenesis might be required for the
remodelling of the extracellular matrix (ECM), since the Raf/ERK-MAPK pathway was found to induce transcription of a number of proteases involved in degradation of ECM components, including uPA, uPAR and matrix metalloproteases (MMPs) (reviewed (Chambers and Matrisian, 1997)).

Whereas expression of activated PI3K is sufficient to induce tubulogenesis in MDCK cells, constitutive activation of the ERK-MAPK pathway by expression of Raf-CAAX or V12Ras led to formation of small cell clusters in collagen gels. Thus, activation of the Raf/ERK-MAPK pathway decreases cell-cell contacts which abrogates the formation of complex multicellular structures. This overrides the morphogenic effects of PI3K in MDCK cells expressing V12Ras. Raf-MAPK needs to be sufficiently activated to promote proliferation and allow remodelling of intercellular contacts. However, excessively strong activation results in loss of cell-cell adhesion and this seems to prevent formation of branching tubular structures.

While expression of activated PI3K was sufficient to induce scattering in MDCK cells, the expression of the PI3K downstream effectors Akt and the small GTPase Rac did not mimic this response. MDCK cells expressing activated forms of Akt or Rac or both grow as cystic structures in collagen gels (Khwaja et al., 1998). Rac has been found previously to affect cell motility by inducing lamellipodia formation and dominant negative Rac (N17Rac1) is able to block HGF/SF and Ras induced disruption of adherens junctions and scattering in MDCK cells (Ridley et al., 1995); (Potempa and Ridley, 1998). This suggests that other PI3K downstream effector pathways might be involved in scattering and tubulogenesis in MDCK cells. PI3K is known to activate a number of other kinases such as p70S6-kinase (Pullen et al., 1998), p21 activated kinase PAK (King et al., 2000) and p90Rsk2 (Jensen et al., 1999), suggesting that further investigation will be required to elucidate the relevance of other signalling pathways. Alternatively, the activation of different Rac family members might be essential and might not have been achieved in this experimental setup.

These results support the biological effects of HGF/SF in vivo. HGF/SF mediated invasiveness, which has been observed in a number of tumours, might be reflected by the Ras phenotype, supported by the finding that oncogenic ras often co-operates with HGF/SF in promoting invasiveness (Di Renzo et al., 1995). On the other hand, activation of the PI3K pathway seems to be the key regulator for tubulogenesis. Activation of PI3K can also be achieved independently of Ras directly through the HGF/SF receptor c-Met, and blocking of this pathway was shown to abolish tubulogenesis (Maroun et al., 1999).
Chapter 4

4 Role of the Raf/MAPK pathway in MDCK cell transformation

4.1 Introduction

In a high proportion of tumours of epithelial origin the ras oncogene has acquired activating mutations which can promote cell transformation. Many carcinomas with activated ras have undergone an epithelial to mesenchymal transition, termed EMT, in which the epithelial phenotype, characterised by strong cell-cell adhesion and cell polarity, is lost and a mesenchymal phenotype is acquired, characterised by reduced cell-cell contacts and increased motility (Schoenenberger et al., 1991); (Birchmeier et al., 1993); (Hay, 1995). While the mechanism by which Ras promotes EMT is not fully understood, it is presumably involved in the establishment of tumour invasiveness a prerequisite for metastasis. It has been reported that mouse mammary epithelial cells expressing activated Ras (Ep2Ras), respond to transforming growth factor (TGF)β by undergoing EMT and then continue to maintain the mesenchymal phenotype by producing TGFβ in an autocrine manner (Oft et al., 1996). Furthermore, there is evidence that TGFβ functions to promote tumour progression from a squamous cell to spindle cell carcinoma in a mouse skin cancer model through co-operation with the Ras signalling pathway (reviewed in (Akhurst and Balmain, 1999)). Moreover, many advanced human tumours were found to produce TGFβ. In apparent contradiction to the role of TGFβ in cancer progression, it is also a well documented tumour suppressor since TGFβ can inhibit cell growth and promotes apoptosis (Oberhammer et al., 1992). TGFβ inhibits cell cycle progression by inducing expression of the CDK inhibitors p21Cip1, p21Kip1 and p15INK4B (Hannon and Beach, 1994); (Reynisdottir et al., 1995) and by repression of cyclinA (Feng et al., 1995) and the CDK activating phosphatase cdc25A (Iavarone and Massague, 1997).

The aim of the following work was to study the contribution of the Raf-MAPK pathway to changes in epithelial cell morphology during cell transformation, since Ras was found to induce disruption of cell-cell junctions which is a prerequisite for EMT. There is evidence that TGFβ can promote cell transformation by co-operating with activated Ras, but the mechanism is still unclear. It has been reported that the ERK-MAPK pathway activated by
oncogenic ras antagonises TGFβ/Smad signalling by blocking Smad translocation into the nucleus (Kretzschmar et al., 1999). However, this model leaves little room for effects of TGFβ on the promotion of tumourigenesis such as the induction of invasive growth and metastasis formation, which required the integrity of the TGFβ signalling pathway (Oft et al., 1998); (Yin et al., 1999). Because of these apparent contradictions, the interactions between the Ras/Raf/MAPK pathway and TGFβ signalling was analysed. To selectively examine the ERK-MAPK pathway, an inducible Raf-1 fusion protein was stably expressed in MDCK cells, leading to activation of ERK-MAPK by addition of 4-hydroxy-tamoxifen (Woods et al., 1997). A major advantage of this inducible form of Raf-1 compared to MDCK cells stably expressing Raf-CAAX is the possibility to study the progression of Raf induced effects.

4.2 Results

4.2.1 Activation of Raf:ER leads to rapid phosphorylation of p42MAPK in MDCK cells.

To investigate the role of Raf-1 kinase in the regulation of epithelial cell morphology, we generated epithelial MDCK cells stably expressing an inducible EGFP-ΔRaf-1:hbER(DD) fusion protein (Woods et al., 1997). This chimeric protein (abbreviated Raf:ER) comprises the kinase domain of Raf-1 containing the conserved region 3 (CR3) but lacking the regulatory regions with the Ras binding site, which functions as a constitutively active protein (ΔRaf-1). In addition the two tyrosine phosphorylation sites Y340 and Y341 are substituted by aspartic acid residues (DD), which potentiates the activity of Raf-1 (Bosch et al., 1997). ΔRaf-1 was made regulatable by fusion of the hormone binding domain (hb) of the human estrogen receptor (ER) to its the C-terminus (Samuels et al., 1993). Addition of 4-hydroxy-tamoxifen (4HT), an 17-β-estradiol analogue, releases the inhibition of Raf-activity by the hormone binding domain causing rapid activation of the chimeric protein. At the N-terminus Δ-Raf-1 is fused to enhanced green fluorescent protein (EGFP) allowing detection of the fusion protein in live cells.

Firstly, MDCK Raf:ER cells were tested for activation of the ERK-MAPK pathway. Cells were starved for 24 hours and treated with 100 nM 4-hydroxy-tamoxifen (4HT) for different times. Lysates were assayed for phosphorylation of p42ERK2-MAPK (p42MAPK) and
expression of the Raf:ER fusion protein by SDS-PAGE and Western blotting (Figure 4.1A).

Activation of the Raf:ER fusion protein results in a rapid and sustained phosphorylation of p42MAPK, as demonstrated by reduced electrophoretic mobility (MAPK shift), which was already detectable 15 min after stimulation and increased with time. In contrast, stimulation with EGF led to a more rapid but only transient phosphorylation of MAPK (data not shown). Interestingly, the abundance of the Raf:ER fusion protein increased after 16 hours of 4HT treatment, correlating with a further increase of phosphorylated p42MAPK. This increase in Raf:ER levels probably reflects stabilisation of the fusion protein by 4HT binding (Samuels et al., 1993). Control cells carrying empty vector (mock) showed no phosphorylation of MAPK in response to 4HT treatment (Figure 4.1B). Pre-treatment of the MDCK Raf:ER cells with the MEK inhibitor PD98059 completely abolished MAPK phosphorylation (Figure 4.1B). In contrast, MDCK cells stably expressing Raf-CAAX, which is constitutively activated due to membrane targeting (Khwaja et al., 1997), showed a low level of MAPK phosphorylation, similar to the signal resulting from 30 min 4HT treatment of MDCK Raf:ER (Figure 4.1C). After additional exposure to EGF, a further increase of MAPK phosphorylation was detected. In order to compare the Raf:ER system with a physiological activator of the Raf-MAPK, MDCK cells were treated with HGF/SF for different times and the kinetics of MAPK phosphorylation was examined. Figure 4.1D shows that stimulation with HGF/SF led to a much weaker and only transient activation of MAPK phosphorylation relative to Raf:ER activation. This transient activation of MAPK might be due to internalisation of the HGF/SF receptor c-Met, which occurs after long-term exposure to the ligand and renders MDCK cells insensitive to HGF/SF stimulation (Naka et al., 1993).

4.2.2 Induction of morphological changes following Raf activation in MDCK cells.

Having established the inducible Raf:ER fusion protein in MDCK cells, the morphological effects caused by Raf-1 activation were examined (Figure 4.2). Since HGF/SF is a physiological activator of the Raf/MAPK pathway that causes cell scattering, HGF/SF induced morphological effects were compared to Raf:ER effects. MDCK Raf:ER cells were treated with 4HT or HGF/SF and phase contrast pictures were taken before and after Raf-1 activation. While MDCK Raf:ER cells in the absence of 4HT grow in compact islands like wild type cells, activation of Raf:ER with 4HT for 24 hours leads to cell scattering, similar to that observed with HGF/SF. The observation that activation of Raf:ER causes scattering, but expression of Raf-CAAX in MDCK cells only leads to disruption of cell-cell contacts (see
Figure 4.1. Activation of Raf leads to rapid phosphorylation of p42MAPK in MDCK cells. (A) MDCK cells expressing Raf:ER were serum starved in DMEM + 0.5% BSA for 24 hours prior to treatment with 100 nM 4-hydroxy-tamoxifen (4HT) for the indicated times. Cytoplasmic lysates were assayed by Western blotting for the expression of Raf:ER fusion protein using an anti-estrogen receptor antibody and for the mobility shift of p42MAPK to the phosphorylated active form. (B) Control MDCK cells carrying empty vector (ev) and MDCK Raf:ER cells were serum starved for 24 h and either untreated or treated with 100 nM 4HT for 1 h. The MEK inhibitor PD98059 (30 μM) was applied 20 min prior to 4HT treatment where indicated. p42MAPK activity was assayed by immunoblotting. (C) MDCK cells stably expressing Raf-CAAX or Raf:ER were serum starved for 24 h and either untreated or treated with 20 ng/ml EGF for 5 min. (D) MDCK Raf:ER cells were serum starved for 24 h and stimulated with 10 ng/ml HGF/SF for the indicated time periods. p42MAPK activity was assayed by Western blotting as above.
Figure 4.2. Activation of Raf leads to induction of morphological changes in MDCK cells. MDCK Raf:ER cells were either untreated or treated with 100 nM 4HT for 24 h or 14 days or with 10 ng/ml HGF/SF for 24 h. MDCK cells expressing V12Ras were examined by phase contrast microscopy. Shown are representative phase contrast images.
Chapter 3), might be due to the strength of MAPK activation by Raf:ER (see Figure 4.1). Strong and sustained activation of the MAPK pathway is sufficient to cause disruption of intercellular contacts and cell migration.

Interestingly, the onset of both HGF/SF and Raf:ER mediated morphological changes occurred with similar kinetics as observed by time lapse video microscopy (data not shown). Treatment with HGF/SF or 4HT led to disruption of cell-cell contacts by 8 hours and cell motility increased within 12 hours. While the HGF/SF mediated scattered phenotype is completely reverted after 3 days in the presence of ligand (data not shown), sustained Raf-1 activation leads to further changes in cell morphology. Cells become elongated and spindle-shaped, indicating a conversion from the epithelial to a mesenchymal phenotype, which was maintained after 14 days of 4HT treatment. Pre-treatment of the cells with the MEK inhibitor PD98059 (30μM), the PI3K inhibitor LY294002 (20μM) (Figure 4.3) or the protein synthesis inhibitor cycloheximide (10 ng/ml) (data not shown) completely blocked the Raf-1 induced scattering response. These findings suggest that basal PI3K activity as well as protein synthesis is required for Raf-induced cell scattering (Boyer et al., 1997); (Khwaja et al., 1998).

In addition, to test whether the acquisition of the mesenchymal phenotype due to sustained Raf:ER activation resembles cell transformation, cells were tested for their ability to grow in soft agar. Growth in soft agar reflects anchorage-independent growth, which is considered as a criteria for cell transformation (reviewed in (Hanahan and Weinberg, 2000)). As a positive control for anchorage independent growth, MDCK cells expressing V12Ras were used (Rodriguez-Viciana et al., 1997). It has been shown previously that H-Ras expression in MDCK cells has transforming potential, since these cells are able to form tumours in nude mice (Mareel et al., 1991). Sustained activation of Raf:ER in MDCK cells resulted in colony formation in soft agar, similar to MDCK cells expressing V12Ras, indicating that sustained Raf:ER activation in MDCK cells is sufficient to mediate cell transformation.

<table>
<thead>
<tr>
<th>Raf:ER</th>
<th>Raf:ER + 4HT&gt;14d</th>
<th>V12Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of colonies</td>
<td>26 ± 2</td>
<td>421 ± 21</td>
</tr>
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</table>

Table 1. Long-term activation of Raf:ER in MDCK cells enables growth in soft agar.
4.2.3 Effects of Raf:ER expression in MCF-10A cells

In order to compare the morphological effects of Raf:ER in another epithelial cell system, the chimeric protein was expressed in the untransformed human luminal mammary epithelial cell line MCF-10A (Soule et al., 1990). MCF-10A Raf:ER cells were generated using ecotropic retroviral infection as described above. After selection with puromycin and cell sorting for GFP, a cell pool expressing Raf:ER was obtained. To confirm that Raf:ER was functionally active, MCF-10A Raf:ER cells were grown in the absence of growth factors for 24h and subsequently treated with 100 nM 4HT for different times (Figure 4.4.A). Standard cell lysates were separated by SDS-PAGE and analysed for activation of MAPK by Western blotting, using an antibody which recognises both phospho-ERK2-p42MAPK and phospho-ERK1-p44MAPK. As a loading control, the expression of total ERK2-p42MAPK was analysed. Addition of 4HT led to a rapid and sustained activation of ERK-MAPK. In contrast, stimulation with EGF led to a more rapid but only transient phosphorylation of ERK2-p42MAPK and ERK1-p44MAPK. To test whether activation of ΔRaf:ER affects the epithelial cell morphology MCF-10A Raf:ER cells were treated with 4HT and phase contrast pictures were taken before and after Raf-1 activation (Figure 4.4 B). While MCF-10A Raf:ER cells in the absence of 4HT grow in colonies with tight cell-cell contacts, activation of Raf:ER with 4HT for 24 hours leads to cell scattering. This indicates that sustained activation of the MAPK pathway is sufficient to cause disruption of intercellular contacts and cell migration in MCF-10A cells.

4.2.4 Activation of Raf:ER leads to repression of epithelial marker proteins and induction of a mesenchymal marker protein

Conversion from an epithelial to a mesenchymal phenotype is characterised by decreased organisation of intercellular junctions, loss of epithelial characteristics and gain of mesenchymal characteristics (Hay, 1995); (Birchmeier et al., 1993). To investigate Raf:ER-mediated changes in cell morphology, immunostaining of epithelial marker proteins and a mesenchymal marker protein was performed and analysed by confocal laser scanning microscopy (Figures 4.5 and 4.6). Optical sections were obtained at 0.25 μm intervals and three-dimensional stacks of images were generated.

The immunostaining pattern showed that in unstimulated MDCK Raf:ER cells the adherens junction protein E-cadherin and the tight junction component ZO-1 are localised to
Figure 4.3. Morphological changes induced by activation of Raf are blocked by the MEK inhibitor PD98059 or the PI3-kinase inhibitor LY294002. MDCK Raf:ER cells were either untreated or treated with 100 nM 4HT for 24 h. The MEK inhibitor PD98059 (30 µM) or the PI3-kinase inhibitor LY294002 (20 µM) were applied 20 min before 4HT treatment. Cells were examined by phase contrast microscopy.
Figure 4.4. Activation of Raf:ER in MCF-10A cells leads to rapid phosphorylation of p42MAPK and induction of morphological changes. (A) MCF-10A Raf:ER cells were treated with 100 nM 4HT or 20 ng/ml EGF as indicated. Total lysates were separated by 10% SDS-PAGE. Phosphorylation of p42MAPK and p44MAPK was detected with a phosphospecific antibody (upper panel) by Western blotting. Expression of p42MAPK was monitored with a pan ERK antibody (lower panel). (B) MCF-10A Raf:ER cells were either untreated or treated with 100 nM 4HT for 24 h and examined by phase contrast microscopy.
intercellular junctions (Figure 4.5 a and e). Moreover, in vertical sections (Figure 4.7), uninduced MDCK cells exhibited a polarised phenotype as demonstrated by localisation of ZO-1 at the apical cell borders above the E-cadherin localisation.

Within 4 days of 4HT treatment (Figure 4.5b), ZO-1 was largely removed from the cell-cell junctions and diffusely expressed over the cell surface, which was already visible after 48 hours of 4HT treatment (data not shown). This effect was even more pronounced after 6 days of 4HT treatment (Figure 4.5c) and in Raf-1 transformed MDCK Raf:ER cells following sustained Raf-1 activation (Figure 4.5d). After 4HT addition for 4 days E-cadherin was present at the intercellular contacts (Figure 4.5f), which were now much more loosely formed. E-cadherin was also present on the cell surface, as observed in the vertical section (Figure 4.7). In contrast, after sustained Raf-1 activation the localisation of E-cadherin became diffuse and much reduced (Figures 4.5g and h). These results indicate that activation of Raf:ER in MDCK cells leads to loss of adherens junctions and tight junctions.

As another marker for epithelial cells, the localisation of intermediate filament proteins was examined by using an antibody detecting cytokeratin 8 and 18. In the absence of 4HT (Figure 4.6a) cytokeratin forms a network of filaments inside the cell borders. When cells were treated with 4HT for 4 days (Figure 4.6b) or 6 days (Figure 4.6c), cytokeratin was removed from the cell borders and only a few filaments across the cell body were visible. Localisation of cytokeratin was completely diffuse and levels much reduced in the Raf-1 transformed MDCK Raf:ER cells (Figure 4.6d). Immunostaining for the mesenchymal marker protein vimentin, a cytoskeletal intermediate filament protein, shows low expression in the uninduced MDCK cells (Figure 4.6e), consistent with the observation that MDCK cells express some vimentin in contrast to many other epithelial cell lines (Zuk et al., 1989). Strong vimentin staining was visible after 6 days of 4HT treatment (Figure 4.6f) as well as in the Raf-1 transformed cells (Figure 4.6h). Similar findings were obtained by analysing the expression of E-cadherin and vimentin by Western blotting (Figure 4.8). In the absence of 4HT MDCK Raf:ER cells express high levels of E-cadherin but only little vimentin. After sustained Raf-1 activation for 14 days E-cadherin expression is reduced and completely abrogated after more than 14 days of 4HT treatment (RafT). This suggests that Raf-activation leads first to relocation of E-cadherin without changing its expression level. Only after prolonged Raf-1 activation E-cadherin expression is completely repressed (see figure 6.3). Expression of vimentin is strongly increased after sustained Raf-1 activation for more than 14 days (RafT).
Figure 4.5. Activation of Raf leads to repression of epithelial marker proteins. MDCK Raf:ER cells either untreated (a,e) or treated with 100 nM 4HT for 4 days (b,f), 6 days (c,g) or after sustained Raf activation (>14 days; d,h) were immunostained with antibodies recognising E-cadherin or ZO-1 and examined by confocal laser scanning microscopy. Confocal images show three-dimensional stacks of horizontal sections.
Figure 4.6. Activation of Raf leads to repression of cytokeratin and expression of vimentin. MDCK Raf:ER cells either untreated (a,e) or treated with 100 nM 4HT for 4 days (b,f), 6 days (c,g) or after sustained Raf activation (>14 days; d,h) were immunostained with antibodies recognising cytokeratin or vimentin and examined by confocal laser scanning microscopy. Confocal images show three-dimensional stacks of horizontal sections.
**Figure 4.7. Effect of Raf activation on the polarized epithelial phenotype of MDCK cells.** MDCK Raf:ER cells either untreated (upper panel) or treated with 4HT for 4 days (lower panel) were immunostained with antibodies directed against E-cadherin (green signal) or ZO-1 (red signal) and examined by confocal laser scanning microscopy. Confocal images show three-dimensional stacks of vertical sections.

**Figure 4.8. Effects of sustained Raf activation on expression of E-cadherin and vimentin.** Lysates of MDCK cells carrying empty vector (C) and MDCK Raf:ER cells untreated or treated with 4HT for 14 days or more than 14 days (RafT) were assayed for the expression of E-cadherin and vimentin by Western blotting.
Taken together, these results show that a strong and sustained Raf-1 signal is sufficient to induce epithelial to mesenchymal transition (EMT), characterised by loss of apical-basal polarity, loss of epithelial marker proteins (E-cadherin, ZO-1, cytokeratin) and expression of the mesenchymal marker protein vimentin.

4.2.5 Activation of Raf in MDCK cells induces secretion of TGFβ

It has been shown that the process of EMT is accompanied by secretion of TGFβ, which is required to maintain the transformed phenotype and promote invasion (Oft et al., 1996). Therefore it was studied whether Raf:ER activation is able to induce secretion of TGFβ. MDCK Raf:ER cells were treated with 4HT for different times and cell culture supernatants were analysed for the presence of TGFβ by ELISA (Figure 4.9A). Treatment with 4HT for 48 hours led to a 3-fold up-regulation of TGFβ in the medium compared to the levels in medium conditioned by uninduced MDCK Raf:ER cells or control cells. After treatment of MDCK Raf:ER cells with 4HT for 6 days and in Raf-1 transformed MDCK Raf:ER cells the level of TGFβ protein in the medium was strongly increased (8-fold). Secretion of TGFβ in response to Raf:ER activation was also demonstrated by immunoblotting of concentrated cell culture supernatants (Figure 4.9B, lower panel). Up-regulation of TGFβ protein was detectable 24 hours after 4HT treatment relative to basal TGFβ level in uninduced MDCK Raf:ER cells or in control cells. Since it has been shown that the Raf/MAPK pathway can activate ECM degrading proteases which are required for cell invasiveness, it was examined whether Raf:ER activation can lead to secretion of the protease urokinase-type Plasminogen Activator (uPA). Activation of Raf:ER by 4HT induces secretion of uPA protein as analysed by Western blotting of the concentrated cell culture supernatants (Figure 4.9.B, upper panel). The onset of uPA secretion was already detectable after 8 hours treatment with 4HT and increased with time in a similar manner as observed for TGFβ secretion.

The biological activity of the secreted TGFβ induced by Raf-1 was assayed using an electrophoretic mobility shift assay (EMSA) of a 32P-labeled c-jun probe, shown to bind TGFβ-induced activated Smad3 in complex with Smad4 (Wong et al., 1999). The ability of 3-times concentrated cell culture supernatant from untreated MDCK Raf:ER cells (cm) and Raf-transformed MDCK Raf:ER cells (cmRafT) to induce TGFβ signalling in control MDCK cells was compared. The results (Figure 4.10.A, lanes 1-4) showed that supernatant from Raf-transformed cells induced formation of a complex that binds the c-jun probe (compare
Figure 4.9. Activation of Raf:ER in MDCK cells induces secretion of TGFβ1. (A) MDCK cells carrying empty vector (ev), MDCK Raf:ER cells treated with 100 nM 4HT as indicated and MDCK Raf:ER cells transformed by long-term treatment with 4HT (RafT) were cultivated in DMEM + 2% FCS for 24h. Subsequently, cells were cultured in DMEM + 0.5% BSA for a further 24h before collecting the supernatant. Cell culture supernatants were examined for TGFβ1 by ELISA. The values shown are normalised for cell numbers and represent the average of three experiments performed in duplicates.

(B) MDCK cells carrying empty vector (ev), MDCK Raf:ER cells treated with 100 nM 4HT as indicated and MDCK RafT cells were cultured in DMEM + 2% FCS. The cell culture supernatant was concentrated 10 times by ultrafiltration and equal aliquots were separated by SDS-PAGE under non-reducing conditions and analysed for TGFβ1 and urokinase-type Plasminogen Activator (uPA) by Western blotting.
Figure 4.10. Activation of Raf leads to secretion of functionally active TGFβ. MDCK cells carrying empty vector (ev) were either untreated or treated with 2 ng/ml TGFβ for 1 h, with conditioned medium of unstimulated MDCK Raf:ER cells (cm) or with conditioned medium of MDCK RafT cells (cmRafT). Conditioned medium used was concentrated 3 times by ultrafiltration. Nuclear extracts were assayed for the binding of activated Smad3/Smad4 complexes to a 32P-labelled c-jun oligonucleotide probe by EMSA (lanes 1-4). Untreated MDCK Raf:ER cells were stimulated with 2 ng/ml TGFβ for 1 h, and nuclear extracts were assayed in supershifts for the presence of Smads in complexes bound to the c-jun probe with following antibodies (lanes 5-11): anti-Smad3, anti-Smad4, anti-Smad2, cross-reacting with Smad3 (Smad2/3 (TL)) and anti-Smad2 (SED). Peptide competition was performed with anti-Smad3 (Smad3+pep). (B) The specificity of the anti-Smad2 antibody (TL) was examined by in vitro transcription/translation of Xenopus Smad2 (XSmad2) and human Smad3 (hSmad3) in reticulocyte lysate.
lanes 1 and 4). The same complex is induced by exogenously added TGFβ (compare lanes 2 and 4). To confirm that this complex was formed by TGFβ-activated Smad3 and Smad4, specific antibodies directed against Smad3, Smad4 and Smad2 were used (Figure 4.10.A, lanes 5-11). The TGFβ-induced complex was quantitatively supershifted in the presence of anti-Smad3 antibodies (lane 7), which was prevented by addition of the peptide used to raise the anti-Smad3 antibody (lane 8). The presence of Smad4 in the complex was confirmed using an anti-Smad4 antibody (lane 9).

Two different anti-Smad2 antibodies were used as a control to show that Smad2 is absent in the DNA-binding complex. Interestingly, the anti-Smad2 antibody (Transduction Laboratories) supershifted the Smad3/4 complex (lane 10), indicating that Smad2 is also present. Alternatively the antibody might cross-react with Smad3 since it was raised against the linker region of Smad2, a region of high homology shared by Smad2 and Smad3. In vitro transcription and translation of human Smad3 (hSmad3) and X.laevis Smad2 (Xsmad2) in reticulocyte lysate analysed by Western blotting revealed that this Smad2 antibody also detects Smad3 with a similar affinity (Figure 4.10.B), which explains its ability to supershift the Smad3/4 complex. However, a more specific anti-Smad2 antibody (SED), supershifted only a very small proportion of the complex (lane 11) indicating that it has only very weak cross-reactivity with Smad3 or, alternatively, that a small amount of Smad2 is present in the DNA-binding complex (Nakao et al., 1997).

Taken together these results indicate that Raf-1 induces secretion of the ECM degrading protease uPA and biologically active TGFβ, which are components known to play important roles in invasiveness and cell transformation.

4.2.6 Activation of Raf in MDCK cells leads to establishment of an invasive phenotype in collagen gels dependent on autocrine TGFβ1 stimulation

Since TGFβ is secreted during Raf-1 induced EMT, the role of TGFβ in this process was analysed. MDCK Raf:ER cells were cultured in collagen gels in the presence or absence of 4HT. Whereas wild type MDCK cells (Figure 4.11) and untreated MDCK Raf:ER cells (a, b) grew in typical lumen containing cysts (Khwaja et al., 1998), treatment with 4HT led to elongated, branched structures, growing in an invasive manner (c). Moreover, Raf-1 transformation also led to formation of invasive structures with cells growing in a more elongated cord-like manner (d). Interestingly, strong Raf:ER activation seems to be required
Figure 4.11. Activation of Raf:ER in MDCK cells leads to induction of an invasive phenotype in collagen gels dependent on autocrine TGFβ effects. Wild type MDCK cells (WT; a,e,i), MDCK Raf:ER cells either untreated (b,f,j) or treated (c,g,k) with 200 nM 4HT and MDCK Raf:ER cells transformed by long-term 4HT stimulation (RafT; d,h,l) were grown in type I collagen matrices for 6 days in the absence (a-d) or presence of 5 ng/ml TGFβ (i-l) or neutralising TGFβ antibodies (e-h) for a further 6 days in the absence of serum. Structures were photographed at 20x magnification. (Figure kindly provided by E.Janda).
for the formation of invasive structures, since MDCK cells expressing Raf-CAAX show
disruption of cell-cell contacts leading to growth in dispersed cell clusters (see Chapter 3,
Figure 3.3). To examine if blockade of TGFβ signalling would cause reversion of the
invasive phenotype, the ability of neutralising TGFβ antibodies to inhibit this process was
tested. MDCK RafiER cells were treated with 4HT for 6 days before TGFβ neutralising
antibodies were added. After growth for 6 days in the presence of antibodies, invasive cord-
like structures reverted into cystic structures (g, h), showing that TGFβ secretion is required
to sustain the invasive phenotype.

Since TGFβ is known to inhibit growth and to induce apoptosis in epithelial cells grown in
collagen (Oft et al., 1996) TGFβ effects on wild type and untreated MDCK Raf:ER cells that
had been cultivated in collagen gels for 6 days were examined. When cells were grown in the
presence of TGFβ for a further 6 days, the cystic structures were degraded and dissociated
into small cellular bodies (i, j). In contrast, Raf-1 expressing cells (k, l) maintained their
invasive phenotype in the presence of TGFβ. The induction of apoptosis by TGFβ and the
protection by Raf-1 will be further analysed and discussed in Chapter 5.

Together, these results indicate that activation of Raf:ER induces invasive growth and alters
cellular plasticity and renders cells insensitive to inhibitory TGFβ effects. Moreover,
autocrine TGFβ stimulation is required to maintain the Raf-1 transformed mesenchymal
phenotype.

4.2.7 Short-term activation of Raf does not prevent TGFβ induced cell cycle
arrest.

It was demonstrated that activation of Raf:ER overcomes the inhibitory effects of TGFβ in
collagen gels. In order to characterise which downstream effects initiated by TGFβ are
inhibited by Raf-1 activation, it was investigated whether Raf-1 activation interferes with
TGFβ-mediated growth arrest. Cell cycle distribution was examined in MDCK Raf:ER cells
24 hours after exposure to TGFβ (Figure 4.12.A). In MDCK Raf:ER cells pre-treated with
4HT for 24 or 48 hours, the exposure to TGFβ led to an increased percentage of cells in G1
(67%) and a reduced percentage of cells in S-phase (9%), comparable to the TGFβ effect in
4HT untreated cells (G1:63%, S:20%). In contrast, cells transformed by prolonged activation
of Raf (RafT) were insensitive to TGFβ-induced growth arrest and no significant changes in
cell cycle distribution were observed relative to untreated cells. Since it has been shown that
Figure 4.12. TGFβ induced cell cycle arrest is not prevented by short-term activation of Raf but is in Raf transformed MDCK cells. (A) MDCK Raf:ER cells either untreated or pretreated with 100 nM 4HT for 24 h or 48 h and MDCK Raf:ER cells transformed by long-term exposure to 4HT (RafT) were stimulated with TGFβ (7.5 ng/ml) for 24 h. The cell cycle distribution was assayed by flow cytometry after propidium iodide staining. (B) MDCK Raf:ER cells either untreated or pretreated with 100 nM 4HT for 24 h and MDCK Raf:ER cells transformed by long-term treatment with 4HT (RafT) were stimulated with TGFβ (7.5 ng/ml) for the indicated time periods. Total cell lysates were assayed for cyclin A expression by Western blotting.
TGFβ induces down-regulation of cyclin A in epithelial cells (Feng et al., 1995) the influence of TGFβ on expression of cyclin A was investigated (Figure 4.12.B). In proliferating cells, cyclin A is up-regulated in late G1 and indicates cell cycle progression into S-phase. Asynchronously growing MDCK Raf:ER cells were treated with 4HT for 24 hours or left untreated before adding TGFβ for 24 or 48 hours and cell lysates were analysed by Western blotting. Cyclin A was highly expressed in the absence of TGFβ and pre-treatment with 4HT for 24 hours did not alter the expression level, whereas addition of TGFβ led to a dramatic decrease in cyclin A expression in both 4HT treated and untreated cells. Consistent with the data obtained above, no growth arrest was induced in Raf-1 transformed cells (RafT), where the expression of cyclin A was not changed by TGFβ.

Together, these findings suggest that TGFβ-induced growth arrest is not perturbed by Raf-1 activation over a period of a few days, whereas long term Raf-1 transformed MDCK cells show a loss in the anti-proliferative response to TGFβ.

4.2.8 Activation of Raf does not affect the TGFβ-induced Smad-dependent transcriptional response.

As shown before activation of Raf:ER in MDCK cells can lead to secretion of TGFβ which is necessary to maintain the transformed state. At the same time activated Raf-1 renders the cells insensitive to the pro-apoptotic effects of TGFβ, suggesting that activation of Raf-1 might directly affect the TGFβ/Smad signalling pathway. A study by Kretzschmar et al. showed that the ERK-MAPK pathway activated by oncogenic ras in a mouse epithelial cell line (EpRas) antagonises TGFβ/Smad signalling by blocking Smad translocation into the nucleus and thus inhibits the TGFβ-induced transcriptional response (Kretzschmar et al., 1999). But inhibition of TGFβ/Smad signalling would contradict the finding that TGFβ is required for invasive growth in collagen gels. Furthermore TGFβ can promote invasiveness in many advanced tumours (Derynck et al., 1985); (Arrick et al., 1992). It was therefore important to analyse whether activation of Raf-1 interferes directly with the TGFβ-Smad pathway in MDCK Raf:ER cells. Several aspects of TGFβ signalling were studied, namely the translocation of Smads into the nucleus (Figure 4.13), the binding of Smads to DNA (Figure 4.14) and finally the Smad-dependent transcriptional response to TGFβ (Figure 4.15).

To examine the nuclear translocation of Smads MDCK Raf:ER cells, untreated (mock) or pre-treated with 4HT for the indicated times and MDCK V12Ras cells, stably expressing
constitutively activated Ras, were stimulated with TGFβ for 1 hour (Figure 4.13). Nuclear extracts were prepared and immunoblotted with antibodies directed against Smad2, 3 and 4. A clear TGFβ-dependent Smad4 signal was observed in nuclear extracts that have been treated with TGFβ for 1 hour (Figure 4.13, top panel). Only very little Smad4 was detected in the nucleus in the absence of a TGFβ signal, whereas exposure to TGFβ led to high accumulation of Smad4 in the nucleus and the same result was obtained for Smad2 and Smad3 (Figure 4.13, second panel). Pre-treatment of the MDCK Raf:ER cells with 4HT for 1, 8 or 24 hours did not impair TGFβ-dependent Smad translocation into the nucleus. MDCK cells constitutively expressing the activated form of Ras (MDCK V12Ras) also showed clear nuclear translocation of Smad2, Smad3 and Smad4 in response to TGFβ. As a control for Raf:ER and Ras activation in these cells, the phosphorylation of p42MAPK in nuclear extracts was assayed by Western blotting (Figure 4.13, third panel). Upon Raf-1 activation, the phosphorylated form of p42MAPK is readily detectable in the nucleus and is not affected by exposure to TGFβ. The expression of PCNA, a nuclear protein involved in DNA replication, was detected by Western blotting (Figure 4.13, lower panel) to show equal protein loading of nuclear extracts. In addition, Western blotting analysis of nuclear extracts showed no contamination with the cytosolic adapter protein Grb2 (data not show). This experiment shows that activation of the Ras/Raf/MAPK pathway does not prevent TGFβ-induced Smad translocation into the nucleus.

Next, the same nuclear extracts were used to examine the ability of the translocated Smads to bind DNA, a prerequisite for gene activation. Therefore the 32P-labeled c-jun probe was used to detect Smad3/4 complexes bound to DNA in an electrophoretic mobility shift assay (EMSA). A Smad3/4-DNA complex was only observed in nuclear extracts from cells which had been treated with TGFβ for one hour (Figure 4.14, lanes 2, 4, 6, 8 and 10). Again, Raf-1 or Ras activation did not prevent nuclear Smad3 and Smad4 binding to DNA. Furthermore, the mouse mammary epithelial cell line expressing activated Ras (Ep2Ras) and control cells (EpH4), which were used in the study by Kretzschmar et al. (Kretzschmar et al., 1999) were also tested in this assay. Nuclear extracts from EpH4 and Ep2Ras cells treated with TGFβ for one hour were analysed by EMSA. Expression of activated Ras did not impair binding of Smad3 and Smad4 to DNA (Figure 4.14, lanes 11-14).

Finally, it was studied whether activation of Raf:ER affects the transcriptional activity of Smads in response to TGFβ (Figure 4.15). It has been shown that activated Smad2 and Smad4 are recruited by transcription factors to regulatory regions of TGFβ target genes. For
Figure 4.14. TGFβ induced DNA binding of Smad3/Smad4 complexes is not affected by Raf or Ras activation. MDCK Raf:ER cells either untreated or pretreated with 100 nM 4HT for the indicated time periods, MDCK V12Ras cells, EpH4 cells and Ep2Ras cells were stimulated with TGFβ (2 ng/ml) for 1h. Activated Smad3/Smad4 binding to a 32P-labelled c-jun probe was examined in nuclear extracts by EMSA.
Figure 4.13. Activation of Raf does not affect TGFβ-induced nuclear translocation of Smad2, Smad3 and Smad4. MDCK Raf:ER cells pretreated with 100 nM 4HT where indicated and MDCK V12Ras cells were exposed to TGFβ (2 ng/ml) for 1 h. Nuclear translocation of Smad4, Smad2 and Smad3 and activation of p42MAPK were detected in nuclear extracts by Western blotting. Expression of the nuclear protein PCNA was assayed to show equal loading of nuclear extracts.
Figure 4.15. TGFβ induced transcriptional activity is not affected by Raf activation.
MDCK Raf:ER cells were transiently transfected with ARE-reporter, pEF-XFast1 and pEF-lacZ. After treatment with 4HT for the indicated periods of time, cells were exposed to 2 ng/ml TGFβ for 6 h and activation of the ARE-Luc reporter was measured. Luciferase activity was normalized to the activity of the cotransfected β-galactosidase control plasmid. Data shown are the mean and average deviation from a representative of three independent experiments performed in duplicates.
example, activated Smad2 and Smad4 can be recruited to the activin/ TGFβ responsive element (ARE) of the Mix.2 promoter by the winged helix transcription factor FAST-1 and stimulate transcription in a signal-dependent manner (Huang et al., 1995); (Chen et al., 1996). To assay the transcriptional activity of the Smads in the presence of activated Raf, MDCK Raf:ER cells were transiently transfected with a plasmid encoding the luciferase reporter gene driven by three copies of the ARE. MDCK Raf:ER cells were pre-treated with or without 4HT for the indicated times and stimulated, or not, with TGFβ for 6 hours (Fig. 9). In 4HT untreated MDCK cells, luciferase activity was strongly increased (4.5-fold) in response to TGFβ, compared to relatively low basal luciferase activity in the absence of ligand. In MDCK Raf:ER cells pre-treated for 1, 8 or 24 hours with 4HT the TGFβ-induced luciferase activity was not changed.

Together, these data indicate that expression of V12Ras or Raf:ER activation in MDCK cells does not prevent the TGFβ-activated Smads from translocating into the nucleus, binding to DNA and activating gene expression indicating that the TGFβ-Smad signalling pathway is fully functional.

4.3 Discussion

4.3.1 Effects of an inducible form of Raf-1 on the cell morphology of epithelial MDCK cells.

As a way to assess the contribution of the Raf/ERK-MAPK pathway on epithelial cell morphology, the 4-hydroxy-tamoxifen (4HT) inducible Raf:ER fusion protein was stably expressed in the untransformed immortalised dog kidney epithelial line MDCK. Activation of Raf:ER which consequently activates the ERK-MAP kinases is sufficient to cause a change from an epithelial to a mesenchymal phenotype. Within 24 hours the cells spread and move apart in a scattering response similar to that seen with HGF/SF treatment. Time lapse microscopy revealed that the onset of disassembly of cell-cell contacts occurs as early as eight hours after Raf-1 activation, with similar kinetics as observed with HGF/SF. In contrast to the 4HT induced Raf:ER activation, MDCK cells expressing Raf-CAAX (Chapter3), (Khwaja et al., 1997); (Khwaja et al., 1998) did not display a scattered phenotype, although cell-cell contacts were partially disrupted. This difference might be caused by different levels of ERK activation. As shown in Figure 4.1 ERK2 activation in Raf:ER cells following 24
hours treatment with 4HT is much higher than the constitutive level in Raf-CAAX cells. Activation of Raf:ER provides a very strong ERK activation signal and it is possible that some of its effects may go beyond what the endogenous ERK pathway could normally achieve. Since activation of Raf:ER in MDCK cells is sufficient to induce anchorage-independent growth in soft agar, it might be possible that activation of Raf:ER resembles the function of oncogenic v-raf (Heidecker et al., 1990); (Huggett et al., 1990). In addition, differences in expression levels of either Raf-construct or their sub-cellular localisation might account for the distinct cellular responses.

4.3.2 Raf and EMT

Sustained activation of Raf:ER leads to an epithelial to mesenchymal transition (EMT) thereby losing expression of the adherens junction protein E-cadherin normally found expressed in epithelial cells and increased expression of the mesenchymal intermediate filament protein vimentin (reviewed in (Hay, 1995)). Furthermore, delocalisation of the tight junction protein ZO-1 and the intermediate filament cytokeratin is observed. The disruption of cell-cell junctions is accompanied by loss of the polarised epithelial phenotype resulting in loosely associated and non-polarised cells. For the disruption of cell-cell contacts by activation of Raf:ER a basal level of PI3K activity is required as well as de novo gene transcription since the PI3K inhibitor LY294002 and cycloheximide block the onset of the morphological changes induced by Raf:ER activation.

The disruption of cell junctions and the delocalisation of E-cadherin occurred well before changes in its expression level. This might be due to post-translational modifications such as tyrosine phosphorylation of E-cadherin and β-catenin, which leads to delocalisation from the cell junctions (Behrens et al., 1993); (Kinch et al., 1995). Furthermore, disruption of the integrity of tight junctions might also induce loss of adherens junctions, since a truncated form of ZO-1 which does not localise to the membrane was able to mediate disruption of adherens junctions subsequently inducing EMT (Reichert et al., 2000). A study by Li et al. showed that activation of Raf:ER leads to down-regulation of expression of the tight junction protein occludin which is suggested to mediate delocalisation of ZO-1 and E-cadherin (Li and Mrsny, 2000). Furthermore, the adherens junction protein β-catenin might play an important role during EMT, since epithelial cells which have undergone EMT showed increased β-catenin signalling (Eger et al., 2000). The role of β-catenin during Raf:ER induced EMT still needs to be elucidated. So far, loss of E-cadherin seems to play a key role in the
establishment of EMT and tumour progression in vivo (Behrens, 1999). Loss of E-cadherin expression was found to be induced either by mutational inactivation of the E-cadherin gene (Birchmeier and Behrens, 1994) or by DNA methylation of the promotor (Graff et al., 1995), a common mechanism for silencing of gene expression (Baylin and Herman, 2000). There is evidence that Raf-1 can associate with vimentin, and this interaction requires the kinase domain of Raf-1. Moreover, Raf-1 can regulate phosphorylation of vimentin, which is important for filament integrity. Interestingly, the phosphorylation of vimentin was shown to be ERK-MAPK independent (Janosch et al., 2000). Whether activation of Raf:ER might mediate these events in MDCK cells is not known so far.

Within two days following Raf:ER activation cells begin to synthesise and secrete TGFβ which is biologically active. Secretion and activation of TGFβ might be promoted by the accompanying up-regulation of urokinase-type plasminogen activator (uPA) expression. uPA activation of plasmin induces the activation of TGFβ from its latent form and increases the expression of matrix-metalloproteases (MMP) (Matrisian, 1992). This is required for the degradation of extracellular matrix components which is a prerequisite for invasive growth of tumour cells (Niedbala and Sartorelli, 1989); (Rosenthal et al., 1998); (Ghiso et al., 1999). Invasive growth of MDCK cells expressing activated Raf-1 in collagen gels can be blocked by neutralising antibodies against TGFβ. This indicates that autocrine TGFβ is required to maintain the invasive phenotype and Raf-1 induced EMT. There is a large body of evidence that TGFβ is an important modulator of EMT and cell invasion in advanced human tumours (reviewed in (Akhurst and Balmain, 1999)). Expression of a dominant negative form of the TGFβ receptor-typeII in highly metastatic mesenchymal carcinoma cells (Oft et al., 1998) and a metastatic keratinocyte cell line (Portella et al., 1998) inhibited invasive growth, led to the reversion of a mesenchymal phenotype to an epithelial one, or inhibited formation of bone metastasis in vivo (Yin et al., 1999).

The mechanism of Smad signalling for promoting cell invasion in advanced human tumours is not fully understood. It has been shown that TGFβ leads to expression of components of the extracellular matrix (ECM) and proteases involved in remodelling of the ECM, such as fibronectin, collagenase and matrix-metalloproteases and plasminogen-activator inhibitor I (PAI-I), some of which are also target genes for the Raf-1/MAPK pathway. These genes contain Smad3/Smad4 binding sites (CAGA) in their promotor region, but whether they are preferentially regulated by Smads or in co-operation with other transcription regulators is not clear, since these promoters contain additional transcription factor binding sites (reviewed in...
(Massague and Wotton, 2000)). Since TGFβ can induce JNK and JNK was found to activate a fibronectin reporter in a Smad-4 independent manner, a role of TGFβ induced JNK activation in the regulation of invasiveness might be an interesting alternative to Smad signalling.

This function was found to be exerted by co-operation with oncogenic ras, and the results presented here suggest that strong activation of Raf:ER can mimic Ras function (Oft et al., 1996)

4.3.3 Raf and TGFβ/Smad signalling

Both the EpRas and the MDCK Raf:ER cells provide clear examples of synergistic interactions between the Raf-1 and TGFβ pathways in establishing an invasive phenotype in epithelial cells. However, since TGFβ is also known to inhibit cell growth in epithelial cells, it is likely that mechanisms exist that allow a cell to evade the inhibitory TGFβ effects, thereby promoting the pro-malignant effects of TGFβ. This biphasic action of TGFβ is also found during multistage skin carcinogenesis, where it acts early as a tumour suppressor but later enhancing the malignant phenotype, thereby losing the sensitivity to the growth inhibitory effects of TGFβ. In the case of the MDCK Raf:ER cells, the change in the response to TGFβ growth inhibitory effects relative to the parental cells depends on the endpoint studied. Activation of Raf-1 for up to 48 hours prior to the addition of TGFβ did not effect the inhibition of cell cycle progression. In contrast, MDCK Raf:ER cells that have undergone EMT due to sustained Raf:ER activation become completely resistant to the growth inhibitory effects of TGFβ. This indicates that Raf-1 activation initiates long-term changes in MDCK cells, which might be a result of transcriptional events. However, the continued dependence of Raf-1 transformed cells on the TGFβ autocrine loop for invasive growth in collagen gels shows that they are still responsive to TGFβ. It has been reported recently that Ras induced ERK-MAPK activation leads to phosphorylation of Smad2 and Smad3 in the linker region which connects their DNA-binding and transcriptional activation domains (Kretzschmar et al., 1999). This study concluded that the ERK-phosphorylated Smads do not translocate to the nucleus in response to TGFβ, which would lead to a general inhibition of a Smad-mediated TGFβ response. The results presented here, however, confirm the integrity of a functionally active TGFβ/Smad pathway in the presence of activated Raf:ER. This was demonstrated by the ability of TGFβ to induce the nuclear translocation of Smad2, Smad3 and Smad4, the ability of Smad3 and Smad4 to bind to a c-jun oligonucleotide probe and the
trans-activation of a reporter construct by Smad2 and Smad4. The ability of Smad3 and Smad4 to bind DNA in the presence of active MAPK was also shown in Ep2Ras cells, the cell line studied by Kretzschmar and co-workers. In addition, Hu et al. found that expression of a constitutively active MEK as well as a dominant negative MEK in HaCAT cells have no effect on TGFβ-induced Smad translocation and DNA binding (Hu et al., 1999). Furthermore, TGFβ and Ras can co-operate in the presence of functionally intact TGFβ/Smad signalling in Ep2Ras cells (Oft et al., 1998) as well as in the prostate cancer cell line TSU-Pr1 (Park et al., 2000).

The data presented here strongly suggest that TGFβ signalling can synergise with the Ras/Raf/ERK pathway to promote EMT, characterised by the conversion of an epithelial to a mesenchymal phenotype. MDCK cells which have undergone EMT evade the growth inhibitory effects of TGFβ and become invasive.
Chapter 5

5 The effects of the Raf-MAPK pathway on cell survival

5.1 Introduction

The results presented in chapter 4 have shown that sustained activation of the 4-hydroxy-tamoxifen (4HT) inducible Raf:ER fusion protein in MDCK cells was sufficient to induce an epithelial to mesenchymal transition (EMT), which is relying on the induction of autocrine TGF\(\beta\) signalling. Sustained Raf-1 activation and TGF\(\beta\) signalling were shown to co-operate in causing invasive growth of MDCK cells in collagen gels. In the absence of Raf-1 activation, TGF\(\beta\) led to disruption of cystic structures of MDCK cells grown in collagen gels, reflecting the inhibitory effects of TGF\(\beta\) on epithelial cells. Beside the regulation of cell cycle, it has been found that TGF\(\beta\) can induce apoptosis in a variety of cell types including hepatocytes, B-lymphocytes and epithelial cells (Chaouchi et al., 1995); (Hsing et al., 1996); (Selvakumaran et al., 1994) but signalling pathways involved in TGF\(\beta\) mediated apoptosis are incompletely understood.

Apoptosis is a major event to prevent expansion of aberrant cells, for example after DNA damage or oncogene activation and to maintain tissue homeostasis. Several morphological changes are characteristic for apoptosis, which include chromatin condensation and DNA fragmentation. These effects are mediated by activation of a family of cysteine proteases, known as caspases (Earnshaw et al., 1999). Upon a pro-apoptotic stimulus initiator caspases, such as caspase-8 and caspase-9, are activated, which mediate proteolytic cleavage and activation of downstream effector-caspases, which cleave a specific subset of proteins thereby disrupting cellular survival pathways and destroying the structural integrity of the cell. Many apoptotic stimuli, such as cellular stress and death receptor activation, induce release of cytochrome c from the mitochondria into the cytoplasm, which is required for apoptosome formation to activate caspase-9 (Zou et al., 1999). Cytochrome c release plays also an important role during detachment induced apoptosis (anoikis) which occurs when cells lose their contact to the extracellular matrix (Frisch and Francis, 1994). Acquired resistance towards apoptosis is a hallmark of tumour development and evasion of apoptosis by cancer cells is frequently induced by cell transformation with oncogenic ras, which has acquired activating mutations in about 30% of human tumours of epithelial origin. Ras
regulates major cell survival pathways via the activation of Akt and the Raf-MAPK pathway (Downward, 1998). Furthermore, activation of Ras can induce the expression of anti-apoptotic proteins, such as Bcl-2 and the related Bcl-X<sub>L</sub> (Sanchez-Garcia and Martin-Zanca, 1997), thereby promoting a survival signal (Antonsson and Martinou, 2000). There is evidence that TGFβ leads to activation of caspases, (Buchmann et al., 1999), since treatment with a broad spectrum caspase inhibitor completely blocked the TGFβ-induced apoptotic response in lymphoma and hepatoma cell lines (Chen and Chang, 1997); (Brown et al., 1998); (Schrantz et al., 1999); (Inman and Allday, 2000).

The aim of the work described in this chapter was to analyse whether TGFβ can induce apoptosis in the epithelial cell line MDCK and whether activation of Raf:ER promotes cell survival. Therefore several aspects of apoptosis were studied, such as the activation of initiator and effector caspases, the involvement of the mitochondria-dependent death pathway and the acquisition of a characteristic apoptotic cell morphology. Since activation of Ras can protect from anoikis and from death receptor and drug induced apoptosis, it was investigated whether activation of Raf:ER is providing a survival signal for different pro-apoptotic stimuli.

5.2 Results

5.2.1 TGFβ-induced apoptosis can be blocked by activation of Raf:ER

As demonstrated in chapter 4 (Figure 4.11), TGFβ was able to induce disruption of cystic structures of MDCK cells grown in collagen gels. Closer microscopic examination revealed the induction of an apoptotic morphology. In contrast, the invasive phenotype of MDCK cells expressing activated Raf:ER was not altered after TGFβ addition, suggesting that Raf-1 provided a survival signal. Therefore the morphological and biochemical features of TGFβ induced apoptosis were examined. Furthermore, the influence of Raf:ER activation on this process was studied. MDCK Raf:ER cells grown as a monolayer were treated with TGFβ for 24 hours and analysed by phase contrast microscopy. As shown in Figure 5.1 MDCK cells undergoing apoptosis acquire a rounded cell shape and subsequently detach from the dish, visible as floating cell bodies. This was prevented in the presence of activated Raf:ER and in Raf-1 transformed MDCK Raf:ER cells due to long-term treatment with 4 HT for more than 14 days (RafT).
Figure 5.1. Induction of an apoptotic cell morphology by TGFβ. MDCK Raf:ER in the presence or absence of 4HT and MDCK RafT cells were grown in DMEM + 2% serum for 24 h before treatment with TGFβ (7.5 ng/ml) for a further 24 h. The cell morphology was examined by phase contrast microscopy.
Apoptosis in MDCK cells was scored following TGFβ treatment for 24 hours by determining the percentage of nuclei with condensed chromatin following staining with Hoechst33342 (Figure 5.2A). The initial titration of TGFβ concentration from 2.5 to 10 ng/ml is shown in Figure 5.2B. Also different serum conditions from 0.5 to 5% FCS in the cell culture medium were tested (Figure 5.2C). Treatment with 7.5 ng/ml TGFβ in the presence of 2% FCS resulted in 7% apoptosis compared to 0.2% apoptosis in control cells not exposed to TGFβ. This TGFβ induced apoptosis is blocked by Raf:ER activation, since pre-treatment with 4HT for 24 hours significantly reduced the percentage of apoptotic cells (Figure 5.2C). Cells pre-treated with 4HT for 48 hours or Raf-1 transformed MDCK cells (RafT) completely abolished TGFβ induced apoptosis (Figure 5.3). The ability of activated Raf:ER to promote cell survival in the presence of TGFβ was also demonstrated in the epithelial cell line MCF-10A. MCF-10A Raf:ER cells (introduced in chapter 4, Figure 4.4) were pre-treated with 4HT for 24 hours, which led to full protection from TGFβ-induced cell death.

5.2.2 TGFβ-mediated apoptosis involves caspase activation which is blocked by Raf activation

Next the activation of caspases in response to TGFβ was examined. Cytosolic lysates were affinity labelled with the Z-EK(bio)D-aomk peptide, which binds covalently to activated effector caspases (Martins et al., 1997) and analysed by SDS-PAGE and Western blotting. TGFβ stimulation led to strong effector caspase activation, which was prevented by pre-treatment of the cells with the broad spectrum caspase inhibitor z-VAD-fmk (Figure 5.4A). Raf-1 activation prior to the addition of TGFβ also blocked caspase activation. Moreover, we investigated the effects of TGFβ on activation of the initiator caspase-8 (Figure 5.4B). It has been shown that upon cellular death receptor stimulation, the initiator caspase-8 becomes cleaved and thereby activated (reviewed in (Wolf and Green, 1999)). Alternatively, caspase-8 activation can also occur downstream of effector caspases as part of an amplification loop (Schulze-Osthoff et al., 1998); (Slee et al., 1999). Western blot analysis showed that TGFβ treatment led to strong activation of caspase-8, which was abolished in MDCK Raf:ER cells pre-treated with 4HT. As a control for Raf-1 activity, phosphorylation of MAPK was analysed. 4HT treatment led to strong MAPK activation in both the TGFβ stimulated and unstimulated cells (Figure 5.4C). Taken together, these findings indicate that TGFβ provides a pro-apoptotic stimulus to MDCK cells by activating the caspase cascade. However, TGFβ-
Figure 5.2. TGFβ induces apoptosis in MDCK cells. MDCK cells treated with TGFβ under different conditions were analysed for the percentage of apoptotic cells, determined by counting cells with condensed nuclei after staining with Hoechst33342. (A) MDCK cells were treated with different concentrations of TGFβ for 24h. Cells were cultured in DMEM + 2% FCS for 24 h prior to TGFβ treatment. Data shown are the mean and standard deviation of three independent experiments performed in duplicate. (B) MDCK Raf:ER prestimulated with 4HT for 24 h as indicated were untreated or treated with 7.5 ng TGFβ for 24 h. Cells were cultured in different serum conditions as indicated. Data shown are the average of two experiments performed in duplicate. (C) MDCK cells were cultured in DMEM + 2% FCS for 24h before treatment with 7.5 ng/ml TGFβ for 24h. Cells with condensed nuclei (arrows) were counted after staining with Hoechst33342 and examined by fluorescence-microscopy.
Figure 5.3 Short-term activation of Raf prevents TGFβ induced apoptosis in MDCK and MCF-10A cells. (A) MDCK Raf:ER cells untreated or treated with 100 nM 4HT as indicated and MDCK RafT cells were grown in DMEM + 2% serum for 24 h before stimulation with 7.5 ng/ml TGFβ for a further 24 h. The percentage of apoptotic cells was determined by counting cells with condensed nuclei after Hoechst33342 staining. Data shown are (A) the mean and standard deviations of three independent experiments performed in duplicate (B) MCF-10A Raf:ER cells were untreated or treated with 4HT as indicated for 24 h before treatment with 7.5 ng/ml TGFβ for a further 24 h. The percentage of apoptotic cells was determined by counting cells with condensed nuclei after Hoechst33342 staining. Data shown represent the mean of one experiment performed duplicate.
Figure 5.4. Short-term activation of Raf is sufficient to block TGFβ-induced apoptosis. (A) MDCK Raf:ER cells either untreated or pretreated with 100 nM 4HT for the indicated time periods and MDCK RafiER cells transformed by long-term exposure to 4HT (RafT) were grown in DMEM + 2% FCS for 24 h before stimulation with 7.5 ng/ml TGFβ for 24 h. To inhibit caspase activation cells were pretreated with 100 μM z-VAD-fmk for 20 min. Lysates were incubated with ZEK(bio)D-aomk peptide and assayed by Western blotting. As a control for the activation of caspases, MDCK cells were placed into suspension for 8 h (C). (B) MDCK Raf:ER cells were treated with 100 nM 4HT as indicated and grown in DMEM + 2% FCS for 24 h. After stimulation with TGFβ (7.5 ng/ml) for 24 h, total lysates were analysed for the activation of caspase-8 by Western blotting using an antibody recognising the cleaved p20 subunit (C) Same lysates as in (B) were used to detect activation of p42MAPK by Western blotting.
induced caspase activation was completely blocked by short-term Raf-1 activation, showing that the Raf/MAPK pathway can antagonise TGFβ-mediated apoptosis.

5.2.3 TGFβ mediated apoptosis involves cytochrome c release

Having shown that TGFβ leads to activation of the caspase cascade in MDCK cells, which can be abolished by activation of Raf, another important aspect of apoptosis was examined: the release of mitochondrial protein cytochrome c into the cytoplasm, which is a key event in many types of apoptosis. Therefore, it was investigated whether TGFβ is able to induce cytochrome c release and whether Raf-1 has any effect on this process (Figure 5.5). Therefore, MDCK cells, treated or untreated with 4HT, were stimulated with TGFβ for 24 hours and digitonin extracts containing only the cytosolic fraction were analysed for the presence of cytochrome c by Western blotting. In response to TGFβ, cytochrome c was released into the cytoplasm. Interestingly, activation of Raf:ER for 24 hours prior to TGFβ treatment did not prevent cytochrome c release, although the activation of caspase-8 was completely abolished. This suggests that Raf-1 activation effects the apoptotic pathway downstream of cytochrome c release, but upstream of caspase-8. Surprisingly, in MDCK cells transformed by Raf-1 due to long-term treatment with 4HT (RafT), cytochrome c release was completely blocked in the presence of TGFβ. This effect is also found in MDCK cells expressing activated Ras (V12Ras), suggesting that during MDCK cell transformation the molecular basis for cell survival may change.

5.2.4 Involvement of anti-apoptotic proteins in protection from TGFβ-induced apoptosis

Since anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-XL, are known to prevent cytochrome c release into the cytosol, it was asked whether they play a role in Raf-1 induced cell survival. Western blotting showed that the expression level of Bcl-XL is not altered during TGFβ induced apoptosis (Figure 5.6A). Furthermore, the presence of activated Raf-1 following 24 hours (data not shown) or 72 hours of 4HT treatment had no effect on the Bcl-XL expression level. Interestingly, MDCK cells transformed by Raf-1 showed increased Bcl-XL expression. These findings strongly indicate that the protection mechanisms underlying short-term Raf-1 activation or Raf-1 transformation are functionally different. Whereas short term Raf-activation acts downstream of cytochrome c release, protection following Raf-1
Figure 5.5. Release of cytochrome c from mitochondria is not prevented by short-term Raf activation, but in Raf transformed MDCK cells. MDCK Raf:ER cells untreated or treated with 100 nM 4HT for 24 h, MDCK RafT cells and MDCK V12Ras cells were grown in DMEM + 2% FCS for 24 h. Cells were treated with 7.5 ng/ml TGFβ for 24h. Cytoplasmic lysates obtained from digitonin treatment were analysed for cytochrome c release (upper panel) and for the activation of caspase-8 by Western blotting.
Figure 5.6. Bcl-X$_I$ is upregulated in Raf transformed MDCK cells. (A) MDCK Raf:ER cells either untreated or treated with 100 nM 4HT for 72 h and MDCK RafT cells were grown in DMEM + 2% FCS for 24 h. Cells were stimulated with 7.5 ng/ml TGFβ where indicated. Total lysates were assayed for Bcl-X$_I$ (upper panel) and activated caspase-8 (lower panel) by Western blotting. (B) MDCK Raf:ER cells either untreated or treated with 100 nM 4HT for 24 h, MDCK cells carrying empty vector (ev) and MDCK cells stably expressing dn FADD, Bcl-X$_I$, or Bcl-2 were grown in DMEM + 2% FCS for 24 h before treatment with 7.5 ng/ml TGFβ for a further 24 h. Cells with condensed nuclei were counted after staining with Hoechst33342. Values show the mean of two experiments performed in duplicate.
transformation appears to provide cell survival by blocking cytochrome c release through expression of Bcl-X<sub>L</sub>. Both survival mechanisms share the ability to prevent caspase-8 activation.

To investigate whether expression of anti-apoptotic Bcl-2 family members directly affect TGFβ induced apoptosis, MDCK-cell lines stably expressing Bcl-2 and Bcl-X<sub>L</sub> were stimulated with TGFβ and analysed by counting nuclei with condensed chromatin after Hoechst33342 staining (Figure 5.6). In addition, the ability of a dominant negative version of the Fas adapter protein FADD (dnFADD) to prevent apoptosis in response to TGFβ was tested. Stable expression of dnFADD in MDCK cells was reported to abolish apoptosis which was induced via activation of the death receptor Fas (Rytömaa et al., 1999). MDCK cells stably expressing Bcl-2, Bcl-X<sub>L</sub> and dnFADD were protected from TGFβ induced apoptosis and the level of protection is similar to the one obtained with activated Raf:ER (24h). Interestingly, the fact that dnFADD is capable of protecting cells from a pro-apoptotic TGFβ signal suggests that TGFβ-induced apoptosis might involve death receptor signalling.

5.2.5 Effects of Raf on protection from detachment-induced apoptosis (anoikis) in MDCK and MCF-10A cells

To investigate whether activation of Raf-1 leads to protection from other pro-apoptotic stimuli than TGFβ, the effect of activated Raf-1 on detachment-induced apoptosis (anoikis) in MDCK cells and MCF-10A cells stably expressing Raf:ER was examined. When cell attachment was prevented by plating cells on poly-HEMA coated dishes, MDCK cells expressing empty vector and uninduced MDCK Raf:ER cells exhibited a 5.5 to 7.2 fold increase in apoptosis within 8 hours in suspension (Figure 5.7A). MCF-10A cells carrying empty vector and MCF-10A RafiER cells showed a 6 to 7 fold increase in apoptosis after 24 hours in suspension (Figure 5.8A). When Raf-1 was activated by treatment with 4HT in both cell lines, induction of apoptosis was prevented. Furthermore, MDCK Raf:ER cells transformed by sustained treatment with 4HT (RafT) were protected even more strongly. In addition, MCF-10A cells expressing V12Ras were also protected from detachment induced apoptosis (Rytömaa et al., 1999). As shown in Figure 5.8B, activation of Raf:ER is able to activate p42MAPK in response to 4HT treatment in both attached cells and cells grown in suspension. This was also shown for MCF-10A cells expressing activated Raf, in which p42MAPK and p44MAPK phosphorylation was analysed.
Figure 5.7. Activation of Raf in MDCK cells induces survival from detachment induced apoptosis. (A) MDCK cells carrying empty vector (ev), MDCK Raf:ER cells pretreated with 100 nM 4HT as indicated and MDCK RafT cells were trypsinised and plated on normal (A) or poly-HEMA coated dishes (S) in the absence or presence of 4HT for 8 h. DNA fragmentation was measured by Cell Death ELISA. Data show represent two independent experiments performed in duplicate. (B) Cell lysates from cells prepared in parallel to (A) were analysed for p42MAPK activation by Western blotting. (C) MDCK Raf:ER cells untreated or pretreated with 100 nM 4HT were detached and cultivated on normal (A) or poly-HEMA coated dishes (S) for 8 h. Cells were assayed for cytochrome c release (upper panel) and for the activated p18 subunit of caspase-8 (lower panel) by Western blotting. (Figure 5.7 C kindly provided by M. Rytomaa).
Figure 5.8. Activation of Raf in MCF-10A cells induces survival from detachment induced apoptosis and prevents release of cytochrome c from mitochondria.

(A) MCF-10A cells carrying empty vector (ev), MCF-10A Raf:ER cells or MCF-10A V12Ras cells were pretreated with 100 nM 4HT as indicated, trypsinised and plated on normal (A) or poly-HEMA coated dishes (S) in medium containing 5% horse-serum in the presence or absence of 4HT for 24 h. DNA fragmentation was measured by Cell Death ELISA. Data are shown as increase in cell death relative to adherent control cells (ev) and represent mean and standard deviation of three independent experiments. (B) Phosphorylation of p42MAPK and p44MAPK was detected with a phosphospecific antibody using lysates from cells prepared in parallel to the experiment shown in (A). (C) MCF-10A Raf:ER cells untreated or pretreated with 100 nM 4HT were detached and cultivated on normal (A) or poly-HEMA coated dishes (S) for 24 h. Cells were assayed for cytochrome c release (upper panel) and for the activated p20 subunit of caspase-8 by Western blotting. (Figure kindly provided by A.Schulze).
The mechanism by which apoptosis is triggered in response to loss of adhesion to the extracellular matrix are not completely understood. It was investigated whether detachment leads to cytochrome c release from the mitochondria into the cytoplasm and whether Raf-1 activation might affect this. Cytoplasmic extracts obtained by digitonin lysis of the plasma membrane were analysed by Western blotting for the presence of cytochrome c. It was shown that cytochrome c is released in MDCK cells after 8 hours in suspension and caspase-8 is activated (Figure 5.7C). Interestingly, pre-activation of Raf:ER for 24 hours was not sufficient to prevent cytochrome c release in MDCK cells grown in suspension, although caspase-8 activation was blocked. The release of cytochrome c into the cytoplasm was also observed in MCF-10A cells following culture in suspension. Interestingly, in contrast to MDCK cells, activation of Raf:ER in MCF-10A cells was sufficient to prevent cytochrome c release as well as caspase-8 activation (Figure 5.8C) (Schulze et al., 2001).

5.2.6 Effects of Raf activation on drug and death receptor induced apoptosis

A rapid induction of apoptosis occurs by activation of death receptor signalling after binding of their appropriate death ligands. An example is the activation of the TNF-receptor by TNFα. To investigate whether strong activation of Raf-1 provides a general survival signal, the capability of Raf-1 activation to prevent TNFα-induced apoptosis in MDCK cells was examined (Figure 5.9). Furthermore, the influence of Raf-1 on apoptosis induced by etoposide, an inhibitor of Topoisomerase-II, and staurosporine, a broad spectrum kinase inhibitor, were analysed. MDCK Raf:ER cells were treated with different concentrations of TNFα for 4 hours in the presence of the protein synthesis inhibitor cycloheximide, which is required to block the anti-apoptotic NF-κB transcriptional response to TNFα. TNFα induced a dose-dependent increase of the percentage of apoptotic cells as demonstrated by Hoechst33342 staining (Figure 5.9A). TNFα also induced strong activation of caspase-8 in a dose-dependent manner (Figure 5.9B). In contrast, Raf-1 activation in MDCK cells by pre-treatment with 4HT for 24 hours was able to protect cells from TNFα induced apoptosis and activation of caspase-8 was prevented. Activation of Raf:ER in MDCK cells was also sufficient to protect cells from etoposide induced apoptosis (Figure 5.9C) but not from staurosporine induced apoptosis (Figure 5.9A and B). These results indicate that strong Raf-1 activation does not only inhibit TGFβ induced death signalling, but renders cells insensitive to a variety of pro-apoptotic stimuli in different epithelial cell lines, suggesting a more general anti-apoptotic function for Raf. However, Raf-1 was not able to protect from staurosporine
Figure 5.9. Activation of Raf blocks TNFα and Etoposide induced apoptosis. 

(A) and (C) MDCK Raf:ER cells were either untreated or pretreated with 4HT for 24 h and stimulated with with the indicated concentrations of staurosporine (ST) or TNFα in the presence of 2 ng/ml cycloheximide (CX) or cycloheximide alone for 4 h. The percentage of apoptotic cells was determined by counting cells with condensed nuclei after staining with Hoechst33342. Data shown represent at least three experiments performed in duplicate. (B) The activated p18 subunit of caspase-8 was detected by Western blotting using lysates from cells prepared in parallel to the experiment shown in (A).
induced apoptosis, since staurosporine may inhibit kinase activity of Raf and other kinases mediating survival pathways.

5.3 Discussion

5.3.1 TGFβ-induced apoptosis is blocked by activation of Raf:ER

Presented in this chapter is strong evidence for the ability of TGFβ to induce apoptosis in the epithelial cell lines MDCK and MCF-10A, which can be rapidly blocked by activation of Raf:ER. TGFβ triggers apoptosis by activation of caspase-8 and effector caspases and by mediating cytochrome c release from the mitochondria into the cytoplasm. Interestingly, the mechanism by which short-term Raf-1 activation prevents apoptosis in MDCK Raf:ER cells differs from the one in MDCK Raf:ER cells, which have undergone EMT (RafT). Short-term activation of Raf-1 protects from TGFβ induced apoptosis by blocking activation of caspase-8 and effector caspases, but release of cytochrome c from the mitochondria is not prevented. However, in RafT cells caspase activation as well as release of cytochrome c from the mitochondria is blocked. Interestingly, expression of the anti-apoptotic protein Bcl-XL is increased in RafT cells, but not after short-term Raf-1 activation or in uninduced MDCK Raf:ER cells. This clearly demonstrates that a change in the anti-apoptotic mechanism occurs during the process of EMT.

The mechanism by which TGFβ induces the caspase cascade leading to apoptosis is still unclear. In MDCK cells, the kinetics of TGFβ induced apoptosis is slow, leaving room for the possibility that transcriptional regulation by TGFβ might occur, which could contribute to the apoptotic effects. For example, expression of anti-apoptotic proteins, such as Bcl-2 and XIAP, was found to be reduced in response to pro-apoptotic TGFβ signal in a hepatoma cell line (Shima et al., 1999). Recently it was shown that TGFβ can induce caspase activation and cytochrome c release in a hepatoma cell line mediating the formation of an Apaf-1/caspase-9 apoptosome complex (Freathy et al., 2000). In this study it was shown that the broad spectrum caspase inhibitor z-VAD blocked caspase activation, but not formation of the apoptosome, indicating that cytochrome c release occurs in a caspase-independent manner. The direct link connecting TGFβ with caspase activation and cytochrome c release still needs to be elucidated. It is not clear whether TGFβ/Smad signalling is involved or whether the pro-apoptotic signal is transduced independently of Smads. Several studies have shown that
TGFβ can activate downstream effectors other than Smads, which might be implicated in the pro-apoptotic response. It was shown that TGFβ can activate JNK in a Smad-independent manner (Atfi et al., 1997). TGFβ was also found to directly activate TAK, a member of the MAPK kinase kinase family, leading to rapid activation of p38 MAPK in vitro (Yamaguchi et al., 1995); (Hanafusa et al., 1999). In vivo, expression of TAK is associated with JNK activation and apoptosis (Takatsu et al., 2000). Interestingly, both activation of JNK and p38 have been implicated in contributing to stress induced apoptosis. Two reports show that apoptosis induced by peroxide (Zhuang et al., 2000) or by UV (Assefa et al., 2000) activates p38 MAPK, caspases, such as caspase-3 and caspase-8, and induces release of cytochrome c. Inhibition of p38MAPK activity by the inhibitor SB203580, was found to prevent cytochrome c release and caspase-3 activation without affecting the activation of caspase-8. This indicates that activation of caspase-8 is independent from p38 MAPK and is not downstream of cytochrome c release during stress induced apoptosis (UV, peroxides). Furthermore, the broad spectrum caspase inhibitor z-VAD blocked UV induced apoptosis by inhibiting caspase activation, without preventing cytochrome c release and p38MAPK activity. Therefore, it would be interesting to investigate whether p38 or JNK are involved in TGFβ induced cytochrome c release.

There is a large body of evidence that caspase-8 activation controls cytochrome c release. Active caspase-8 cleaves and thereby activated the pro-apoptotic protein Bid, which is able to directly induce cytochrome c release. However, in MDCK cells it seems that cytochrome c release might be uncoupled from caspase-8 activation, since short-term activation of Raf-1 can block its activation, but does not prevent cytochrome c release. It is therefore very likely that caspase-8 dependent cleavage of Bid is not the main mediator of cytochrome c release in response to TGFβ.

The results presented here show that TGFβ-induced apoptosis in MDCK cells can be blocked by expression of a dominant negative version of FADD (dnFADD), Bcl-2 and Bcl-XL. While the anti-apoptotic Bcl-2 family members are known to promote cell survival by blocking cytochrome c release through dimerisation with pro-apoptotic Bcl-2 family members, the role for the death receptor binding protein FADD in TGFβ induced apoptosis is not clear at present. FADD might also interact via its protein interaction domain (death domain) with other proteins than Fas or TNF-receptor. Since caspase-8 is known to be primarily activated via death receptor signalling, the involvement of death receptor signalling would provide an explanation for caspase-8 activation in response to TGFβ. Another likely mechanism for the
activation of caspase-8 downstream of cytochrome c release has been described: caspase-8 activation can also occur downstream of effector caspases as a part of an amplification response to cytochrome c release (Schulze-Osthoff et al., 1998); (Slee et al., 1999). This would allow the inhibition of caspases by Raf-1 in MDCK cells without affecting cytochrome c release.

In a study by Yeh et al. over-expression of the Raf downstream effector M KK1 was found to increase the expression of c-Flip, a FADD binding protein, which can bind to death receptor associated FADD and antagonised activation of caspase-8 (Irmler et al., 1997); (Yeh et al., 1998). This might suggest that TGFβ induced apoptosis could lead to caspase-8 activation via death receptor signalling. This might possibly be antagonised by Raf-1 induced expression of c-Flip, but whether this mechanism is relevant for the protection from TGFβ induced apoptosis is presently unclear.

The mechanism, which protects RafT cells from TGFβ induced apoptosis seems to be distinct from the one induced by short-term Raf-1 activation. Since Bcl-XL expression is increased and cytochrome c release is blocked in these cells, it seems likely that Bcl-XL prevents cytochrome c release by binding to pro-apoptotic members of the Bcl-2 family on the outer membrane of the mitochondria, thereby preventing their functionality (Antonsson and Martinou, 2000). RafT cells seem to be protected by a similar mechanism as observed in ras transformed MDCK cells (Rytomaa et al., 2000). In addition, it was reported that the anti-apoptotic Bcl-2 protein can directly bind to Raf-1 (Wang et al., 1994) and over-expressed Raf-1 targeted to the mitochondria was reported to increase cell survival (Wang et al., 1996). However, a study performed by Olivier et al. could not find evidence for Bcl-2 to require Raf-1 interaction for mediating cell survival in fibroblasts (Olivier et al., 1997). At present, there is no evidence for a physiological role of Raf-1 interacting with Bcl-2.

5.3.2 Activation of Raf:ER prevents anoikis and protects from other pro-apoptotic stimuli

Raf:ER induced protection from apoptosis in MDCK cells is not specific for TGFβ since it could also been shown for protection from detachment-induced apoptosis (anoikis) in MDCK Raf:ER cells and MCF-10A Raf:ER cells. Activation of caspase-8 during anoikis is blocked by Raf-activation in both cell lines. Interestingly, Raf-1 activation does not prevent cytochrome c release in MDCK cells (Rytomaa et al., 2000), while Raf-1 was found to act
upstream of cytochrome c release in MCF-10A cells (Schulze et al., 2001). Furthermore, Raf:ER activation was also found to protect MDCK cells from TNFα and etoposide induced apoptosis, demonstrating that Raf-1 provides a more general anti-apoptotic signal in MDCK cells.

Consistent with the data presented here, a study by LeGall et al. also showed that activation of Raf:ER protects from anoikis in MDCK cells (Le Gall et al., 2000). The strong activation of the Raf/MAPK pathway might account for the efficient protection from detachment induced apoptosis. It has been shown that expression of an oncogenic ras can induce the synthesis of autocrine growth factors which promote cell survival (Hamilton and Wolfman, 1998). Furthermore, Raf-1 has been found to increase the expression of growth factors which can act in an autocrine manner, such as hbEGF (McCarthy et al., 1995). Autocrine activation of EGF receptor has been found to be important for protection from detachment induced apoptosis in MCF-10A cells which results in the activation of the PI3K/Akt survival pathway (Schulze et al., 2001).

However, there seems to be a difference in the survival mechanisms induced by Raf-1 in different cell lines. While in MCF-10A cells, Raf:ER activation prevents the release of cytochrome c from the mitochondria, short-term activation of Raf:ER in MDCK cells fails to block cytochrome c release. It has been suggested that activation of Akt might lead to the inhibition of cytochrome c release (Kennedy et al., 1999). This would explain why cytochrome c release is prevented in MCF-10A cells in the presence of activated Raf:ER. In contrast, Akt activation might not be involved in MDCK cells protected from anoikis and TGFβ-induced apoptosis by short-term activation of Raf:ER, since cytochrome c release from mitochondria is not blocked in these cells.

5.3.3 A general survival response by Raf:ER

Activation of Raf:ER can promote survival in MDCK cells via different mechanisms: post-translational modification, thereby inhibiting pro-apoptotic proteins, and by induction of transcription of pro-survival genes. It was shown that Ras-induced MAPK activation leads to activation of the kinase p90Rsk2. p90Rsk2 can either induce phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family and can also activate the transcription factor CREB, which mediates survival in neuronal cells (Bonni et al., 1999). It might be possible that activation of Raf:ER in MDCK cells leads to inactivation of Bad thereby providing a
general survival mechanism, since ERK-MAPK induced Bad phosphorylation was found in a number of different studies (Scheid and Duronio, 1998); (Wang et al., 1996); (Fang et al., 1999).

A common survival mechanism for the different apoptotic stimuli might also be provided by Raf/MAPK dependent activation of IAPs (inhibitors of apoptosis), since Raf-1 seems to interfere with the apoptotic pathway downstream of cytochrome c release possibly by inhibiting apoptosome function. Some members of the IAP family were found to bind and inhibit activated caspase-3 and caspase-9 in mammalian cells (Deveraux et al., 1998); (Bratton et al., 2001). Although there is no direct link between expression of IAPs and Ras/Raf signalling at present, the expression of some IAPs appears to be induced in some types of cancer (Deveraux and Reed, 1999). However, compelling genetic evidence came from studies in D. melanogaster demonstrating a key role for the Ras/Raf/MAPK pathway in the protection from apoptosis. It was found that Ras/Raf/MAPK signalling blocked the pro-apoptotic function of Hid (Bergmann et al., 1998); (Kurada and White, 1998). Hid antagonises the anti-apoptotic function of DIAP1, the IAP homologue in D.melanogaster, thereby liberating activated caspases and inducing apoptosis (Goyal et al., 2000). In mammalian cells, Smac/DIABLO is the only inhibitor for IAPs known so far which functionally overlaps with Hid. Smac/DIABLO is released from the mitochondria during apoptosis. It was found to bind to and thereby inhibit XIAP which leads to the release of activated caspase-9 (Ekert et al., 2001). It would be interesting to investigate whether the Raf/MAPK pathway interferes with Smac/DIABLO function.

Taken together the results presented here show that the anti-apoptotic function of Raf:ER activation in MDCK cells, together with the inhibition of TGFβ induced growth arrest occurring at later stages when cells have undergone EMT, renders the cells insensitive to the inhibitory effects of TGFβ, which is a feature of many advanced human tumours (Akhurst and Balmain, 1999). Furthermore, strong activation of the Raf/MAPK pathway provides a more general rather than a TGFβ specific survival mechanism against different apoptotic stimuli.
Chapter 6

6 Role of Smad3 in mediating TGFβ resistance in RafT cells

6.1 Introduction

In chapter 3 it was revealed that MDCK Raf:ER cells which have undergone EMT due to sustained Raf-1 activation (RafT) are refractory to the anti-proliferative effects of TGFβ. In contrast, short-term activation of Raf-1 in MDCK Raf:ER cells does not prevent the growth inhibitory TGFβ effects.

This biphasic responsiveness of cells to TGFβ is also found during tumourigenesis. While TGFβ acts as a tumour suppresser by inhibiting cell growth at early tumour stages, it was found to promote growth and invasiveness during late stages of tumour development. This is based on the finding that TGFβ-induced growth arrest is prevented in many advanced human tumours of epithelial origin, which have often undergone an epithelial to mesenchymal transition (EMT) (Cui et al., 1996); (Fynan and Reiss, 1993). The loss of sensitivity to TGFβ induced growth inhibition is associated with tumour progression, such as the development of invasive properties (Welch et al., 1990). The mechanisms, which render these advanced tumour cells insensitive to TGFβ-induced growth arrest are still unclear. Some cells lose responsiveness by down-regulation of TGFβ receptor-typeII expression (Zhao and Buick, 1995), while others show mutations in components of the TGFβ pathway. These involve mutations in TGFβ receptor typeI, typeII, Smad2 and Smad4, but no mutations in Smad3 have been observed so far (reviewed in (Akhurst and Balmain, 1999)). However, most advanced tumours do not show mutations of the receptor or Smads leaving the integrity of the TGFβ signalling pathway intact. Many advanced tumours in mouse or human are found to over-express TGFβ required to promote malignancy (Derynck et al., 1985); (Krieg et al., 1991).

Since many advanced tumours which express activated mutant Ras protein have undergone EMT, this leads to the suggestion that Ras and TGFβ pathways can co-operate in rendering tumour cells resistant to anti-proliferative TGFβ effects. However, only little is known about the mechanism.
In this chapter, the MDCK Raf:ER cells which have undergone EMT (RafT) were used as a model system to study the molecular basis which allows these cells to evade TGFβ mediated growth control.

6.2 Results

6.2.1 Altered Smad expression in RafT cells

MDCK Raf:ER cells which have undergone EMT due to sustained activation of Raf:ER (RafT) are refractory to TGFβ induced growth arrest (compare Chapter 4, Figure 4.12). Since Smads are the major effectors of TGFβ in regulating gene transcription, it was examined whether Smad functions were altered during EMT in MDCK cells. Therefore, the ability of Smads to translocate into the nucleus in response to TGFβ was analysed in MDCK Raf:ER cells pre-treated with 4HT for 24 hours or for 14 days, MDCK RafT cells and MDCK V12Ras cells, stably expressing constitutively activated Ras (Figure 6.1). Activation of Raf:ER for 14 days in MDCK cells induced EMT and these freshly transformed MDCK cells were used as a control to show that effects observed in the RafT cells are not due to further genetic alterations acquired during further cell passages. While MDCK RafT cells have lost the ability to express E-cadherin, it is not down-regulated in MDCK V12Ras expressing cells. These cells still exhibit many features of epithelial cells, although they have been transformed by expression of V12Ras. Following TGFβ treatment, nuclear extracts were prepared and immunoblotted with antibodies directed against Smad2, 3 and 4. In all cell lines examined, exposure to TGFβ led to accumulation of Smad4 in the nucleus (Figure 6.1A, upper panel). The same result was obtained for Smad2 translocation (Figure 6.1A, middle panel). In contrast, Smad3 accumulation in response to TGFβ was impaired in MDCK cells, which had undergone EMT (14 days of 4HT and RafT). As a control for Raf:ER and Ras activation in these cells, the phosphorylation of p42MAPK in nuclear extracts was assayed by Western blotting (Figure 6.1A, third panel). Upon Raf-1 activation, the phosphorylated form of p42MAPK is detectable in the nucleus and in the presence of sustained Raf:ER activation all endogenous p42MAPK is phosphorylated. The expression of PCNA, a nuclear protein involved in DNA replication, was detected by Western blotting to show equal protein loading of nuclear extracts (Figure 6.1B, upper panel). In addition, Western blotting analysis of nuclear extracts showed no contamination with the cytosolic
Figure 6.1. Sustained Raf activation leads to reduced nuclear translocation of Smad3. MDCK Raf:ER cells untreated or treated with 100 nM 4HT for the indicated time periods, MDCK RafiER cells transformed by long-term exposure to 4HT (RafT) and MDCK V12Ras cells were treated with 2 ng/ml TGFβ for 1 h as indicated.

(A) Nuclear extracts were assayed for Smad2, Smad3 and Smad4 and for p42MAPK by Western blotting. (B) Nuclear lysates were examined for contamination with the cytoplasmic protein Grb2. Total lysate from MDCK Raf:ER cells was used as a control (T). To show equal loading of nuclear extracts the expression of the nuclear protein PCNA was assayed by Western blotting.
adapter protein Grb2 (Figure 6.1B, lower panel). These results show that the amount of Smad3 accumulating in the nucleus in response to TGFβ is reduced in MDCK cells, which have undergone EMT.

To determine whether reduced Smad3 translocation in RafT cells might quantitatively affect DNA binding, nuclear extracts were prepared from MDCK V12Ras cells, MDCK RafT cells and from MDCK cells treated with 4HT for the indicated times. The 32P-labeled c-jun probe was used to detect Smad3/4 complexes bound to DNA in electrophoretic mobility shift assays (EMSA). A shifted Smad3/4-DNA complex was only observed in nuclear extracts, which had been treated with TGFβ (Figure 6.2). Interestingly, following EMT less Smad3/4 complexes were shifted (lanes 14 and 16) compared to the other nuclear extracts.

To further investigate whether Smad3 expression levels are reduced or whether only nuclear translocation of Smad3 is impaired following EMT, cells were treated as described above and total protein lysates were analysed by Western blotting for the abundance of Smad4, Smad3 and Smad2 (Figure 6.3A). In MDCK RafT cells as well as in MDCK Raf:ER cells which have been treated with 4HT for 14 days Smad3 expression is reduced compared to MDCK V12Ras cells and untreated or 4HT treated MDCK Raf:ER cells. The expression of E-cadherin was monitored to demonstrate that the freshly transformed MDCK Raf:ER cells had fully undergone EMT after 14 days of Raf-1 activation (Figure 6.3B). No E-cadherin protein was detectable in MDCK cells which had undergone EMT, confirming their transformed status. Expression of PCNA and Grb2 was detected to show equal loading. This experiment showed that Smad3 expression is reduced in MDCK cells which have undergone EMT.

6.2.2 Repression of Smad3 and E-cadherin gene transcription during EMT in MDCK cells

To further analyse whether Smad3 expression is down-regulated at the transcriptional level, the mRNA levels of Smad2, Smad3 and Smad4 were directly measured by RNAse protection assay. As a control for EMT of MDCK cells, an E-cadherin RNAse protection probe was used. Total RNA was prepared from MDCK RafT cells, untreated MDCK Raf:ER cells and after 24 hours of 4HT treatment and MDCK V12Ras cells.

As shown in Figure 6.4A, the level of Smad3 mRNA is significantly reduced in RafT cells compared to the other cell lines demonstrating that during EMT, expression of Smad3 is down-regulated. However, Smad2 and Smad4 mRNA levels were not altered in these
Figure 6.2. Sustained Raf activation leads to reduced DNA binding of Smad3/4 complexes. MDCK Raf:ER cells untreated or treated with 100 nM 4HT for the indicated time periods and MDCK Raf:ER cells transformed by long-term treatment with 4HT (RafT) and MDCK V12Ras cells were stimulated with 2 ng/ml TGFβ for 1 h where indicated. Nuclear extracts were assayed for the binding of activated Smad3/4 complexes to a 32P-labelled c-jun oligonucleotide probe by EMSA.
Figure 6.3. Sustained Raf activation leads to downregulation of Smad3 expression. MDCK Raf:ER cells untreated or treated with 100 nM 4HT for the indicated time periods, MDCK Raf:ER cells transformed by long-term exposure to 4HT (RafT) and MDCK V12Ras cells were treated with 2 ng/ml TGFβ for 1 h as indicated. (A) Lysates were assayed for expression of Smad2, Smad3 and Smad4 and for activation of p42MAPK by Western blotting. (B) Same lysates as used in (A) were examined for expression of E-cadherin, Grb2 and PCNA by Western blotting. Nuclear extract from MDCK Raf:ER cells was used as a control (N).
Figure 6.4. Long-term activation of Raf leads to loss of E-cadherin and decrease of Smad3 transcription. RNase protection analysis of RNA from MDCK Raf:ER cells either untreated or treated with 100 nM 4HT as indicated, MDCK RafT cells and MDCK V12Ras cells. The RNA was hybridized with probes to E-cadherin, Smad4, Smad3, Smad2 and GAPDH.
MDCK cell lines. The E-cadherin RNAse protection probe did not detect any E-cadherin mRNA in RafT cells compared to the MDCK V12Ras and the uninduced or short-term 4HT treated MDCK Raf:ER cells. This result is consistent with the data from the Western blotting experiment (Figure 6.2 and Chapter 4, Figure 4.8) and confirms that MDCK RafT cells have lost E-cadherin expression due to inhibition of gene transcription during EMT. To get further insights into the onset of reduction in Smad3 and E-cadherin mRNA levels during the EMT process, a time course experiment was performed. MDCK Raf:ER cells were treated with 4 HT for the times indicated and RNA was prepared to analyse the onset of reduction in Smad3 and E-cadherin mRNA abundance (Figure 6.4B). A drop in Smad3 and E-cadherin mRNA expression was observed after 12 days of Raf-1 activation. The decrease of Smad3 mRNA levels seemed to be more continuously and was significantly reduced after 14 days of Raf-1 activation. In contrast, Smad2 mRNA and Smad4 mRNA levels were not altered during EMT.

Taken together, these results demonstrate that sustained Raf-1 activation leads to a repression of Smad3 and E-cadherin gene transcription after 12 to 14 days during the process of EMT.

6.2.3 TGFβ-dependent p21Cip1 expression is abrogated in RafT cells

It has been shown that in response to TGFβ the expression of the cyclin-dependent kinase inhibitor p21Cip1 is increased due to transcriptional activation of the promotor, which contains a TGFβ responsive element (Datto et al., 1995). Furthermore, the p21Cip1 promotor was found to be specifically activated via Smad3/Smad4 and expression of a mutant Smad3 protein abrogated p21Cip1 promotor induction (Moustakas and Kardassis, 1998).

To test whether Smad3 repression affects transcriptional activation of the p21Cip1 gene, a RNAse protection assay was performed using a p21Cip1 probe. RNA was prepared from MDCK RafT cells, untreated MDCK Raf:ER cells and MDCK Raf:ER cells after 24 hours of 4HT treatment and from MDCK V12Ras cells which were all untreated or treated with TGFβ for 3 hours (Figure 6.5 A). Induction of p21Cip1 mRNA was observed in response to TGFβ stimulation in untreated MDCK Raf:ER cells. In the presence of activated Raf:ER following 24 hours 4HT treatment, p21Cip1 mRNA was already induced in the absence of TGFβ and no further p21Cip1 mRNA induction occurred following TGFβ stimulation. Similar results were obtained following detection of expression levels of p21Cip1 protein in MDCK Raf:ER and RafT cells using Western blotting. Induction of p21Cip1 protein expression was
observed in untreated MDCK Raf:ER cells in response to TGFβ (Figure 6.5B). In the presence of activated Raf-1 following 4HT treatment for 72 h, MDCK cells showed a clear induction of p21Cip1 protein, which was not altered in the presence of TGFβ. Furthermore, p21Cip1 mRNA levels were not altered in the presence of TGFβ. In RafT cells only little p21Cip1 was detectable, similar to untreated MDCK Raf:ER cells and treatment with TGFβ did not increase p21Cip1 protein expression.

These results show that the expression of p21Cip1 is not induced in RafT cells upon TGFβ treatment, whereas short-term activation of Raf-1 or expression of V12Ras is sufficient to increase p21Cip1 levels in the absence of TGFβ and no further increase in these cells is observed following TGFβ treatment. It seems that down-regulation of Smad3 expression in RafT cells correlates with the inability to increase p21Cip1 expression in response to TGFβ.

6.2.4 Re-expression of Smad3 in MDCK RafT cells restores sensitivity to TGFβ growth inhibitory effects

To further investigate whether there is a molecular link between a decrease of Smad3 protein expression in RafT cells and the resistance of these cells to TGFβ-induced growth arrest, MDCK RafT cells expressing full length human Smad3 were generated and analysed for their responsiveness to TGFβ. As a control Smad3 was also expressed in MDCK Raf:ER cells. For each cell line, two cell populations were generated expressing different levels of Smad3. A control cell population for RafT and Raf:ER cells was generated carrying empty vector. Figure 6.6A shows total lysates analysed for efficient re-expression of Smad3 by Western blotting (upper panel). Smad3 protein expression in RafT cells was restored and expression levels were significantly increased compared to MDCK Raf:ER cells carrying empty vector. As a control for Raf:ER activation in RafT cells expressing Smad3, the phosphorylation of p42MAPK in total lysates was assayed by Western blotting (Figure 6.6A, lower panel). In RafT cells, expression of Smad3 does not alter the ability to strongly activate p42MAPK.

To analyse whether re-expression of Smad3 in RafT cells restores the sensitivity to growth inhibitory effects of TGFβ, cell cycle distribution was examined in MDCK RafT and MDCK Raf:ER cells expressing Smad3 or empty vector. Cells were analysed 24 hours after treatment with TGFβ (Figure 6.7B). In all three MDCK Raf:ER cell lines exposure to TGFβ led to an increased percentage of cells in G1 (66-71%) and a reduced percentage of cells in S-phase (14-15%), comparable to TGFβ untreated cells (G1: 34-40%, S: 31-35%). In contrast,
Figure 6.5. TGFβ-dependent induction of p21CIP transcription is abrogated in Raf transformed MDCK cells. (A) RNase protection analysis of RNA from MDCK RafiER cells treated with 100 nM 4HT where indicated, Raf transformed MDCK RafiER cells (RafT) and MDCK V12Ras cells untreated or treated with 2 ng/ml TGFβ for 3 h. The RNA was hybridized with probes to p21CIP1 and γ-actin. Bands were quantitated using a Phosphorimager. Expression of p21CIP1 is shown as the fold change of the value for the untreated MDCK RafiER cells corrected for the γ-actin expression. (B) MDCK RafiER cells either untreated or treated with 4HT for 72h and MDCK RafT cells were stimulated with 2 ng/ml TGFβ for 24 h. Lysates were analysed for the expression of p21CIP by Western-blotting.
Figure 6.6. Expression of Smad3 in MDCK RafT cells restores sensitivity to TGFβ induced growth arrest. (A) MDCK Raf:ER cells untreated or transformed by long-term 4HT treatment (RafT) stably expressing Smad3 (Smad3-hy, Smad3-bl) or empty vector (ev-hy) were assayed for Smad2 and Smad3 expression and for activation of p42MAPK by Western blotting. (B) MDCK Raf:ER cells and MDCK RafT cells stably expressing Smad3 (Smad3-hy, Smad3-bl) or empty vector (ev-hy) were stimulated with TGFβ (7.5 ng/ml) for 24 h. The cell cycle distribution was assayed by flow cytometry after propidium iodide staining.
in RafT cells carrying empty vector no significant changes in cell cycle distribution in response to TGFβ were observed. However, re-expression of Smad3 into RafT cells rendered these cells sensitive to TGFβ and led to a reduction in S phase (16-19%) and an increase in G1 (55-56%). Since the re-expression of Smad3 in RafT cells resulted in high Smad3 levels compared to MDCK Raf:ER cells, it is not clear whether this reflects a physiological response. In order to address this question, RafT clones expressing Smad3 were isolated and will be analysed in a similar manner.

These data indicate a positive correlation between Smad3 expression and the TGFβ induced cell cycle regulation, since re-expression of Smad3 was able to restore the sensitivity to growth inhibition by TGFβ in MDCK Raf:ER cells which have undergone EMT (RafT).

6.3 Discussion

MDCK Raf:ER cells which have undergone EMT due to sustained Raf-1 activation are refractory to anti-proliferative effects of TGFβ. During EMT, gene expression of Smad3 and E-cadherin is strongly down-regulated at a very late stage of this process, possibly due to impaired gene transcription. It is possible that Smad3 repression causes MDCK RafT cells to evade TGFβ induced growth inhibition, since TGFβ dependent p21Cip1 expression is also abolished in RafT cells. This is an interesting observation, since p21Cip1 is a target gene for both the TGFβ pathway and the Raf/MAPK pathway. Re-expression of Smad3 in RafT cells is able to restore the sensitivity to TGFβ leading to inhibition of cell cycle progression.

A possible mechanism for the repression of Smad3 and E-cadherin relatively late during EMT might be promoter methylation. Methylation of gene promoter regions results in gene silencing and thus induces loss of gene function, a common epigenetic event during cell transformation and cancer progression. An example for methylation and gene inactivation is the CDK inhibitor p16INK4a gene which is mutated or silenced by methylation in a large number of human tumours (Herman et al., 1995). The E-cadherin gene expression is also found to be silenced in a variety of human carcinomas (Graff et al., 1998); (Yoshiura et al., 1995). Since activation of the Raf/MAPK pathway by oncogenic Ras was found to enhance expression and activity of DNA methyltransferase (MacLeod et al., 1995); (Deng et al., 1998), it will be of interest to determine whether Raf might induce DNA methylation causing down-regulation of Smad3 and E-cadherin during EMT.
RafT cells which have down-regulated Smad3 expression are resistant to TGFβ induced growth arrest. This might imply a role for Smad3 in regulating TGFβ induced growth response. Evidence that Smad3 function is involved in the regulation of cell cycle came from studies in Smad3 knockout mice. In contrast to Smad2 and Smad4 null mice which are embryonic lethal due to severe developmental defects (Nomura and Li, 1998); (Weinstein et al., 1998); (Sirard et al., 1998), Smad3 knockout mice are viable and survive into adulthood (Datto et al., 1999); (Yang et al., 1999); (Zhu et al., 1998). Mouse embryonic fibroblasts (MEFs) derived from the Smad3 null mice generated by Datto et al. show loss in their responsiveness to TGFβ-mediated growth inhibition which is also observed in Smad3 null keratinocytes and Smad3 null astrocytes (Datto et al., 1999). Smad3 null mice generated in a different study exhibit immune system dysfunction based on the loss of TGFβ mediated growth inhibition in primary splenocytes (Yang et al., 1999). Interestingly, these mice also exhibit accelerated cutaneous wound healing characterised by an increased rate of re-epithelialisation as a result of loss of TGFβ responsiveness and cell cycle arrest (Ashcroft et al., 1999). Furthermore, Smad3 mutant mice generated by Zhu et al. form highly invasive colorectal carcinomas, indicating that inactivation of this TGFβ pathway relieves some epithelial cells from the differentiation signal leading to maintenance of the proliferative state (Zhu et al., 1998). However, tumour formation has not been observed in any other Smad3 deficient mouse lines. Taken together, the results obtained from Smad3 knockout mice strongly suggest a role for Smad3 in negative cell cycle regulation.

In MDCK RafT cells, which are resistant to TGFβ-induced growth arrest, down-regulation of Smad3 seems to be responsible for the inhibition of p21Cip1 expression, a TGFβ target gene product. Furthermore, re-expression of large amounts of Smad3 in RafT cells increases the sensitivity to TGFβ-induced cell cycle arrest. This might be supported by a study showing that the p21Cip1 promotor is preferentially activated via Smad3/Smad4 in response to TGFβ and expression of a mutant Smad3 protein abrogated p21Cip1 promotor induction (Moustakas and Kardassis, 1998). Another suggestion, why RafT cells are resistant to TGFβ-induced growth arrest, might involve the proto-oncogene product c-Myc. It was found that TGFβ leads to a decrease c-Myc expression in epithelial cells resulting in an increase in p21Cip1 and p15INK4b synthesis thereby causing cell cycle arrest (Hannon and Beach, 1994); (Reynisdottir et al., 1995); (Claassen and Hann, 2000). This decrease in c-Myc expression might be due to inhibition of c-Myc transcription by a Smad repressor complex (Chen et al., 2001), which is directly associated with down-regulation of p21Cip1 expression.
(Claassen and Hann, 2000). Thus, in MDCK RafT cells that have reduced levels of Smad3, TGFβ-induced down-regulation of c-Myc might be impaired and elevated c-Myc levels could lead to p21Cip1 repression, conferring resistance to anti-proliferative TGFβ effects. However, the relevance of c-Myc in rendering RafT cells resistant to TGFβ-induced cell cycle arrest still needs to be investigated.

In addition, activation of the Raf/MAPK pathway can also induce expression and increase protein stability of c-Myc (Kerkhoff et al., 1998); (Sears et al., 1999). However, the Raf/MAPK pathway is also known to induce p21Cip1 expression (Woods et al., 1997); (Sewing et al., 1997); (Zhu et al., 1998). It might be possible that the p21Cip1 promoter is repressed in a c-Myc-dependent manner, which might be regulated by a similar mechanism as observed for the p15INK4b promoter (Seoane et al., 2001). This could prevent p21Cip1 expression via activation of the Raf/MAPK pathway.

In contrast to RafT cells, in MDCK cells expressing activated Ras (V12Ras) p21Cip1 regulation is not blocked in response to TGFβ. It seems that these cells respond to TGFβ in a similar manner as observed following short-term Raf-1 activation in MDCK cells: p21Cip1 mRNA expression is already increased in the absence of TGFβ. MDCK V12Ras cells do not show reduced levels of Smad3 expression, which might explain the expression p21Cip1 and it might be speculated that they are still responsive to TGFβ-induced growth arrest. MDCK V12 Ras cells are transformed, but they have not undergone EMT and still express E-cadherin. Transformed epithelial cells expressing activated ras oncogenes can undergo EMT in co-operation with TGFβ which renders them resistant to TGFβ-mediated growth inhibition, thereby promoting tumour progression and cell invasion (Oft et al., 1996); (Oft et al., 1998); (Yin et al., 1999). Therefore it might be of interest to analyse, whether MDCK V12Ras cells would respond to TGFβ treatment with EMT.
Chapter 7

7 Final Discussion

7.1 Raf-induced EMT and Cancer

To study the process of epithelial cell transformation, the untransformed immortalised dog kidney epithelial line MDCK (Madin-Darby canine kidney) was used as an epithelial model system. MDCK cells form a polarised epithelial monolayer in vitro and show many features of an epithelial phenotype (Barker and Simmons, 1981). The initial investigations of this project were centred around the contribution of Ras and its downstream effectors PI3K and Raf-1 in their ability to affect epithelial morphology of MDCK cells. An important result from this study came from expression of a constitutively activated form of Raf-1 (Raf-CAAX) in MDCK cells, which leads to disruption of cell-cell junctions. This indicated that activation of the Raf-MAPK pathway is required to regulate the reduction of cell-cell contacts. From this initial observation further studies were focussed on the contribution of the Raf/ERK-MAPK pathway on changes in cell-cell adhesion and epithelial cell morphology during cell transformation.

Using a 4-hydroxy-tamoxifen (4HT) inducible Raf:ER fusion protein stably expressed in MDCK cells, activation of Raf:ER is sufficient to cause a change in cell morphology within 24 hours of Raf-1 activation which is similar to a cell scattering response observed with HGF/SF: it is characterised by reduced cell-cell adhesion, loss of cell polarity and increased cell motility. Furthermore, sustained Raf-1 activation for more than 14 days leads to cell transformation by mediating an epithelial to mesenchymal transition (EMT). This is characterised by delocalisation of the junctional proteins E-cadherin and ZO-1 as well as the intermediate filament protein cytokeratin occurring within the first days of Raf-1 activation. Only after sustained Raf-1 activation is expression of the adherens junction protein E-cadherin abolished and expression of the mesenchymal intermediate filament protein vimentin induced. Loss of E-cadherin expression is frequently observed in human tumours of epithelial origin either due to mutations in the E-cadherin gene (Birchmeier and Behrens, 1994) or due to methylation of CpG dinucleotides in the promotor region (Graff et al., 1995). Methylation as a mechanism of gene inactivation has been frequently found to regulate tissue specific gene expression. It has also been suggested to play an important role in
tumourigenesis for example by inactivation of tumour suppressor genes, such as pRb and p16INK4a (Mostoslavsky and Bergman, 1997). Furthermore, loss of E-cadherin expression might contribute to tumour cell proliferation. The molecular link might be provided by the signalling function of β-catenin which has been found to regulate expression of the \textit{c-myc} proto-oncogene (He et al., 1998) and the \textit{cyclinD1} gene (Tetsu and McCormick, 1999). It seems likely that β-catenin signalling plays an important role in advanced tumours, since an increase in β-catenin/LEF-1 complexes was observed mediating transcriptional activity in mouse mammary epithelial cells which had undergone EMT due to sustained activation of an inducible form of c-Fos (Fos:ER) (Eger et al., 2000). Therefore, loss of E-cadherin expression during Raf-1 induced EMT in MDCK cells might be regulated by promotor methylation, which could lead to activation of β-catenin signalling. Promotor methylation might also be involved in mediating changes in the overall gene transcription pattern during EMT in MDCK cells, since oncogenic Ras protein was found to increase expression and activity of DNA methyltransferase presumably via activation of the Raf/MAPK pathway (MacLeod et al., 1995); (Deng et al., 1998).

Unlike the MDCK cells in which Raf:ER was activated by exposure to 4HT, MDCK cells expressing Raf-CAAX do not display a mesenchymal phenotype and do not exhibit invasive growth in collagen gels, although they do show partial disruption of cell-cell contacts, the initial event required for EMT. The likely critical difference between these two cell lines is the strength of Raf-1 activity. ERK-MAPK activation in Raf:ER expressing cells following treatment with 4HT is much higher than the constitutive level in Raf-CAAX expressing cells. Since the observation that quantitative changes in signal input can lead to qualitatively different cellular responses is widely known for some growth factors and intracellular signalling molecules (Wennstrom and Downward, 1999); (Whitman and Melton, 1992). Different threshold levels of Raf-1 activity might lead to different biological effects on cell morphology and cell transformation. It seems that the effects of strong and sustained Raf-1 activation in MDCK cells resemble those of murine breast epithelial cell line expressing activated mutant H-Ras (Ep2Ras) which also undergo EMT in the presence of TGFB (Oft et al., 1996). Furthermore, expression of activated mutant H-Ras results in EMT and tumour progression in a mouse skin cancer model (Cui et al., 1996). High constitutive levels of MAPK activation is also characteristic for Ep2Ras cells, and activity levels almost reach those of strong Raf:ER activation in MDCK cells (Janda et al. manuscript in preparation). Similar high levels of MAPK activity might be achieved for example by gene amplification of the
mutant ras or loss of heterocigosity (LOH) which have been observed frequently during tumour progression in a mouse skin cancer model (Bremner and Balmain, 1990).

During the process of EMT, MDCK cells begin to produce and secrete TGFβ. This autocrine factor is required to maintain invasive growth of MDCK cells expressing activated Raf-1 in collagen gels. There is evidence that TGFβ functions as an important mediator of EMT resulting in cell invasion, which is also found in advanced human tumours. It has been demonstrated in vitro and in vivo that TGFβ can synergise with activated H-Ras. For example, in Ep2Ras cells addition of exogenous TGFβ induced EMT by co-operation with the activated Ras protein (Oft et al., 1996). In a mouse skin model of tumour progression, treatment with a chemical carcinogen results in h-ras mutations, initiating benign squamous tumours (Bremner and Balmain, 1990). During tumour progression the gene dosage of mutant H-Ras is stepwise amplified which leads to EMT, resulting in progression from benign squamous cell carcinomas to malignant spindle cell carcinomas in vivo. This process is dependent on autocrine TGFβ signalling (Caulin et al., 1995); (Portella et al., 1998). Both the Ep2Ras cells and the skin cancer model provide clear examples of synergistic interactions between the Ras and TGFβ pathways in establishing EMT resulting in an invasive phenotype and tumour progression. The MDCK Raf:ER model for EMT now provides further support for the importance of the Raf/MAPK pathway during cell transformation.

The fact that TGFβ promotes invasive growth in the presence of activated Ras or Raf-1 seems to be in apparent contradiction to the well documented finding that TGFβ can act as a tumour suppressor by inhibiting cell growth and promoting apoptosis of epithelial cells and of benign tumour cells (Alexandrow and Moses, 1995). However, TGFβ seems to exert a dual role during tumour development, since it enhances tumour progression and invasiveness at late stages of tumourigenesis (Welch et al., 1990); (Fynan and Reiss, 1993); (Cui et al., 1996). This biphasic responsiveness to TGFβ is also found in the case of MDCK Raf:ER cells, in which the response to TGFβ depends on the endpoint studied. Activation of Raf-1 for up to 48 hours prior to the addition of TGFβ did not prevent inhibition of cell cycle progression, indicating that the integrity of the TGFβ pathway is not altered. However, MDCK Raf:ER cells that have undergone EMT due to sustained Raf-1 activation become completely resistant to anti-proliferative TGFβ effects. It seems that this is not the result of early or intermediate Raf-1 regulated signalling, but a result of long-term transcriptional changes. However, the continued dependence of the Raf-1 transformed cells on the TGFβ autocrine loop for invasive growth in collagen gels shows that they are still responsive to some of the effects of TGFβ.
Different mechanisms can account for loss of sensitivity to growth inhibitory TGFβ effects. For instance, rat intestinal epithelial cells were found to lose responsiveness by down-regulation of TGFβ receptor type II expression (Zhao and Buick, 1995). Alternatively, mutations in components of the TGFβ pathway can occur. These involve mutations in TGFβ receptor type I, type II (Markowitz and Roberts, 1996), Smad2 and Smad4 (Schutte, 1999). Inactivation of Smad4 occurs in about 50% of pancreatic cancers, but it is relatively rare in other tumour types. Interestingly, mutations in Smad3 have not been observed so far. Mutations in TGFβ signalling components seem to allow tumour cells to evade TGFβ-induced growth arrest, thereby promoting tumour progression. In contrast, there is evidence that tumour cells carrying mutations in Smad4 can still respond to TGFβ (Fink et al., 2001). This might be due to signalling through Smad-independent pathways (Hocevar et al., 1999) or, alternatively, other functional Smad4 homologues might exist, which have been described for X. laevis (Howell et al., 1999); (Newman and Krieg, 1999). However, most advanced tumours do not show mutations of the receptor or Smads leaving the integrity of the TGFβ signalling pathway intact. It is clear that in many circumstances TGFβ can promote the malignant phenotype of transformed cells in human tumours or animal cancer models (Akhurst and Balmain, 1999). It is therefore likely that mechanisms exist that allow the tumour cells to avoid the inhibitory effects of TGFβ while continuing to take advantage of the positive, pro-malignant effects.

It should be noted that beside the effects of TGFβ on the tumour cell itself, it might also affect cells of the surrounding tissue. This could for example be important for promoting angiogenesis and for mediating immunosuppression.

7.2 Raf and Smad signalling

MDCK cells still undergo cell cycle arrest in response to TGFβ following short-term activation of the Raf/MAPK pathway, which reflects that the functional integrity of the TGFβ signalling pathway is not altered in response to Raf-1 activation. This is also confirmed by having a closer look at Smad function: after short term-activation of Raf, nuclear translocation, DNA binding and transcriptional activity of Smads2, Smad3 and Smad4 are not altered in response to TGFβ in MDCK cells. This is in contrast to a study by Kretzschmar et al., showing that in Ras-transformed Ep2Ras cells ERK-MAPK can phosphorylate Smad2 and Smad3 thereby blocking nuclear translocation. This mechanism would result in the
inhibition of Smad-mediated TGFβ signalling (Kretzschmar et al., 1999). However, it seems that this model can only lead to a partial inhibition in Smad translocation, since in Ep2Ras cells the DNA binding ability of Smad3 is not impaired (see Chapter 4). Furthermore, some TGFβ response is retained in Ep2Ras cells which is required for EMT and invasive growth (Oft et al., 1996). Complete inhibition of TGFβ signalling by expression of a dominant negative TGFβ receptor in Ep2Ras cells abrogates EMT (Oft et al., 1998). An intact TGFβ pathway is also required for the progression of benign skin tumours to invasive spindle carcinomas in mice expressing activated $ras$ with keratinocyte-targeted expression of TGFβ (Cui et al., 1996). How oncogenic Ras co-operates with TGFβ to prevent its growth inhibitory function and to promote invasiveness is still unclear, although blocking of Smad2 and Smad3 translocation into the nucleus does not seem to be a likely mechanism. However, it has to be considered that alternative TGFβ pathways might exist which could contribute to biological effects in a Smad-independent manner. For example, TGFβ is still able to induce increased expression of fibronectin, a matrix component often found to promote cell invasiveness, in tumour cell lines lacking functional Smad4 through a JNK-dependent mechanism (Hocevar et al., 1999).

An interesting model for the inhibition of anti-proliferative TGFβ effects in MDCK cells, which have undergone EMT due to sustained Raf-1 activation (RafT) has been revealed in this study. The results show that in MDCK RafT cells the expression of Smad3 is strongly suppressed. Furthermore, TGFβ-regulated expression of p21Cip1 is also abolished in RafT cells. Interestingly, re-expression of Smad3 protein in these RafT cells restores the sensitivity towards TGFβ growth inhibition.

Evidence supporting a function of Smad3 in transducing the anti-proliferative TGFβ signal comes from Smad3 knockout studies in mice (Datto et al., 1999); (Yang et al., 1999); (Zhu et al., 1998). The observation that Smad3 knockout mice develop highly invasive and metastatic adenocarcinomas does not only implicate a role for Smad3 in controlling cell growth (Zhu et al., 1998), but it also might indicate effects of a changed equilibrium of Smad3 and Smad2, shifted towards Smad2 function. This might suggest a role for Smad2-dependent transcription in the modulation of invasiveness in MDCK cells. Since it has been demonstrated that Smad3 can directly inhibit Smad2 action at the transcriptional level (Labbe et al., 1998), it seems that down-regulation of Smad3 expression might enhance a Smad2 mediated TGFβ response. Since Smad2 is not able to bind DNA itself, additional transcription factors are required to reach specific target genes. Moreover, in the presence of
an activated Raf/MAPK pathway different sets of co-factors might be available to co-operate with Smads. These could possibly be provided by activation of gene expression by the Raf/MAPK pathway, thereby leading to a co-operation with Smad2 on the level of gene transcription. Since there is evidence that Smads can regulate transcription in co-operation with β-catenin (Nishita et al., 2000), it would be interesting to investigate in the EMT model in MDCK cells whether sustained Raf-1 activation could positively affect β-catenin/LEF-1 mediated gene transcription and whether β-catenin could co-operate with Smads.

Interestingly, since mutations in Smad3 in connection with tumourigenesis in mice or humans have not been observed so far, epigenetic down-regulation, possibly via methylation of the Smad3 promotor during cell transformation might be one possible mechanism to achieve resistance towards anti-proliferative TGFβ effects. This might directly affect p21Cip1 expression, since the p21Cip1 promotor is preferentially activated by Smad3 complexes rather than by Smad2 (Datto et al., 1995); (Moustakas and Kardassis, 1998). Another mechanism which might lead to loss of p21Cip1 expression in response to TGFβ could involve expression of the proto-oncogene c-myc, since expression and protein stability of c-Myc were found to be regulated via the Raf/MAPK pathway (Kerkhoff et al., 1998); (Sears et al., 1999). c-Myc was found to repress directly transcription of the p21Cip1 gene (Claassen and Hann, 2000) and p15INK4b gene (Seoane et al., 2001). In a breast epithelial cell line transformed by H-Ras (MCF-7) loss of TGFβ-responsiveness was found to correlate with loss of c-myc repression (Chen et al., 2001). Since c-myc expression can be inhibited by a Smad repressor complex (Chen et al., 2001), it might be suggested that down-regulation of Smad3 expression in RafT cells prevents c-Myc repression. This could enhance Raf/MAPK induced c-Myc expression and allow evasion of TGFβ-induced cell cycle arrest. Other studies have shown that Raf-1 can directly induce expression of p21Cip1 (Woods et al., 1997); (Sewing et al., 1997); (Zhu et al., 1998). However, it might be possible that the p21Cip1 promotor is rendered insensitive to Raf-1 activation due to c-Myc mediated p21Cip1 repression.

In vivo studies have shown that tumour progression in mice from squamous cell carcinomas to spindle cell carcinomas in the presence of high expression of oncogenic H-Ras is associated with irreversible loss of the INK4 locus [ ]. This might explain resistance towards growth-inhibitory TGFβ function in these carcinomas (Linardopoulos et al., 1995). Loss of the INK4 locus, which encodes for the CDK inhibitors p16INK4a and p15INK4b has been associated with the evasion of growth control in a number of tumours (Chin et al., 1998).
However, loss of the INK4 locus does not seem to be involved in MDCK RafT cells, since re-expression of Smad3 renders these cells again sensitive for anti-proliferative TGFβ effects. It can be suggested that strong activation of Ras or Raf during cell transformation mediates the down-regulation of CDK inhibitors which allows evasion of anti-proliferative TGFβ effects (see Figure 7.1).

**Figure 7.1.** Model showing the effects of Raf-1 activation on TGFβ-induced apoptosis and cell cycle arrest. Co-operation between the Raf-1 pathway and TGFβ signaling is required for EMT.

### 7.3 Raf and TGFβ-induced apoptosis

In MDCK cells TGFβ induces apoptosis which is accompanied by activation of the initiator caspase-8, effector caspases and by induction of cytochrome c release from mitochondria. However, the ability of TGFβ to induce apoptosis is rapidly blocked by Raf-1 activation in MDCK cells as well as in the human mammary epithelial cell line MCF-10A. The mechanism by which short-term Raf-1 activation prevents apoptosis in MDCK cells differs from the one in MDCK cells, which have undergone EMT due to sustained Raf-1 activation (RafT). While short-term Raf-1 activation does not abolish cytochrome c release, it is blocked by sustained activation of Raf-1 in MDCK cells. However, in both situations, TGFβ-induced caspase activation is prevented.

At present it is not clear how TGFβ induces caspase activation and cytochrome c release. It is possible that activation of death receptor signalling is involved, since TGFβ-induced
apoptosis can be blocked by dominant negative FADD in MDCK cells. FADD is known to associate with a number of death receptors, thereby mediating the formation of a death inducing signalling complex (DISC) which leads to caspase-8 activation (Schulze-Osthoff et al., 1998). However, other reports found that TGFβ induced apoptosis in lymphoma cells does not seem to involve death receptor signalling but whether this is cell type specific is not clear at present (Inman and Allday, 2000).

As a result of this project, two mechanisms have been evolved for the induction of apoptosis by TGFβ supported by the findings of Raf-1 protecting from TGFβ-induced apoptosis (Figure 7.2). Since short-term activation of Raf-1 prevents caspase activation but does not block cytochrome c release it does not seem likely that caspase-8 activation occurs upstream of cytochrome c release. This suggests that caspase-8 activation could occur downstream of effector caspases as part of an amplification response to cytochrome c release (Figure 7.2, model A). The other mechanism suggests that caspase-8 activation and cytochrome c release are regulated via different pathways (Figure 7.2, model B), which would provide an explanation for the involvement of death receptor signalling. Activation of death receptors could directly lead to activation of caspase-8, while cytochrome c release could be directly induced independently by another mechanism. It might be possible that TGFβ induced apoptosis could involve the activation of TAK, a MAPKKK, thereby inducing the stress activated MAPK p38 (Yamaguchi et al., 1995); (Hanafusa et al., 1999). In vivo, activation of TAK is associated with JNK activation and apoptosis (Takatsu et al., 2000). Activation of p38 and JNK have been implicated in the induction of stress activated apoptosis (UV, peroxide), thereby directly mediating cytochrome c release, resulting in apoptosome formation (Tournier et al., 2000); (Assefa et al., 2000); (Zhuang et al., 2000). TGFβ might activate p38 or JNK, possibly via TAK, to induce release of cytochrome c. TGFβ-induced apoptosis does not seem to involve caspase-8 mediated cleavage of Bid, which is known to induce cytochrome c release (Li et al., 1998). Otherwise inhibition of caspase-8 by Raf-1 activation would possibly prevent cytochrome c release.

How does short-term activation of Raf-1 protect MDCK cells from TGFβ-induced apoptosis? Activation of Raf-1 can induce post-translational modifications of proteins or de novo transcription. Both might be involved in the regulation of epithelial cell survival by Raf-1 activation. Raf-1 might promote survival through phosphorylation of pro-apoptotic proteins,
Figure 7.2. Hypothetical models for TGFβ-induced apoptosis. A Caspase-8 is activated downstream of effector caspases via an amplification loop. B TGFβ-induced apoptosis involves death receptor signalling, which might lead directly to caspase-8 activation.

such as Bad (Bonni et al., 1999); (Fang et al., 1999), a member of the Bcl-2 family, which mediates cytochrome c release (Antonsson and Martinou, 2000). Involvement of phosphorylation and thus inhibition of Bad in Raf-1 mediated survival of MDCK cells seems unlikely, since inhibition of Bad should lead to prevention of cytochrome c release. Activation of Raf-1 might also mediate survival via activation of IAP proteins, which inhibit apoptosis through direct interaction with processed caspases, acting downstream of cytochrome c release from mitochondria (Deveraux and Reed, 1999). Although a direct molecular link between the Raf/MAPK pathway and IAPs is still missing, there is genetic evidence from studies in D. melanogaster. Activation of the Raf/MAPK pathway blocks the apoptotic function of Hid (Kurada and White, 1998); (Bergmann et al., 1998), an inhibitor of DIAP1, the IAP homologue in D. melanogaster. Inhibition of Hid by the Raf/MAPK pathway results in IAP activation and protection from apoptosis (Goyal et al., 2000). The only IAP
inhibitor identified in mammalian cells so far is Smac/DIABLO (Du et al., 2000); (Verhagen et al., 2000). A recent study showed that Smac/DIABLO promotes apoptosis in NLE cells by binding to the IAP family member XIAP, which resulted in the release of processed caspase-9 (Ekert et al., 2001). Therefore, it would be important to examine whether the Raf/MAPK pathway can inhibit factors like Smac/DIABLO and induce IAP dependent inhibition of caspases.

In MDCK RafT cells the mechanism leading to cell survival seems to be different from the one observed following short-term Raf-1 activation. In RafT cells caspase activation as well as release of cytochrome c from the mitochondria is blocked in response to TGFβ. Moreover, expression of the anti-apoptotic protein Bcl-XL is increased in RafT cells, which is not observed after short-term Raf-1 activation. The explanation for the establishment of a distinct survival mechanism is presently unclear, but might be a result of changes in the overall protein expression during Raf-1 induced cell transformation. This could lead to expression of anti-apoptotic proteins to allow the evasion from apoptosis. Up-regulation of Bcl- XL protein levels might explain the prevention of cytochrome c release in RafT cells. Over-expression of Bcl- XL in MDCK cells has been shown to prevent cytochrome c release and protect from detachment induced apoptosis (anoikis) (Rytomaa et al., 2000). An example for Ras induced down-regulation of expression of pro-apoptotic proteins has been shown in fibroblasts and epithelial cells, where Ras activation leads to repression of Fas transcription, which conferred resistance to Fas-ligand induced apoptosis (Peli et al., 1999).

The results presented here indicate that TGFβ induced apoptosis and TGFβ induced cell cycle arrest in MDCK cells seem to be mediated via separate pathways. This is concluded from the observation that apoptosis is prevented by short-term activation of Raf-1 at a point where growth arrest still occurs and where the integrity of of the Smad signalling pathway is still ensured. This observation is also supported by two reports showing that inhibition of TGFβ induced apoptosis by treatment with a broad spectrum caspase inhibitor still allows these cells to undergo growth arrest in response to TGFβ (Inman and Allday, 2000); (Brown et al., 1998). While TGFβ induced cell cycle arrest involves Smad signalling, it is not clear so far whether TGFβ induced apoptosis requires Smad function. It cannot be ruled out that the apoptosis pathway might act downstream of Smad-mediated transcription since the onset of TGFβ induced apoptosis in MDCK cells is slow enough, to allow Smad mediated transcriptional events.
Protection from TGFβ induced apoptosis by Raf-1 at a time where MDCK cells are still able to undergo TGFβ induced growth arrest might be an important cellular protection mechanism, mediating cell survival in the presence of pro-apoptotic signals. This might allow the establishment of the autocrine TGFβ loop, which is required for EMT.

7.4 Raf and anoikis

The protection from apoptosis by Raf-1 activation is, however, not specific for TGFβ, since it can also be seen for TNFα-induced apoptosis, matrix detachment induced (Rytomaa et al., 2000); (Le Gall et al., 2000) and etoposide-induced apoptosis. It therefore appears that activation of the Raf/ERK pathway provides a more general survival signal in epithelial cells. Recently, the role of the Raf/MAPK pathway in protection from a number of apoptotic stimuli became more evident (Bonni et al., 1999); (Kazama and Yonehara, 2000).

In MDCK cells activation of Raf-1 is sufficient to protect from anoikis via a mechanism acting downstream of cytochrome c release. Previous work in Ras transformed MDCK cells showed that Ras activation protects from anoikis through the ability of Ras to activate the PI3K pathway, thereby preventing cytochrome c release from the mitochondria (Rytomaa et al., 2000). The functions of Raf-1 as an inducer of cell survival are less well documented than for the Ras downstream effector Akt/PKB, which is primarily known for its function in mediating cell survival (Downward, 1998); (Datta et al., 1999). Activated Akt/PKB has been reported to inhibit cytochrome c release from the mitochondria in response to matrix detachment (Rytomaa et al., 2000), UV, etoposide or upon c-Myc expression in the absence of serum (Kennedy et al., 1999).

Interestingly, short-term activation of Raf:ER in the human epithelial cell line MCF-10A protects these cells from anoikis via a mechanism involving Akt/PKB activation (Schulze et al., 2001). In this cell line, activation of Raf:ER induces the expression of hbEGF, TGFα and amphiregulin which leads to establishment of an autocrine growth factor loop. Activation of EGF receptor was found to activate the PI3K/Akt survival pathway. Several reports show that cell survival mediated by Akt/PKB seems to affect the cell death machinery upstream of mitochondria, thereby blocking cytochrome c release (Kennedy et al., 1999); (Rytomaa et al., 2000); (Page et al., 2000). However, preliminary data suggest that in MDCK cells autocrine EGF growth factor signalling does not play a major role in providing a survival signal in response to TGFβ or matrix detachment. This might explain the fact that cytochrome c release
is not blocked by activation of Raf-1 in MDCK cells. It seems that the survival mechanism regulated by Raf functions primarily downstream of cytochrome c release from the mitochondria. This is supported by a study showing that B-Raf can protect Rat-1 fibroblasts from serum deprivation induced apoptosis downstream of cytochrome c release (Erhardt et al., 1999). Although cytochrome c is an essential component of the mitochondrial respiratory chain its release is not necessarily the commitment step for apoptosis. Cells can recover from cytochrome c release, for example when caspase activation is blocked (Deshmukh and Johnson, 1998); (Martinou et al., 1999). There is evidence that the commitment to cell death may be regulated downstream of cytochrome c release, since cytochrome c is essential for apoptosome formation and caspase-9 activation. It has been shown that mitochondria whose outer membrane has been permeabilised by pro-apoptotic Bcl-2 family members can be restored to normal function (von Ahsen et al., 2000).

Regulation of apoptosis by Raf seems to be largely dependent on the threshold of Raf activation. For example, expression of Raf-CAAX, which is a less potent activator of the ERK-MAPK pathway, is unable to protect MDCK cells from anoikis (Khwaja et al., 1997), whereas activation of Raf:ER seems to reach the required degree of Raf-1 activity. Furthermore, activation of a particular survival pathway might be dependent on the survival stimulus. For example, strong activation of EGF receptor was found to inhibit TGFβ-induced apoptosis in rat fetal hepatocytes via a PI3K-dependent pathway, which impaired cytochrome c release and caspase3 activation (Fabregat et al., 2000).

Furthermore, it has been shown that GM-CSF-induced survival in haematopoietic cells requires Raf-MAPK activation but is PI3K-independent. In the same cell type, IL-3 induces survival via the PI3K/Akt pathway (Scheid and Duronio, 1998). Further investigations are required to characterise the contribution of the Raf/MAPK pathway to the network of survival pathways, which could lead to a better understanding of tumourigenesis.

7.5 Future directions

During the last years, progress has been made in delineating the mechanisms by which activation of oncogenic ras allows cells to evade apoptosis and cell cycle control leading to cell transformation and tumour development. Therefore it is of great importance to understand which Ras downstream effector pathways are involved at particular times during tumour progression. In this thesis the functions of the Ras downstream effector Raf-1 in promoting
epithelial cell survival and cell transformation to an invasive mesenchymal phenotype have been characterised. The in vitro EMT model in MDCK cells established here reflects important features of in vivo models of tumour progression and could therefore be employed to study the progression of molecular events during cell transformation. It seems that a high level of Raf/MAPK activity is required to promote not only epithelial cell transformation but also to induce a cellular dedifferentiation process (EMT), which is a prerequisite for the development of metastasis in vivo.

To investigate the contribution of TGFβ signalling in EMT by specific inhibition of Smad2 or Smad3 should help to untangle, at least in part, the complex signalling network, allowing the study of specific Smad2 or Smad3 downstream effects. Targeting Smad2 and Smad3 by an antisense strategy might allow to specifically block Smad-mediated gene expression. Alternatively, Smad signalling could be targeted by dominant negative Smad2 and Smad3 proteins. However, it has to be taken into account that they might lead to a general inhibition of TGFβ receptor signalling. Functional analysis of Smad interacting transcriptional co-factors in the presence of activated Raf/MAPK could help to understand the co-operation of both pathways in the regulation of transcription.

In addition, it would be of particular interest to analyse whether TGFβ induced apoptosis is Smad-dependent and whether the activation of stress activated MAPKs (p38, JNK) is involved. Furthermore, the mechanisms of Raf/MAPK providing a survival signal should be analysed in more detail: are post-translational modifications alone involved, or are transcriptional targets contributing in cell survival? Are IAPs regulated by activation of the Raf/MAPK pathway? Furthermore, the study of potential interplay between different survival pathways and their molecular mechanisms is of great importance since a network of signalling events seems to conduct cell survival upon oncogene activation.

To investigate transcriptional changes during EMT, comparative gene expression analysis could be performed using cDNA microarray technology. This would allow to monitor the kinetics of Raf-1 induced transcriptional events contributing to EMT, providing insight into the molecular mechanism involved in cell survival, proliferation and invasiveness. The identification of key regulators controlling these dramatic events would be a valuable tool for drug design and therapeutical intervention.
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Raf induces TGFβ production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells

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C-Raf-1 is a major effector of Ras proteins, responsible for activation of the ERK MAP kinase pathway and a critical regulator of both normal growth and oncogenic transformation. Using an inducible form of Raf in MDCK cells, we have shown that sustained activation of Raf alone is able to induce the transition from an epithelial to a mesenchymal phenotype. Raf promoted invasive growth in collagen gels, a characteristic of malignant cells; this was dependent on the operation of an autocrine loop involving TGFβ, whose secretion was induced by Raf. TGFβ induced growth inhibition and apoptosis in normal MDCK cells: Activation of Raf led to inhibition of the ability of TGFβ to induce apoptosis but not growth retardation. ERK has been reported previously to inhibit TGFβ signaling via phosphorylation of the linker region of Smads, which prevents their translocation to the nucleus. However, we found no evidence in this system that ERK can significantly influence the function of Smad2, Smad3, and Smad4 at the level of nuclear translocation, DNA binding, or transcriptional activation. Instead, strong activation of Raf caused a broad protection of these cells from various apoptotic stimuli, allowing them to respond to TGFβ with increased invasiveness while avoiding cell death. The Raf–MAP kinase pathway thus synergizes with TGFβ in promoting malignancy but does not directly impair TGFβ-induced Smad signaling.

[Key Words: Ras; Raf; TGFβ; SMAD; apoptosis]

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Activating mutations in the ras oncogenes occur in a high proportion of human cancers. Ras proteins act through a number of effectors to promote cell transformation, including the serine/threonine kinase Raf, which activates the ERK MAP kinase pathway, and the lipid kinase phosphoinositide 3-OH kinase [PI 3-kinase], which activates the serine/threonine kinase Akt and the small GTPase Rac (Downward 1998). Many carcinomas with activated ras oncogenes have undergone epithelial to mesenchymal transition [EMT], in which the epithelial phenotype, characterized by strong cell–cell junctions and polarity across the epithelial cell layer, is lost and a mesenchymal phenotype is acquired, characterized by weaker cell–cell interactions and increased motility (Schoenenberger et al. 1991; Birchmeier et al. 1993; Hay 1995). While the exact mechanisms by which Ras promotes EMT are not fully understood, it is likely to be important in the behavior of a number of tumor types, in particular in the establishment of invasiveness and metastasis.

Mouse mammary epithelial cells expressing activated Ras [EpRas] respond to TGFβ by undergoing EMT and then continue to maintain the mesenchymal phenotype by producing TGFβ in an autocrine manner (Oft et al. 1996). In addition, several other lines of evidence suggest that TGFβ can act to promote malignant transformation of cells: The factor was originally identified because of its ability to promote transformation of fibroblasts in cooperation with TGFa (Roberts et al. 1981). TGFβ is produced by many advanced human tumors and has been reported to promote progression from squamous cell to spindle cell carcinoma in a mouse model of skin carcinogenesis (for review, see Akhurst and Balmain 1999). Its importance for tumor maintenance is also suggested by the fact that the second copy of the TGFβ1 gene is not lost during chemical carcinogenesis in TGFβ1 heterozygous mice. As TGFβ can promote angiogenesis, wound healing, and immunosuppression, at least some of its positive effects on tumor progression may be through action on cells other than those of the tumor itself.
In apparent contradiction to the body of data implicating TGFβ in cancer progression, it is also a well-documented inhibitor of cell growth and inducer of cell death. TGFβ inhibits progression through the cell cycle via its ability to disregulate the Cdk inhibitors p21^{cip1}, p27^{kip1}, and p15^{ink4b} and is able to induce caspase-mediated apoptosis [Oberhammer et al. 1992; Hannon and Beach 1994; Reynisdottir et al. 1995]. Most tumors are resistant to the inhibitory effects of TGFβ, which in a subset of tumor types has been shown to be caused by loss of TGFβ receptors or the downstream signaling protein Smad4/DPC4 [for review, see Massague 1998]. This suggests that general loss of responsiveness to TGFβ may be important for epithelial cells to form tumors, with the positive effects of TGFβ on tumorigenesis possibly being due entirely to its actions on surrounding normal tissue interacting with the tumor.

Recently, it has been reported that the ERK MAP kinase, acting downstream of Ras, phosphorylates Smad2 and Smad3 in the linker region connecting the MH1 and MH2 domains [Kretzschmar et al. 1999]. This prevents their translocation to the nucleus in response to TGFβ, causing a general inhibition of TGFβ responses in epithelial cells. This model leaves little room for cell-autonomous effects of TGFβ on the promotion of tumorigenesis, such as have been reported recently; for example, expression of a dominant negative TGFβ receptor inhibits metastasis formation in mice by tumor cells that have undergone EMT [Oft et al. 1998; Yin et al. 1999].

These apparent contradictions between the roles of TGFβ as a tumor suppressor and as a tumor promoter prompted us to look in more detail at the interactions between the Ras–MAP kinase pathway and TGFβ signaling. In particular, we have addressed the issue of whether activation of Raf causes a global inhibition of TGFβ signaling, at least through Smads, or whether the effects are more selective, acting only on the tumor-suppressive aspects of the TGFβ response. We find that sustained Raf activation is able to induce EMT in MDCK cells, leading to the establishment of an autocrine TGFβ loop that promotes invasive behavior of the cells in collagen gels in vitro. The ability of TGFβ to cause invasive growth is not inhibited by Raf, but the apoptotic effects of TGFβ are blocked. This inhibition of apoptosis is not restricted to TGFβ but is common to several death stimuli. Raf activation does not impair TGFβ-induced Smad signaling in this system. We propose that activation of the Raf–MAP kinase pathway does not specifically inhibit TGFβ signaling but, rather, allows cells to display only the promalignant aspects of the TGFβ response, such as invasiveness, while broadly blocking apoptotic responses.

Results

Activation of Raf-ER leads to rapid phosphorylation of p42MAPK in MDCK cells

To investigate the role of Raf kinase in the regulation of epithelial cell morphology and in mediating antiapoptotic effects, we generated epithelial MDCK cells stably expressing a 4-hydroxytamoxifen (4HT) inducible EGF-F–Raf-1–hβER(DD) fusion protein [Woods et al. 1997]. This fusion protein (abbreviated Raf-ER) consists of the catalytic domain of Raf-1 lacking the Ras binding site (ARaf-1), with the two tyrosine phosphorylation sites Y340 and Y341 substituted by aspartic acid residues (DD), potentiating its kinase activity [Bosh et al. 1997]. MDCK Raf-ER cells were tested for activation of the MAPK pathway. Cells were starved and treated with 4HT for different times (Fig. 1A), and lysates were assayed for phosphorylation of p42MAPK and expression of the Raf–ER fusion protein by Western blotting. Induction of the Raf–ER fusion protein resulted in a rapid and sustained phosphorylation of p42MAPK, which was already detectable 15 min after stimulation and increased with time. In contrast, stimulation with EGF or HGF/SF led to a more rapid but only transient phosphorylation of MAPK (data not shown). In contrast, control cells carrying empty vector (mock) showed no phosphorylation of MAPK in response to 4HT treatment (Fig. 1B). As expected, pretreatment with the MEK inhibitor PD98059 completely abolished MAPK phosphorylation (Fig. 1B), indicating that the inducible Raf–ER system mirrors the growth factor–induced activation of the MAPK pathway.

Induction of morphological changes following Raf activation in MDCK cells

Having established the inducible Raf–ER fusion protein in MDCK cells, we examined morphological effects caused by Raf activation [Fig. 1C–F]. As HGF/SF is a physiological activator of the Raf–MAPK pathway that causes cell scattering, we compared HGF/SF-induced morphological effects to Raf–ER effects. MDCK Raf–ER cells were treated with 4HT or HGF/SF and phase-contrast pictures were taken before and after Raf activation. While MDCK Raf–ER cells in the absence of 4HT grow in compact islands like wild-type cells (Fig. 1C), activation of Raf–ER with 4HT for 24 h leads to disruption of intercellular contacts and increased cell migration (Fig. 1E), similar to the scattering effect, as observed with HGF–SF [Fig. 1D, Stoker et al. 1987; Gherardi et al. 1989]. Interestingly, the onset of both HGF/SF- and Raf–ER-mediated morphological effects occurred with similar kinetics as observed by time-lapse video microscopy (data not shown). Treatment with HGF/SF or 4HT led to disruption of cell–cell contacts by 8 h, and cell motility increased within 12 h. Pretreatment of the cells with the MEK inhibitor PD98059 (30 μM), the PI 3-kinase inhibitor LY294002 (20 μM), or the protein synthesis inhibitor cycloheximide (10 ng/mL) completely blocked the Raf-induced scattering response (data not shown). These findings suggest that basal PI 3-kinase activity as well as protein synthesis is required for cell scattering [Boyer et al. 1997; Khwaja et al. 1998]. Whereas the HGF/SF–mediated scattered phenotype is completely reverted after 3 d in the presence of ligand (data not shown), sus-
tained Raf activation leads to further changes in cell morphology (Fig. 1F). Cells became elongated and spindle shaped, indicating a conversion from the epithelial to a mesenchymal phenotype, which was stably maintained after 14 d of 4HT treatment. These results and the ability of the converted cells to grow in soft agar (data not shown) indicate that sustained Raf–ER activation in MDCK cells is sufficient to mediate cell transformation.

Activation of Raf–ER leads to repression of epithelial and induction of mesenchymal marker proteins

Conversion from an epithelial to a mesenchymal phenotype is characterized by decreased organization of intercellular junctions, loss of epithelial characteristics, and gain of mesenchymal characteristics (Greenburg and Hay 1986; Boyer et al. 1989). To investigate Raf–ER-mediated changes in cell morphology, immunostaining of epithelial and mesenchymal marker proteins was performed (Fig. 2). Optical sections were obtained at 0.25-μm intervals, and three-dimensional stacks of images were generated.

The immunostaining pattern showed that in unstimulated MDCK Raf–ER cells the adherens junction protein E-cadherin and the tight junction component ZO-1 are localized to intercellular junctions (Fig. 2a,e). Moreover in vertical sections (Fig. 2i), uninduced MDCK cells exhibited a polarized phenotype as demonstrated by localization of ZO-1 at the apical cell borders above the E-cadherin localization.

Within 4 d of 4HT treatment (Fig. 2b), ZO-1 was largely removed from the cell–cell junctions and diffusely expressed over the cell surface. This effect was even more pronounced after 6 d of 4HT treatment (Fig. 2c) and in MDCK Raf–ER cells that were transformed following sustained Raf activation (Fig. 2d). After 4HT addition for 4 d, E-cadherin was present at the intercellular contacts (Fig. 2f), which were now much more loosely formed, as well as on the cell surface, as observed in the vertical section (Fig. 2i). In contrast, after sustained Raf activation the localization of E-cadherin became diffuse and much reduced (Fig. 2g,h). In the absence of 4HT (Fig. 2k), the intermediate filament protein cytokeratin18 forms a network of filaments inside the cell borders. After treatment with 4HT for 4 d (Fig. 2l) or 6 d (Fig. 2m), only a few cytokeratin filaments across the cell body were visible, and levels were much reduced in the Raf transformed MDCK Raf–ER cells (Fig. 2n).

These results show that a strong and sustained Raf signal is sufficient to induce epithelial to mesenchymal transition (EMT), characterized by loss of apical-basal polarity, and epithelial marker proteins and expression of mesenchymal marker proteins (for review, see Birchmeier et al. 1995; Hay 1995).

Activation of Raf in MDCK cells induces secretion of TGFβ

It has been shown that the process of EMT is accompanied by secretion of TGFβ1, which is required to maintain the transformed phenotype (Oft et al. 1996). MDCK Raf–ER cells were therefore treated at different times with 4HT and cell culture supernatants analyzed for the presence of TGFβ1 by ELISA (Fig. 3A). Treatment with 4HT for 48 h led to a threefold up-regulation of TGFβ protein in the medium. An eightfold increase was seen after sustained Raf activation for >14 d, when MDCK cells have undergone EMT. Secretion of TGFβ in response to Raf–ER activation was also demonstrated by
Mechanisms of Raf synergy with TGFβ

Figure 2. Activation of Raf-ER leads to repression of epithelial marker proteins and induction of expression of a mesenchymal marker protein. MDCK Raf-ER cells either untreated [a,e,i,k,o] or treated with 100 nM 4HT for 4 d [b,f,i,l,p], 6 d [c,g,m,q] or after sustained Raf activation (>14 d; d,h,n,i) were immunostained with antibodies recognizing E-cadherin, ZO-1, cytokeratin, and vimentin and examined by confocal laser scanning microscopy. Confocal images show three-dimensional stacks of horizontal sections [a-h,k-r] or of vertical sections [i,j].

immunoblotting of concentrated cell culture supernatants [Fig 3B]. As it has been shown that the biologically active form of TGFβ is released from its cell surface-bound latent proform [LTGFβ] by serine protease urokinase-type plasminogen activator (uPA) induced plasmin activation (Lyons et al. 1990; Godar et al. 1999), we asked whether Raf-ER activation leads to uPA secretion in MDCK cells. The onset of uPA secretion was already detectable after 8 h of treatment with 4HT and increased with time in a manner similar to TGFβ secretion. Activation of uPA increases the expression of matrix-metalloproteases (MMP) and promotes the degradation of extracellular matrix components, a prerequisite for invasive growth of tumor cells (Rosenthal et al. 1998).

We next assayed the biological activity of the secreted TGFβ induced by Raf using the electrophoretic mobility shift assay (EMSA) on a 32P-labeled c-jun probe shown to bind TGFβ induced activated Smad3 in complex with Smad4 (Wong et al. 1999). Concentrated cell culture supernatant from Raf-transformed MDCK Raf-ER cells (cmRafT) was able to induce TGFβ signaling in control MDCK cells (mock), detected by a complex that bound the c-jun probe [Fig 3C, lanes 2, 4]. The same complex is induced by exogenously added TGFβ [Fig 3C, lanes 1, 2]. To confirm that this complex was formed by TGFβ-activated Smad3 and Smad4, we quantitatively supershifted the TGFβ-induced complex in the presence of an anti-Smad4 antibody or an anti-Smad3 antibody [Fig. 3C, lanes 7,9]. Addition of the peptide used to raise the anti-Smad3 antibody prevented the supershift [Fig. 3C, lane 8]. The commercial anti-Smad2 antibody (Transduction Laboratories) that cross-reacts with Smad3 (data not shown) efficiently supershifted the Smad3/4 complex [Fig. 3C, lane 10]. However, a more specific anti-Smad2
Figure 3. Activation of Raf in MDCK cells induces secretion of TGFβ. (A) MDCK Raf-ER cells, control MDCK cells, and MDCK Raf-ER cells transformed by long-term culture in 4HT [RafT] were cultivated in the presence or absence of 100 nM 4HT in DMEM -2.5% FCS for 24 h. Cells were cultured in DMEM -0.5% BSA for a further 24 h before collecting the supernatants. Cell culture supernatants were examined for TGFβ levels by ELISA. The values shown were normalized for cell number. (B) MDCK Raf-ER cells, control MDCK cells, and Raf-transformed MDCK Raf-ER cells [RafT] were cultured in DMEM -2% FCS and stimulated with 100 nM 4HT for the indicated times. The cell culture supernatant was concentrated by ultrafiltration and equal aliquots were analyzed by 12% SDS-PAGE under nonreducing conditions and immunoblotted for TGFβ and Urokinase-type Plasminogen Activator [uPA]. (C) MDCK control cells were either untreated or treated for 1 h with 2 ng/mL TGFβ, with conditioned medium of 4HT untreated MDCK Raf-ER cells [cm-4HT] or with conditioned medium of MDCK Raf-ER cells transformed by long-term exposure to 4HT [cmRafT]. Nuclear extracts were assayed for the binding of activated Smad3/4 complexes to a ^32P-labeled c-jun oligonucleotide probe by EMSA [lanes 1–4]. Untreated MDCK Raf-ER cells were stimulated with 2 ng/mL TGFβ for 1 h, and nuclear extracts were assayed in supershifts for the presence of Smads in complexes bound to the c-jun probe with the following antibodies: anti-Smad3, anti-Smad4, anti-Smad2, cross-reacting with Smad3 (Transduction Laboratories), and anti-Smad2 (SED). Peptide competition was performed with anti-Smad3 (Smad3 - pep).

Antibody [SED] did not modify the mobility of the complex [Fig. 3C, lane 11; Nakao et al. 1997].

Taken together these results indicate that Raf induces secretion of uPA and biologically active TGFβ, which are components known to play important roles in invasiveness and cell transformation.

Activation of Raf in MDCK cells leads to establishment of an invasive phenotype in collagen gels dependent on autocrine TGFβ stimulation

Having shown that TGFβ is secreted during Raf-induced EMT, we set out to analyze its role in this process. MDCK Raf-ER cells were cultured in collagen gels in the presence or absence of 4HT. Whereas wild-type MDCK cells [Fig. 4a] and untreated MDCK Raf-ER cells [Fig. 4b] grew in typical lumen-containing cysts [Khwaja et al. 1998], treatment with 4HT led to elongated, branched structures growing in an invasive manner [Fig. 4c]. Moreover, the Raf-transformed MDCK Raf-ER cells led to formation of invasive growing cord-like structures [Fig. 4d].

As TGFβ is known to inhibit growth and to induce apoptosis in epithelial cells grown in collagen [Oft et al. 1996] we examined the effects of TGFβ on wild-type and untreated MDCK Raf-ER cells that had been cultivated in collagen gels for 12 d. When cells were grown in the presence of TGFβ for 6 d, the cystic structures were degraded and dissociated into small fragments [Fig. 4e,f], probably reflecting apoptotic effects of TGFβ. In contrast, Raf-expressing cells [Fig. 4g,h] maintained their in-vitro phenotype and were still growing in the collagen matrices in the absence of growth factors [Fig. 4i].

Figure 4. Activation of Raf in MDCK cells leads to formation of an invasive phenotype in collagen gels dependent on autocrine TGFβ effects. Wild-type MDCK cells [WT; a,e,i], MDCK Raf-ER cells either untreated [b,f,j] or treated [c,g,k] with 200 nM 4HT for 6 d and MDCK Raf-ER cells transformed by long-term 4HT stimulation [RafT; d,h,l] were grown in type I collagen matrices in the absence [a–d] or presence of 5 ng/mL TGFβ [e–l] or neutralizing TGFβ antibodies [i–l] for a further 6 d under serum-free conditions. Structures were photographed at 20x magnification.
vasive phenotype in the presence of TGFβ. To examine if blocking of TGFβ signaling would cause reversion of the invasive phenotype, we tested the ability of neutralizing TGFβ antibodies to inhibit this process. MDCK Raf-ER cells were treated with 4HT for 6 d before TGFβ-neutralizing antibodies were added for a further 6 d. This led to a reversion of the invasive structures into cysts, indicating that TGFβ secretion is required to sustain the invasive phenotype.

Together, these results indicate that activation of Raf-ER induces invasive growth in collagen gels and that autocrine TGFβ function is required to maintain this Raf-induced phenotype.

**Short-term activation of Raf does not prevent TGFβ-induced cell cycle arrest**

We have demonstrated that activation of Raf-ER overcomes TGFβ inhibitory effects of MDCK cells in collagen gels. In order to characterize whether Raf activation interferes with TGFβ-mediated growth arrest, cell cycle distribution was examined (Fig. 5A). In MDCK Raf-ER cells pretreated with 4HT, the exposure to TGFβ led to an increased percentage of cells in G1 (67%) and a reduced percentage of cells in S phase (9%), comparable to TGFβ effects in 4HT untreated cells (G1: 63%; S: 20%). In contrast, cells transformed by prolonged activation of Raf (RafT) were insensitive to TGFβ-induced growth arrest. As it has been shown that TGFβ directly induces down-regulation of cyclin A in epithelial cells (Ralph et al. 1993), we also investigated the influence of TGFβ on cyclin A expression by Western blotting of total lysates (Fig. 5B). In proliferating cells cyclin A is up-regulated in late G1, indicating cell cycle progression into S phase. In asynchronously growing MDCK Raf-ER cells, cyclin A was highly expressed in the absence of TGFβ, and pretreatment with 4HT for 24 h did not alter the expression level, whereas addition of TGFβ led to a dramatic decrease in cyclin A expression in both 4HT-treated and untreated cells. Consistent with the data obtained above, the Raf transformed MDCK Raf-ER cells showed no changes in cyclin A expression, indicating that they were
no longer sensitive to growth-inhibitory signals mediated by TGFβ.

Together, these findings suggest that TGFβ-induced growth arrest is not perturbed by Raf activation over a period of a few days, whereas long-term Raf-transformed MDCK cells, which have already undergone EMT, show a loss in the antiproliferative response to TGFβ.

**TGFβ-induced apoptosis can be blocked by activation of Raf**

As it has been shown that TGFβ induces apoptosis via activation of the caspase cascade in a variety of cell types [Chen and Chang 1997], we explored both the role of TGFβ in triggering a pro-apoptotic signal and the influence of Raf on this process. Apoptosis in MDCK cells was scored following TGFβ treatment by determining the percentage of nuclei with condensed chromatin. TGFβ1 treatment resulted in a 7.5-fold increase in the percentage of apoptotic cells [Fig. 5C], whereas pretreatment with 4HT for 48 h or long term [RafT] completely abolished TGFβ-induced apoptosis. We next examined the activation of effector caspases in response to TGFβ. Cytosolic lysates were affinity labeled with the Z-EK[bio]D-aoenk peptide, which binds covalently to activated caspases [Martins et al. 1997]. TGFβ stimulation led to strong caspase activation, which was prevented by pretreatment of the cells with the pan-caspase inhibitor z-VAD [Fig. 5D]. Raf activation before the addition of TGFβ was also able to block caspase activation. Moreover, we investigated the activation of caspase-8 in response to TGFβ [Fig. 5E, upper panel]. It has been shown that on cellular death receptor stimulation, the initiator caspase-8 becomes activated, facilitating cleavage and activation of various effector caspases [Muzio et al. 1996; for review, see Wolf and Green 1999]. Caspase-8 can also be activated downstream of effector caspases as part of an amplification loop [Schulze-Osthoff et al. 1998]. Immunoblot analysis showed that TGFβ treatment led to strong activation of caspase-8, which was abolished in MDCK Raf-ER cells pretreated with 4HT. Taken together, these findings indicate that TGFβ provides a pro-apoptotic stimulus to MDCK cells by activating the caspase cascade. However, TGFβ-induced caspase activation was completely blocked by short-term Raf activation, showing that the Raf–MAPK pathway rapidly antagonizes TGFβ-mediated apoptosis.

**Activation of Raf does not affect signal transduction by Smads**

We have shown that the activation of Raf–ER in MDCK cells leads to secretion of TGFβ, which is necessary to maintain a highly transformed state, and at the same time, activated Raf renders the cells insensitive to the pro-apoptotic effects of TGFβ. So far, the mechanism by which activated Raf–ER inhibits TGFβ-mediated apoptosis is not known. Therefore, we studied the impact of Raf activation on TGFβ-mediated Smad signaling. Recently, it has been shown by others [Kretzschmar et al. 1999] that activation of Ras or EGF signaling in mouse epithelial cells blocks the TGFβ–induced translocation of the Smads into the nucleus and thus inhibits the TGFβ–induced transcriptional response. It was therefore important to study in the MDCK Raf–ER cells whether activation of Raf interferes directly with the TGFβ–Smad pathway. We studied several aspects of TGFβ signaling, namely the translocation of Smads into the nucleus, the binding of Smads to DNA, and finally, the Smad-dependent transcriptional response to TGFβ.

MDCK Raf–ER cells, untreated or pretreated with 4HT, and MDCK V12Ras cells were stimulated with TGFβ, and nuclear extracts were immunoblotted with antibodies to detect Smad2, Smad3, and Smad4. Only very little Smad4 was detected in the nucleus in the absence of a TGFβ signal, whereas exposure to TGFβ led to high accumulation of Smad4 in the nucleus [Fig. 6A, top panel]. The same result was obtained with the anti-Smad2/3 antibody [Fig. 6A, second panel], indicating that Smad2, Smad3, and Smad4 translocated to the nucleus in response to TGFβ stimulation, which was not impaired in MDCK Raf–ER cells pretreated with 4HT. MDCK cells constitutively expressing the activated form of Ras (MDCK V12Ras) also showed clear nuclear translocation of Smad2, Smad3, and Smad4 in response to TGFβ. On Raf activation, the phosphorylated form of p42MAPK is readily detectable in the nucleus and is not affected by exposure to TGFβ [Fig. 6A, third panel]. In addition, immunoblotting of the nuclear extracts showed no contamination with the cytosolic adaptor protein Grb2 [data not shown]. From this experiment we conclude that activation of the Ras–Raf–MAPK pathway does not prevent TGFβ-induced Smad translocation into the nucleus.

Next, the same nuclear extracts were used to examine the ability of the translocated Smads to bind DNA, a prerequisite for gene activation. Using the [32P]P-labeled c-jun probe as a readout for the Smad3/4 binding to DNA in the EMSA, we only observed a shifted protein–DNA complex in the nuclear extracts from cells treated with TGFβ [Fig. 6B, lanes 2,4,6,8,10]. Again, Raf or Ras activation did not prevent nuclear Smad3 and Smad4 binding to DNA. Dose-response analysis revealed no effect of Raf stimulation on Smad3 activation even at low concentrations of TGFβ [data not shown].

Finally, we studied whether activation of Raf–ER affects the transcriptional activity of the Smads in response to TGFβ. It has been shown that activated Smad2 and Smad4 are recruited by transcription factors to regulatory regions of TGFβ target genes. For example, activated Smad2 and Smad4 can be recruited to the activin/TGFβ responsive element (ARE) of the Mix.2 promoter by the winged helix transcription factor Fast-1 and then can stimulate transcription in a signal-dependent manner [Huang et al. 1995; Chen et al. 1996]. To assay the transcriptional activity of the Smads in the presence of activated Raf, we transiently transfected MDCK Raf–ER cells with a plasmid encoding the luciferase reporter gene driven by three copies of the ARE.
Mechanisms of Raf synergy with TGFβ

To further investigate whether activation of Raf leads to protection from other pro-apoptotic stimuli than TGFβ, we examined the effect of Raf on TNFa-induced apoptosis (Fig. 7). TNFa acts through its death receptor (TNFRI) to cause apoptosis in many epithelial cell types (Sidoti-de Fraisse et al. 1998). MDCK Raf-ER cells were treated with different concentrations of TNFa for 4 h in the presence of the protein synthesis inhibitor cycloheximide, which is required to block the anti-apoptotic NF-κB transcriptional response to TNFa. TNFa induced a dose-dependent increase of the percentage of apoptotic cells (Fig. 7A) as well as strong activation of caspase-8 (Fig. 7B), as demonstrated by Hoechst staining or in Western blotting, respectively. In contrast, 4HT pretreatment protected cells from apoptosis and caused caspase-8 not to be detectably activated. Moreover, we could show that activation of Raf-ER in MDCK cells blocks detachment-induced apoptosis, "anoikis" (Le Gall et al. 2000; Rytömaa et al. 2000). These results indicate that Raf not only inhibits TGFβ-induced death signaling but renders cells insensitive to a variety of pro-apoptotic stimuli, suggesting a more general anti-apoptotic function for Raf.

Discussion

Raf and epithelial–mesenchymal transition

MDCK cells are an untransformed immortalized dog kidney epithelial line that will form a polarized epithe-

Figure 6. TGFβ-induced nuclear translocation, DNA-binding, and transcriptional activity of Smad3/4 complexes is not affected by Raf activation. MDCK Raf-ER cells either untreated or pretreated with 100 nM 4HT for the indicated time periods and V12Ras MDCK cells were stimulated with TGFβ (2 ng/mL) for 1 h. (A) Nuclear translocation of Smad4, Smad2, Smad3, and activation of p42MAPK were detected in nuclear extracts by Western blotting. Expression of PCNA, a nuclear protein was assayed to show equal loading of nuclear extracts. (B) Activated Smad3/4 binding to a 32P-labeled c-jun probe was examined in nuclear extracts by EMSA. (C) MDCK Raf-ER cells were transiently transfected with ARE-Luc reporter, pEF-XFast1, and pEF-lacZ. After treatment with 100 nM 4HT for the indicated periods of time, cells were stimulated with 2 ng/mL TGFβ for 6 h and activation of the ARE-Luc reporter was measured. Luciferase activity was normalized to the activity of the cotransfected β-galactosidase control plasmid. Data shown are the means ±average deviations of duplicates from one out of three representative experiments.

[Fig. 6C]. In 4HT untreated MDCK cells, luciferase activity was strongly increased in response to TGFβ, compared to relatively low basal luciferase activity in the absence of ligand. In MDCK Raf-ER cells pretreated with 4HT, the TGFβ-induced luciferase activity was not changed.

Together, these data indicate that expression of V12Ras or Raf-ER activation in MDCK cells did not prevent the TGFβ-activated Smads from translocating into the nucleus, binding to DNA and activating gene expression.

Raf inhibits apoptosis induced by TNFa

To further investigate whether activation of Raf leads to protection from other pro-apoptotic stimuli than TGFβ, we examined the effect of Raf on TNFa-induced apoptosis (Fig. 7). TNFa acts through its death receptor (TNFRI) to cause apoptosis in many epithelial cell types (Sidoti-de Fraisse et al. 1998). MDCK Raf-ER cells were treated with different concentrations of TNFa for 4 h in the presence of the protein synthesis inhibitor cycloheximide, which is required to block the anti-apoptotic NF-κB transcriptional response to TNFa. TNFa induced a dose-dependent increase of the percentage of apoptotic cells (Fig. 7A) as well as strong activation of caspase-8 (Fig. 7B), as demonstrated by Hoechst staining or in Western blotting, respectively. In contrast, 4HT pretreatment protected cells from apoptosis and caused caspase-8 not to be detectably activated. Moreover, we could show that activation of Raf-ER in MDCK cells blocks detachment-induced apoptosis, "anoikis" (Le Gall et al. 2000; Rytömaa et al. 2000). These results indicate that Raf not only inhibits TGFβ-induced death signaling but renders cells insensitive to a variety of pro-apoptotic stimuli, suggesting a more general anti-apoptotic function for Raf.

Discussion

Raf and epithelial–mesenchymal transition

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Figure 7. Raf inhibits apoptosis induced by TNFa plus cycloheximide. MDCK Raf-ER cells were either untreated or treated with 100 nM 4HT for 24 h and stimulated with the indicated concentrations of TNFa and cycloheximide [2 μg/mL] for 4 h. (A) The percentage of apoptotic cells was assayed by Hoechst staining. Data shown represent at least three independent experiments performed in duplicates. (B) Activation of caspase-8 was detected by Western blotting, showing the processed and activated p20 subunit of caspase-8.
We previously described an MDCK cell line that constitutively expressed a form of Raf that was activated by localization to the plasma membrane, Raf-CAAX (Khwaja et al. 1997, 1998). Unlike the 4HT-treated Raf-ER MDCK cells, this line did not display a mesenchymal phenotype and did not form invasive growth in collagen gels. It also was not protected from apoptosis in response to 4HT while Raf-CAAX expressing cells are scattered, although they have undergone some morphological changes (Khwaja et al. 1998).

Over the course of a few days following Raf activation the cells begin to produce their own biologically active TGFβ promoted by the accompanying up-regulation of uPA expression. This autocrine TGFβ is responsible for promoting the invasive growth of the activated Raf-expressing MDCK cells in collagen gels, as can be demonstrated by the ability of neutralizing antibodies against TGFβ to block this phenotype in cells where Raf has either been activated for the course of the experiment or for a more prolonged period. There are clear similarities and some differences, between this system and the previously described activated Ras-transformed EpH4 murine breast epithelial cells, EpRas (Oft et al. 1996). Unlike the cells used here, in the EpRas cells, initial addition of exogenous TGFβ was required to set up the autocrine TGFβ loop. However, once established, the autocrine TGFβ would maintain the invasive phenotype and the EMT, presumably in cooperation with the activated Ras protein.

We previously described an MDCK cell line that constitutively expressed a form of Raf that was activated by localization to the plasma membrane, Raf-CAAX (Khwaja et al. 1997, 1998). Unlike the 4HT-treated Raf-ER MDCK cells, this line did not display a mesenchymal phenotype and did not form invasive growths in collagen gels. It also was not protected from apoptosis in response to detachment from extracellular matrix or other apoptotic insults, again unlike the 4HT-treated Raf-ER MDCK cells. The likely critical difference between these two cell lines is the strength of Raf activity achieved by the two constructs: ERK activation in Raf-ER cells is much higher than the constitutive level in Raf-CAAX cells [data not shown]. The strong activity of the Raf-ER construct may also account for why these cells scatter in response to 4HT while Raf-CAAX expressing cells are not scattered, although they have undergone some morphological changes (Khwaja et al. 1998).

**Interplay between Raf and TGFβ signaling pathways**

It has been suggested that certain tumors lose sensitivity to the inhibitory effects of TGFβ, in particular its ability to cause growth arrest and apoptosis, by losing expression of cell surface receptors for TGFβ. Alternatively, there may be loss of Smad4/DP4, the critical common Smad that interacts with the TGFβ receptor-activated Smad2 and Smad3 and translocates with them to the nucleus [Massague 1998]. Both of these mechanisms would give rise to a generalized block in TGFβ signaling. However, it is clear that in many circumstances TGFβ can promote the malignant phenotype of transformed cells in human tumors or animal cancer models (Akhurst and Balmain 1999), so it is likely that mechanisms exist that allow the tumor cells to avoid the inhibitory effects of TGFβ while continuing to take advantage of the positive effects.

In the case of the MDCK Raf-ER cells, the change in the response to TGFβ relative to the parental cells depends on the endpoint studied. Activation of Raf for up to 48 h before the addition of TGFβ did not prevent the slowing down of progression through the cell cycle. TGFβ treatment over 24 h does not arrest the cells completely, but there is a clear reduction in the rate of proliferation. Interestingly, MDCK Raf-ER cells that have been treated with 4HT for several weeks become completely resistant to the growth inhibitory effects of TGFβ, suggesting that this is a result of the cells having undergone complete EMT and consequent major changes in gene expression profile and not the result of early or intermediate Raf-regulated signaling, either direct or transcriptional. However, the continued dependence of the long-term Raf-transformed cells on the TGFβ autocrine loop for invasive growth in collagen gels shows that they are still responsive to some of the stimulatory effects of TGFβ.

By contrast, the ability of TGFβ to induce apoptosis is fairly rapidly blocked by the short-term activation of Raf, well before the onset of EMT, although slowly enough that a transcriptional event could be required. This protection from apoptosis is, however, not specific for TGFβ and is seen equally clearly for TNFα-induced apoptosis and matrix detachment-induced apoptosis [Le Gall et al. 2000; data not shown]. It therefore appears that the short- to medium-term activation of the Raf-ERK pathway inhibits programmed cell death in this system in a nonspecific manner. This, together with the incomplete nature of the inhibition of cell proliferation by TGFβ, allows the cells to largely avoid the inhibitory effects of TGFβ while responding to it with increased invasiveness characteristic of malignancy and induction of EMT. As EMT is established following longer-term activation of Raf, the inhibitory effects of TGFβ on cell proliferation are also lost, giving rise to cells that have no antiproliferative/prodeath responses to TGFβ but do display the pro-invasive responses. At present we do not know the mechanism by which strong Raf activation blocks apoptotic responses in MDCK cells; a similar response seen in another epithelial line, MCF-10A, has been found to be caused by an epidermal growth-factor family autocrine loop resulting in a survival signal (A. Schulze and J. Downward, unpubl.).
Raf and Smad signaling

Recently, it has been reported that ERKs can phosphorylate Smad2 and Smad3 in the linker region connecting the MH1 and MH2 domains [Kretzschmar et al. 1999]. The ERK phosphorylated Smads cannot translocate to the nucleus in response to TGFβ, leading to a general inhibition of Smad-mediated TGFβ responses. The fact that clear biological effects of TGFβ can still be seen in cells following strong and sustained activation of the Raf–ERK pathway suggests the possibility that these effects may be mediated through mechanisms that do not involve Smads. Alternatively, Smad signaling may not be effectively suppressed by active ERK here.

Smad-independent TGFβ signaling has been reported previously: TGFβ can activate TAK1, a member of the MAP kinase kinase kinase family, and this activation has been implicated in the rapid activation of p38 MAP kinase by TGFβ [Yamaguchi et al. 1995; Hanafusa et al. 1999]. In addition, TGFβ is still able to induce increased expression of fibronectin, although not PAI-1, in tumor cell lines lacking functional Smad4 [Hoeveve et al. 1999]: This involves a JNK-dependent mechanism. Although it is not known at present whether the invasiveness promoting effects of TGFβ require JNK or p38 activity, induction of anokiosis in MDCK cells does not need these pathways [Khwaja and Downward 1997]. Uncertainty as to whether Smad-independent signaling could account for the Raf-resistant effects of TGFβ led us to revisit the suppression of Smad2 and Smad3 signaling by ERK. Surprisingly, we were unable to see any inhibitory effect of potent Raf activation on the ability of TGFβ to induce the nuclear translocation of Smad2, Smad3, or Smad4; the DNA binding ability of Smad3 and Smad4; or the transactivation of expression from a reporter construct by Smad2 and Smad4. A lack of effect of expression of activated MEK on nuclear translocation and DNA binding ability of Smad3 has also recently been reported in HaCaT cells [Hu et al. 1999].

At present it is not clear why activation of the Raf–ERK pathway does not block TGFβ signaling to Smad2 and Smad3 in MDCK cells. It is possible that cell type differences account for this, although we also do not see the effect in EpRas cells, a line used in the study by Kretzschmar et al. [data not shown].

Raf, TGFβ, and cancer

The data presented here indicate that TGFβ signaling pathways can synergize with the Ras–Raf–ERK pathway to promote the conversion of normal epithelial cells to highly malignant cells with a mesenchymal phenotype. In MDCK cells, activation of Raf selectively blocks the negative effects but not the positive effects of TGFβ on cell growth. Inhibition of TGFβ effects on apoptosis and cell cycle exit are likely to be well downstream of the TGFβ-specific pathways, with Smad-mediated signaling left fully operational. This is in contrast to many reports in the literature of general loss of responsiveness to TGFβ in some tumor-derived cells. However, there is reason to believe that complete loss of TGFβ signaling in tumors may be restricted to certain types of cancer and be relatively infrequent overall [Riggins et al. 1997].

TGFβ-receptor type II is mutated in human colon cancer cell lines with high rates of microsatellite instability from families with genetic defects in DNA repair (RER) causing hereditary nonpolyposis colon cancer. In addition, similar mutations are seen in familial gastric cancer from families with RER. Further analysis has found some indication of occasional TGFβ-receptor type II mutation in microsatellite-stable colon carcinoma and of TGFβ receptor type I in human cervical carcinoma. Overall, the frequency of TGFβ-receptor mutation in human tumors is low. Moreover, it is apparent that some of the mutations found may not block all aspects of TGFβ-receptor signaling. For example, a mutation in TGFβ-receptor type II in a kindred of hereditary nonpolyposis colorectal cancer without microsatellite instability selectively blocks the signaling of TGFβ to p15INK4B expression but not to PAI-1 expression [for review, see Kim et al. 2000]. Conversely, homozygous mutations in this receptor in two RER positive colorectal cancer cell lines fail to suppress the growth inhibitory response of the cells to TGFβ [Ilyas et al. 1999].

The other major mechanism for inactivation of TGFβ signaling in tumors is through loss of function of Smad4, also known as Dpc4 or Madh4. Smad4 inactivation is a common genetic alteration in pancreatic ductal adenocarcinomas. Inactivation of Smad4 occurs in ~50% of pancreatic cancers, it is relatively rare in other tumor types, although it does occur in a small proportion of cancers from other organs, particularly the colon, and also breast, ovary, and biliary tract [Thiagalingam et al. 1996; Hahn et al. 1998] cancers. Germ-line mutations in the Smad4 gene have also been implicated in juvenile polyposis, [for review, see Weinstein et al. 2000]. The relatively small number of tumors in which TGFβ signaling has been inactivated by mutation of the receptor or Smads might reflect the fact that TGFβ pathways can act to promote malignancy as well as suppress it. During the process of tumor progression it would be expected that there would be strong selection to lose the growth inhibitory effects of TGFβ but, also, to maintain the pro-malignant aspects of the TGFβ response. This could be achieved by inactivating the growth arrest and apoptosis responses to TGFβ well downstream of the TGFβ-specific signaling events. Similarly, from the data presented here we propose that the Ras–MAP kinase pathway acts to suppress the inhibitory effects of TGFβ downstream of Smads, rather than by blanket inhibition of TGFβ responses.

Material and methods

Expression vectors

EGFP tagged ΔRaf-1−hHER (DD) [Raf−ER] cDNA in pBabe-puro was described previously [Woods et al. 1997]. Xenopus Fast-1 in pEF-Flag, Xenopus Smad2 in pEF-myc, and pPTX5 pEF-lacZ were described in Howell and Hill (1997). Human Smad3
Cell culture and retroviral infection

MDCK cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Retroviral vector pBabe-puro-EGFP-ΔRaf-1-hbER (DD) was packaged in GP+E cells and used to infect MDCK cells expressing the ecotropic retrovirus receptor. After selection with 2.5 μg/mL of puromycin (Sigma), cells were pooled and Raf-ER-expressing cells were sorted twice by FACS for EGFP expression. MDCK cell lines stably expressing V12Ras have been described previously (Khwaja et al. 1997).

Collagen gel culture

Cells (3 x 10^4) were suspended in rat collagen type I (Collaborative Medical Products) and overlaid with DMEM containing 5% FCS, 5 ng/mL TGFα (R&D systems), and 0.04 LE/mL Insulin (Novo Nordisk). Neutralizing TGFβ antibodies (100 ng/mL chicken anti-TGFβ1, [R&D systems] and 20 μg/mL mouse anti-TGFβ1,3,3 (Genzyme) were used. Medium was changed every second day.

Confocal fluorescence microscopy

Antibodies were from: E-cadherin (Transduction Laboratories), vimentin V3B (Boehringer Mannheim), ZO-1 [Zymed], Cy3-labeled goat antirabbit IgG (Amersham); Cy5-labeled donkey antimouse IgG H+L (Jackson). Rabbit antiserum to cytokeratin has been described previously (Reichmann et al. 1992). Cells grown on 24-mm Falcon cell culture inserts (0.4 pm pore size; Becton Dickinson) were fixed in methanol/acetone (1:1) and immunostained as previously described (Oft et al. 1996). Samples were examined with a Zeiss LSM510 confocal microscope.

Antibodies, Western blotting, and ELISA

Antibodies were from: ERK2/p42MAPK pan ERK (Transduction Laboratories), estrogen-receptor MC20 (Santa Cruz), TGFβ1 chickens (R&D); Urokinase-type Plasminogen Activator Ab-1 [Neo Markers]; activated caspase-8 C20, cyclin A H432, Smad4 B8 (Santa Cruz), Smad2 (cross-reacting with Smad3), Transduction Laboratories. The anti-PCNA antibody (PC 10) was obtained from the ICRF hybridoma unit. Total cell extracts in SDS-sample buffer and nuclear extracts were prepared as described previously (Wong et al. 1999). The ^32P-labeled c-jun oligonucleotide probe was generated by annealing and filling in the overlapping oligonucleotides GAGGTCGCGGGATCAGGACAGACACACA GACAACACG (sense) and TGCCGAACCTGGCCTGCAGCCGT GCCTTGGTCTGTGCTGTCTG (antisense) by PCR in the presence of [α-^32P]dCTP and [α-^32P]dATP. The EMSA reaction was performed as described previously (Germain et al. 2000) and contained 10–15 μg of nuclear extract. For supershift experiments, the following antibodies and competing peptide were added to the nuclear extracts before probe addition and incubated at room temperature for 5 min: 1 μL mouse anti-Smad4 B8 (Santa Cruz), 1 μL anti-Smad3 without or with 0.5 μL competing peptide (Nakao et al. 1997), 0.5 μL anti-Smad2 (Transduction Laboratories), or 1 μL of anti-Smad2 (SED; Nakao et al. 1997).

Cell cycle analysis

To examine cell cycle distribution, cells were fixed in 70% ethanol, treated with ribonuclease (100 μg/mL) for 5 min at room temperature, stained with 50 μg/mL propidium iodide (Becton Dickinson) for 5 min, and analyzed by flow cytometry using 488 nm excitation.

Nuclear extracts and EMSA

Nuclear extracts were prepared as described previously (Wong et al. 1999). The ^32P-labeled c-jun oligonucleotide probe was generated by annealing and filling in the overlapping oligonucleotides GGAGGTGCGCGGAGTCAGGCAGACAGACAGACACACA GACAACACG (sense) and TGCCGAACCTGGCCTGCAGCCGT GCCTTGGTCTGTGCTGTCTG (antisense) by PCR in the presence of [α-^32P]dCTP and [α-^32P]dATP. The EMSA reaction was performed as described previously (Germain et al. 2000) and contained 10–15 μg of nuclear extract. For supershift experiments, the following antibodies and competing peptide were added to the nuclear extracts before probe addition and incubated at room temperature for 5 min: 1 μL mouse anti-Smad4 B8 (Santa Cruz), 1 μL anti-Smad3 without or with 0.5 μL competing peptide (Nakao et al. 1997), 0.5 μL anti-Smad2 (Transduction Laboratories), or 1 μL of anti-Smad2 (SED; Nakao et al. 1997).

Transfection and luciferase assay

MDCK Raf-ER cells were transiently transfected using Lipofectamine (GIBCO BRL) with ARE-Luc reporter, XFAST-1 expression vector, and lacZ as an internal control for transfection efficiency. Luciferase assays were performed according to the procedures recommended by the supplier (Promega). The β-galactosidase assays were performed using chlorphenol red-β-D-galactopyranoside (Calbiochem) as a substrate and quantified photometrically at 595 nm. All transfections were normalized to β-galactosidase activity.

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