Activator Protein-1 and Epidermal Growth Factor Receptor Interplay - *In Vivo* & *In Vitro* Studies

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (**I-III**).

I. Kajanne R, Miettinen PJ, Mehlem A, Leivonen S-K, Birrer M, Foschi M, Kähäri V-M, Leppä S. EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways. (2007) *J Cell Physiol.* 212(2): 489-97.

II. Kajanne R, Leppä S, Luukkainen P, Ustinov J, Thiel A, Ristimäki A, Miettinen PJ. Hydrocortisone and indomethacin negatively modulate EGF-R signaling in human fetal intestine. (2007) *Pediatr Res.* 62(5): 570-5.

III. Kajanne R, Miettinen PJ, Tenhunen M., Leppä S. Transcription factor AP-1 promotes growth and radioresistance in PC-3 prostate cancer cells. (2009) *Int J Oncol.* (In press).

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2. ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
AP-1	activator protein 1
AR	amphiregulin
ATF	activating transcription factor
BTC	betacellulin
bZIP	basic region-leucine zipper
CDK	cyclin-dependent kinase
CREB	CRE-binding protein
ECM	extracellular matrix
EGF	epidermal growth factor
EGF-R	EGF receptor
EMT	epithelial–mesenchymal transition
EP	epigen
EPR	epiregulin
ErbB	erythroblastic leukemia oncogene homolog; RTK
Erk	extracellular signal-regulated protein kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
Fra	Fos-related antigen
GPCR	G-protein coupled receptor
Grb2	growth factor receptor-bound protein 2
IGF-I	insulin like growth factor
HB-EGF	heparin binding EGF
HC	hydrocortisone
IL	interleukin
Ind	indomethasine
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase

MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEF	mouse embryonal fibroblast
MMP	matrix metalloproteinase
NF-kB	nuclear factor kB
PDGF	platelet-derived growth factor
PDGF-R	platelet-derived growth factor receptor
PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
Ras	rat sarcoma viral oncogene homolog; GTPase
RTK	receptor tyrosine kinase
SH2	Src homology domain 2
Shp1	SH2-containing tyrosine phosphatase
Src	Rous sarcoma viral oncogene homolog; tyrosine kinase
STAT	signal transducer and activator of transcription
TGF-α	transforming growth factor α
TPA	12-*O*-tetradecanoyl-phorbol 13-acetate
TRE	TPA response element
VEGF	vascular endothelial growth factor

3. ABSTRACT

Critical cellular decisions such as should the cell proliferate, migrate or differentiate, are regulated by stimulatory signals from the extracellular environment, like growth factors. These signals are transformed to cellular responses through their binding to specific receptors present at the surface of the recipient cell.

The epidermal growth factor receptor (EGF-R/ErbB) pathway plays key roles in governing these signals to intracellular events and cell-to-cell communication. The EGF-R forms a signaling network that participates in the specification of cell fate and coordinates cell proliferation. Ligand binding triggers receptor dimerization leading to the recruitment of kinases and adaptor proteins. This step simultaneously initiates multiple signal transduction pathways, which result in activation of transcription factors and other target proteins, leading to cellular alterations. It is known that mutations of EGF-R or in the components of these pathways, such as Ras and Raf, are commonly involved in human cancer.

The four best characterized signaling pathways induced by EGF-R are the mitogen-activated protein kinase cascades (MAPKs), the lipid kinase phosphatidylinositol 3 kinase (PI3K), a group of transcription factors called Signal Transducers and Activator of Transcription (STAT), and the phospholipase Cγ (PLCγ) pathway. The activation of each cascade culminates in kinase translocation to the nucleus to stimulate various transcription factors including activator protein 1 (AP-1).

AP-1 family proteins are basic leucine zipper (bZIP) transcription factors that are implicated in the regulation of a variety of cellular processes including proliferation and survival, growth, differentiation, apoptosis, cell migration, and transformation. Therefore, the regulation of AP-1 activity is critical for the decision of cell fate and their deregulated expression is widely associated with many types of cancers, such as breast and prostate cancers.

The aims of this study were to characterize the roles of EGF-R signaling during normal development and malignant growth *in vitro* and *in vivo* using different cell lines and tissue samples. We show here that EGF-R regulates cell proliferation but is also required for regulation of AP-1 target gene expression in fibroblasts in a MAP-kinase mediated manner. Furthermore, EGF-R signaling is essential for enterocyte proliferation and migration during intestinal maturation. EGF-R signaling network, especially PI3-K-Akt pathway mediated AP-1 activity is involved in cellular survival in response to ionizing radiation.

Taken together, these results elucidate the connection of EGF-R and AP-1 in various cellular contexts and show their importance in the regulation of cellular behaviour presenting new treatment cues for intestinal perforations and cancer therapy.

4. REVIEW OF THE LITERATURE

4.1. Introduction

Cancer is a genetic disease arising because of mutations in cancer-susceptibility genes, which can be modified by environmental factors. These cancer-associated mutations are either inherited or somatic and belong to one of the three classes: gatekeepers, caretakers, or landscapers. Of these, gatekeepers directly regulate growth and differentiation pathways in the cell. Gatekeeper genes consist of growth-promoting oncogenes and growth-constraining tumor-suppressor genes. In a normal cell, proto-oncogenes are counterbalanced by growth-constraining tumor suppressor genes, but mutations that potentiate the activities of proto-oncogenes create the oncogenes that force the growth of tumor cells. Caretakers in turn maintain the genomic integrity (prevent mutations) of the cell, and mutations of caretakers can lead to genetic instability. Defects in landscapers generate an abnormal stromal environment. At the tissue level, constancy in cell number results in tissue homeostasis, which reflects a highly regulated balance between the rates of cell proliferation and cell death. If this balance is shifted towards uncontrolled proliferation, cancer occurs [reviewed in (1)].

Actual tumor progression is a multistep process, which enables cells to evolve from benign group of cells to malignant tumors. One of the first steps is autocrine secretion of cancer cells (2, 3), which generally exhibits a reduced requirement for exogenously supplied growth factors to maintain a high rate of proliferation. At present, cancer progression has been suggested to depend on six essential characteristics identified as the hallmarks of cancer which include: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of apoptosis, 4) limitless replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (4).

Classically, basic cancer research has focused on either gain or loss-of-function mutations in oncogenes or tumor-suppressor genes, respectively. Many of the known proto-oncogenes code for proteins that are part of intracellular signaling network and therefore, carcinogenesis and the development of cancer has been said to be a disease of the signaling system. The intracellular signaling network is a highly complicated group of proteins transmitting signals and regulators that fine tune or inhibit this process, resulting in changes in cell proliferation, differentiation, cell migration, survival, and cellular metabolism. Signals received at the cell surface must be properly transmitted to critical targets within the cell to achieve the appropriate biological response. The process is often initiated by receptor tyrosine kinases (RTKs), which function as entry points for many extracellular cues, and play a critical role in recruiting the intracellular signaling cascades that orchestrate a particular response. The focus of this review is delimited to signaling events initiated by epidermal growth factor receptor (EGF-R) and its downstream target AP-1.

4.2 EGF receptor

The EGF-R signaling has been widely studied since its discovery in late 1970´s (5). The EGF-R was the first RTK cloned (6). Interstingly, the EGF-R cytoplasmic domain was found to be the human ortholog of the *v-ErbB* oncogene of the avian erythroblastosis virus, which lacks almost the entire extracellular region leading to constitutive signaling activity (7). This finding identified EGF-R as one of the first proto-oncogenes. During the 1990´s and 2000´s the complex kinase-signaling network underlining EGF-R has been revealed [reviewed in (8, 9)]. Although this research has been very fruitful, it is now clear that a more complete understanding of key regulatory signaling pathways is required, since this receptor family and growth factor ligands play an essential role in the regulation of epithelial cell proliferation.

The EGF-R family (also called HER/ErbB family) consists of four different tyrosine kinases (EGF-R, ErbB-2, ErbB-3, and ErbB-4) that are activated following binding of epidermal growth factor (EGF)-like growth factors (Table I and Figure 1B). A member of EGF-R family has an extracellular region that contains two ligand-binding domains (domains I and III), an extracellular juxtamembrane region, a single hydrophobic transmembrane domain, cytoplasmic tyrosine kinase-containing domain (except ErbB-3), and cytoplasmic tyrosine residues that serve as sites for receptor phosphorylation (Figure 1A) (10). The intracellular tyrosine kinase domains of Erb receptors are highly conserved, whereas their extracellular domains are not, suggesting that they can bind different ligands (See chapter 4.3 EGF-R ligands) (10-12). The EGF-R is expressed in almost all types of non-transformed cells, with the only exception of mature cells of the lymphohematopoietic system (13, 14).

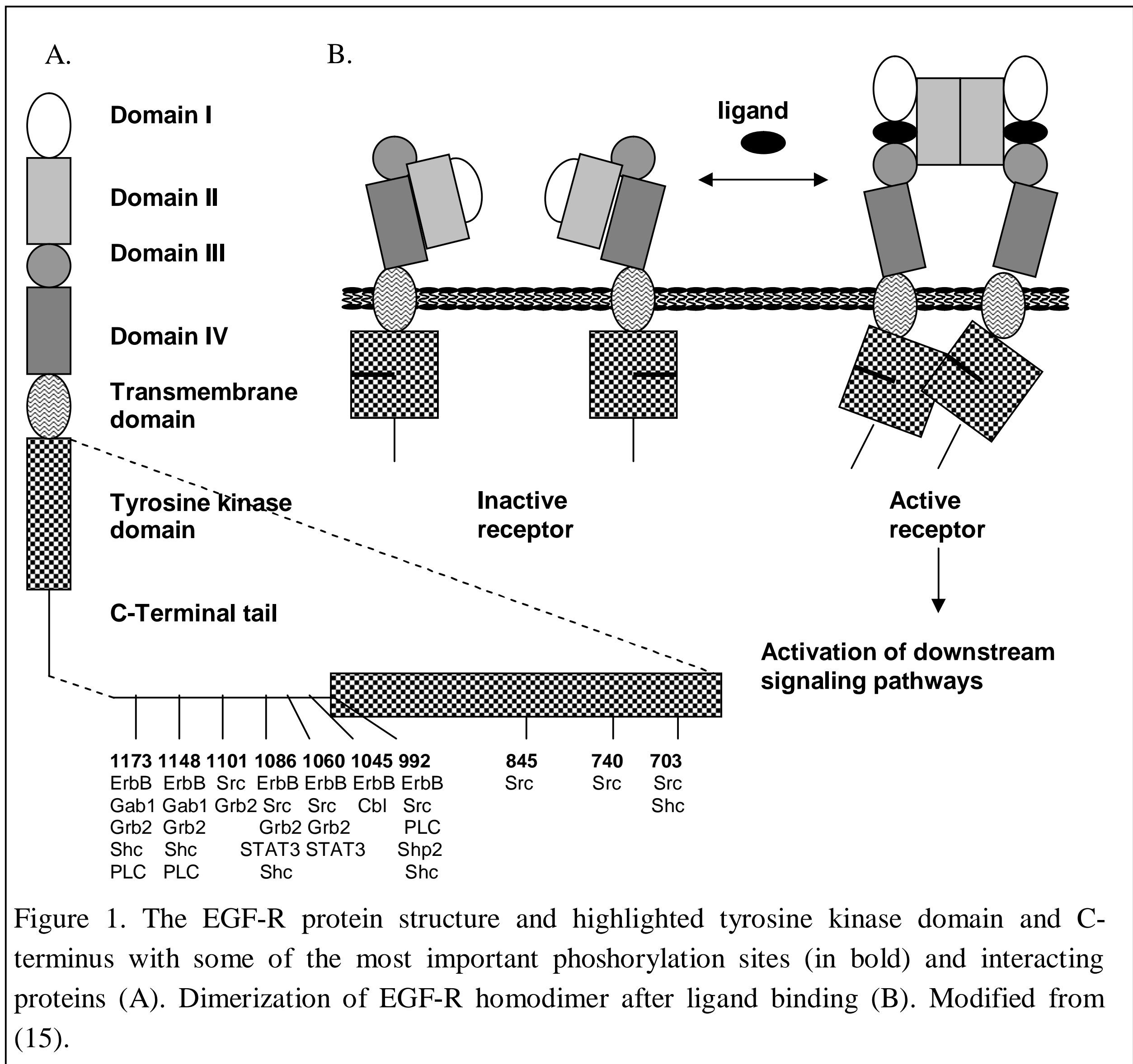

Figure 1. The EGF-R protein structure and highlighted tyrosine kinase domain and C-terminus with some of the most important phoshorylation sites (in bold) and interacting proteins (A). Dimerization of EGF-R homodimer after ligand binding (B). Modified from (15).

4.3 EGF-R ligands

The existence of seven EGF-R ligands [amphiregulin (AR), betacellulin (BTC), epidermal growth factor (EGF), epigen (EP), epiregulin (EPR), heparin binding EGF (HB-EGF), transforming growth factor (TGF-α)], and four ErbB receptors allows numerous combinatorial possibilities of signaling. This signaling diversity is based on several aspects. Firstly, the expression pattern of each ligand and their ability to induce not only EGF-R homodimers but also heterodimers are essential features. Secondly, EGF-R ligands BTC, HB-EGF, and EPR are bivalent binding both EGF-R and ErbB4, which determines which receptor dimers are formed, influencing which signaling pathways are activated (16). Thirdly, each EGF-R ligand has a unique binding affinity, influencing signal strength and duration. Fourthly, the pH stability of the ligand–receptor interaction influences receptor trafficking.

For example, EGF-EGF-R interaction is pH resistant. EGF-R is targeted to lysosomes, whereas TGFα-EGF-R interaction dissociates at endosomal pH, resulting in receptor recycling to the plasma membrane (17, 18). Therefore, TGFα often produces stronger or more prolonged effects than EGF (19). All these interactions play a role in signal potentiation. Thus, in response to EGF-R ligands the cells can proliferate, differentiate, survive, or move, indicating that EGF-R and its ligands have broad roles in different tissues during development, maintaining homeostasis, and regulating injury responses.

EGF is a prototypic member of the family of these growth factors. It is a single chain polypeptide that was first isolated from mouse submaxillary glands (20), which serve as a source for circulating EGF. All EGF-R ligands are synthesized as transmembrane molecules that can release their extracellular domains containing the EGF-like motif through a specialized type of limited proteolysis, known as ectodomain shedding, which is regulated via protein kinase C (PKC) (21). This proteolytic processing by ADAMs (a disintegrin and metalloprotease) regulates the bioavailability of several EGF-R-ligands. ADAM10 is the main processor of EGF and BTC, and ADAM17 the main processor of EPR, EP, TGF-α, AR, and HB-EGF (22, 23).

The neuregulins (NRGs) are additional ligands harboring an EGF-like domain. This domain binds to both the ErbB3- and ErbB4-receptor tyrosine kinases (24) but also HB-EGF, BTC, and EP can bind to ErbB4 (25, 26).

Soluble ErbB ligands generally act over short distances as autocrine or paracrine growth factors, activating EGF-R in the very EGF-R ligand-producing cell or in proximal cells, respectively. If the shedding is prevented the transmembrane forms of ligands also have the ability to activate EGF-R in adjacent cells in a juxtacrine fashion (27).

Table I. The ErbB receptors and their associated ligands. Modified from (28).

EGF-R	ErbB2	ErbB3	ErbB4
Amphiregulin (AR)	None known	Neuregulin 1 (NRG-1)	Betacellulin (BTC)
Betacellulin (BTC)		Neuregulin 2 (NRG-2)	Epigen (EP)
Epidermal growth factor (EGF)			Heparin binding EGF (HB-EGF)
Epigen (EP)			Neuregulin 1 (NRG-1)
Epiregulin (EPR)			Neuregulin 2 (NRG-2)
Heparin binding EGF (HB-EGF)			Neuregulin 3 (NRG-3)
Transforming growth factor (TGF-α)			Neuregulin 4 (NRG-4)
			Tomoregulin

4.4 The EGF-R network during development

In general, EGF-R ligands have important physiological roles in development as stimulators for epithelial tissue growth. However, they can also modify body composition and when overexpressed they have negative effects on growth of an individual. For example, overexpression of EGF, HB-EGF, or BTC leads to decreased body weight and retarded bone development in mice (29-32). The mechanism behind this might be that the EGF-R ligands stimulate proliferation and prevent differentiation of adipocytes and osteoblastic cells (33, 34). Furthermore, the EGF-R ligands are needed in the maturation of the gastrointestinal tract, and subsequent optimal nutrient uptake (35). They can also negatively regulate other growth factors such as insulin like growth factor (IGF-I) (32). In addition, they are chemoattractants for a number of different cell types and can contribute to cell adhesion, cell motility, and angiogenesis [reviewed in (36)].

During development, ErbB receptors and EGF-R ligands show distinct expression patterns that are organ- and developmental stage-specific. As EGF-R ligands act locally as autocrine or paracrine growth factors, the availability of a specific ligand is an essential way of controlling its functional consequence.

The role of an individual gene in development is currently studied by creating knockout (KO) animal models, usually mice, in which the desired target gene has been inactivated by homologous recombination. In general, mice with inactivated ErbB receptors develop multiorgan failure leading to embryonic or prenatal death, whereas inactivation of the ligands does not lead to lethality suggesting redundancy in their action (Table II).

4.4.1 ErbB family knockout-mice

Expression of the EGF-R already at a blastocyst stage indicates its importance during development (37). Indeed, EGF-R$^{-/-}$ mice suffer from impaired epithelial development of several organs, including the skin, lung, and gastrointestinal tract (38, 39). Together with placental defects they either lead to peri-implantation, embryonic, or postnatal lethality depending on genetic background of the EGF-R$^{-/-}$ mice strains (39-41). Furthermore, EGF-R signaling is necessary for normal craniofacial (42) and pancreatic development (38). The natural murine mutation of the EGF-R is called Waved-2. Waved-2 results from a point-mutation in the tyrosine-kinase domain of the EGF-receptor unaffecting protein expression or ligand binding but leading to a reduced ligand-dependent autophosphorylation of EGF-R (43). It has a similar but not as severe phenotype as the EGF-R$^{-/-}$ mice.

ErbB-2$^{-/-}$ mice have arrested oligodendrocyte development and myelin formation (44). In addition, mice have alterations in cardiac and neural structures that cause lethality at embryonic stage E10.5 (45, 46). Also, ErbB-3$^{-/-}$ mice die from severe degeneration of the nervous system at embryonic stage E13.5 (47, 48). Like ErbB-2$^{-/-}$ mice, the ErbB-4$^{-/-}$ mice die during mid-embryogenesis due to cardial and neural defects. The conditional nervous system-specific ErbB-4$^{-/-}$ mice also show altered motor and behavioral activities, suggesting a role for this receptor not only in neuronal development but also in neuronal function (49, 50).

To conclude, these phenotypes of ErbB receptor KO-mice reflect the importance of receptor heterodimerization and co-operation (See Table II). The heart and CNS are defective in all KO-mice implicating that ErbB receptors must co-operate during heart and CNS development. Interestingly, ErbB receptors also play essential roles in the adult organism. This is reflected in the mammary gland, which is an organ that undergoes most of its proliferation and differentiation during pregnancy and lactation. All four ErbB receptors are expressed in the mammary gland in distinct patterns (51) and ErbB receptor KO-mice show defective mammary gland development at different stages.

4.4.2 EGF-family knockout-mice

EGF$^{-/-}$ mice have no clear phenotype. Even their growth rate is normal as compared to wild type littermates. EGF may also contribute to the mammary gland development and lactation

(52). EGF mRNA is expressed in the salivary gland, thyroid gland, mammary gland, and kidney, which serve as major sources of circulating EGF (53).

TGF-$\alpha^{-/-}$ mice have similar hair and eye defects as have been previously associated with the recessive mutation waved-1 (wa-1) (54). They also have abnormal skin architecture (55). In contrast, overexpression of TGFα results in hyperplasia and hyperkeratosis in the epidermis reminiscent to psoriasis (56).

Heparin-binding epidermal growth factor (HB-EGF)$^{-/-}$ mice are viable and fertile but more than half of the HB-EGF$^{-/-}$ mice die during the first postnatal week and the survivors develop severe heart failure (57). HB-EGF is needed in blastocyst implantation (58) and wound healing (59). Furthermore, HB-EGF is reported to be a more potent stimulator of smooth muscle cell proliferation than EGF or TGFα (60). Moreover, HB-EGF also serves as receptor for diphtheria toxin (61).

Amphiregulin$^{-/-}$ (AR) mice show no overt phenotype but have a distinct and essential role for AR in mammary ductal morphogenesis, supporting roles for EGF and TGFα in lactogenesis (52, 62).

Betacellulin$^{-/-}$ (BTC) mice are viable and fertile and display no overt defects but the lifespan of *HB-EGF*$^{-/-}$*/BTC*$^{-/-}$ mice is further reduced, apparently due to accelerated heart failure (63). However, BTC overexpressing mice have several pathological alterations (31). These include abnormalities in the eye, lung, and bone structure suggesting a unique role for BTC in EGF-R signaling.

Epiregulin (EPR)$^{-/-}$ mice do not manifest any abnormal phenotype. Unlike other EGF-R ligands, epiregulin shows dual biological activity; it stimulates proliferation of fibroblasts, hepatocytes, smooth muscle cells, and keratinocytes but inhibits growth of several tumor-derived epithelial cell lines (64, 65). Epiregulin is mainly expressed on peripheral blood macrophages and the placenta (66).

The newest EGF-R ligand is Epigen (EP) (25). No knockout mice have been produced yet but it is known that soluble EP is more mitogenic than EGF. EP expression is detectable in multiple organs of the mouse embryo (67).

Overall, phenotypes of mice deficient in EGF ligands are mostly mild. This suggests redundancy between different ligands, which assures correct development and tissue homeostasis even if the expression of a single EGF-R ligand is lost. Even the triple KO-mice lacking EGF, AR, and TGFα are healthy and fertile, however, they are growth retarded and show impaired gastrointestinal tract development (35). EGF family members are also able to induce their own mRNA production and that of other family members in ligand-specific patterns, which suggests that they also have distinct, non-redundant functions (68).

Furthermore, overexpression studies revealed the importance of optimal levels of both milk-derived and endogenous EGF-R ligands in regulating growth (discussed in the next chapters).

Table II. Summary illustrating the phenotype of ErbB receptor or ligand inactivation.

Target gene	Phenotype outcome	Reference
EGF-R	Impaired epithelial development in the skin, mammary gland, lung, pancreas and intestine	(39)
ErbB2	Impaired cardiac ventricular myocyte differentiation, impaired development of oligodendrocytes, sensory ganglia and motor nerves in the CNS	(44-46)
ErbB3	Defective heart valves, impaired differentiation in the cerebellum (CNS)	(47, 48)
ErbB4	Impaired cardiac ventricular myocyte differentiation, alterations in the hindbrain in the CNS	(49, 50)
EGF		(69)
TGFα	Abnormal skin structure (wavy hair), eye anomalies	(55)
HB-EGF	Enlarged cardiac valves and ventricular chambers, impaired wound healing	(57, 59)
Amphiregulin	Impaired mammary gland development	(69)
Betacellulin	As above	(63)
Epiregulin	As above, susceptibility to mucosal damage	(65)
Epigen	N/A	N/A

4.4.3 EGF-R in the intestine

The epithelial lining of the gastrointestinal (GI) tract is constantly renewed. The homeostasis of the intestinal epithelium results from a highly regulated equilibrium between cell proliferation, migration, differentiation, and apoptosis (70, 71), which originates from a crosstalk between the epithelium and adjacent cell layers (72).

The epithelial lining consists of enterocytes, which are polarized, differentiated epithelial cells. They possess a specialized apical surface facing the intestinal lumen as well as a laterobasal surface exposed to blood and subepithelial cells. EGF is secreted directly into the intestinal lumen from several different cellular sites of origin. Indeed, EGF is known to be present at physiological concentrations within the intestinal cavity but the localization of the EGF-R to the laterobasal membrane (73) or to the apical membrane (74) in enterocytes is still disputed. Also other EGF family members like TGFα, amphiregulin, heparin-binding EGF, and epiregulin are all expressed in the GI tract at some levels but for example in the foetal gut, TGFα is more widely distributed than EGF (75).

There are many reports showing the importance of the EGF-R signaling system in the GI tract. During development, inactivation of the EGF-R results in epithelial immaturity of the GI tract. Similarly, as already mentioned, mice with triple KO mutations lacking AR, EGF, and TGFα show alterations in the GI tract (76). Moreover in a recent study, TGFα inhibits methotrexate-induced enterocyte apoptosis (77).

Defective EGF-R signaling has also been implicated pathogenesis of necrotizing enterocolitis (NEC) (78), which is the most common gastrointestinal disease of prematurely born infants. The molecular mechanisms underlying EGF-mediated protection against NEC include the reduction of intestinal apoptosis (79) and an improvement of intestinal barrier function (80). EGF insufficiency or lack of EGF-R may play an important role in normal cell renewal and healing after cellular damage, since both EGF and TGFα promote cell proliferation and stimulate cell migration. The natural source of EGF and TGFα for newborn infants is milk, which contains high levels of these growth factors (81), which are also needed to ensure the optimal postnatal growth of neonates.

Other examples of the role of the EGF-R signaling system in the GI tract include *Helicobacteria pylori* infection and Zollinger-Ellison Syndrome (ZES). *H. pylori* infection induces mucosal damage, which increases the expression of EGF peptide and EGF-R mRNA in the gastric mucosa (82), whereas patients with ZES typically have hypersecretion of acid and pepsin but also a significantly higher EGF concentration in saliva and gastric juice. This elevated content of salivary and gastric EGF in ZES patients may play a protective role in preventing the development of reflux esophagitis and gastric ulcer induced by gastric acid and pepsin (83). This type of protective but not mitogenic effect of EGF (and TGFα) reducing mucosal damage could result from their capability of inhibiting gastric acid secretion (84).

It has been reported that signaling of EGF-R in GI-tract has a role in intestinal cancer development (85), especially in colon cancer (86), in which EGF-R is over-expressed (87), but also in the pathogenesis of Ménétrier's disease (88). Furthermore, epiregulin$^{-/-}$mice are highly susceptible to cancer-predisposing intestinal damage caused by oral administration of dextran sulfate sodium (89).

4.4.4 EGF-R controlling the ECM and epithelial-mesenchymal interactions

Adult mesenchyme consists of resident cells (such as fibroblasts, adipocytes, and osteoblasts) and wandering cells (such as macrophages and mast cells) embedded in the extracellular matrix (ECM). The composition of the ECM is tissue-specific but the major ECM components include collagens, proteoglycans, and a large number of non-collagenous glycoproteins and proteins (90). One type of the resident cells, the fibroblasts, synthesize and maintain ECM by secreting the precursors of the components of the ECM and by producing enzymes, such as matrix metalloproteinases (MMPs) that, e.g., degrade the epithelial

basement membrane and the ECM (MMPs are discussed in more detail in chapter 4.11). In such way fibroblasts provide a structural framework (stroma) for many tissues.

During development the epithelial-mesenchymal interactions facilitate branching morphogenesis. It is well established that intact EGF-R signaling in fibroblasts is required for epithelial morphogenesis. EGF-R$^{-/-}$ mice have abnormal lung branching due to low expression of MT1-MMP (MMP-14) and reduction in active MMP-2 in the mesenchyme (91). In mammary ductal branching and morphogenesis, stromal EGF-R expressed by fibroblasts but also in this case by adipocytes in fat pads, is required for normal ductal development, which is activated by amphiregulin from mammary epithelial cells expressing ADAM17 (92, 93). In addition the growth of uterine, vaginal, and prostate organ requires EGF-R signaling from stroma (94, 95).

An interesting example of an interplay between ECM and EGF-R function in fibroblasts is featured in Ehlers–Danlos syndrome patients (EDS), who have impaired wound healing due to mutations in genes coding for collagen type III or V (96, 97). Fibroblasts in an *in vitro* wounding assay derived from EDS patients show defective migration and regeneration repair (98). As a consequence of insufficient ECM anchorage, they also undergo growth arrest and anoikis (detachment induced cell death), which can be rescued by αvβ3 integrin-dependent EGF-R activation (99). In normal conditions, MMP3 plays a critical role in skin wound healing by mediating epithelial cell migration (100). In addition, the EGF-R ligands, especially HB-EGF, are essential in stimulating keratinocyte migration and proliferation in epithelialization during wound healing (59).

The interaction between non-malignant stromal cells and tumor cells is known to be involved in cancer growth and progression. The trigger for tumor progression may come from signals in the stromal microenvironment (101, 102). The fibroblasts can modulate tumor cell migration and invasion through secretion of growth factors and cytokines in autocrine and paracrine fashion, and producing MMPs that modulate the ECM (103, 104). It has also been demonstrated that stromal fibroblasts might play a role in tumor associated angiogenesis by producing VEGF (105). As fibroblasts express all ErbB family members, the EGF-R system is an important tumor microenvironmental mediator regulating autocrine and paracrine circuits that contribute to enhanced tumor growth (28).

4.5 Intracellular signaling pathways downstream of EGF-R

Not only the impaired function of RTKs but also their signaling pathways have been linked to severe developmental defects, and various cancers (106). The major pathways downstream of EGF-R include MAPK pathways (Erk, JNK, p38), PI3-K-Akt pathway, STAT pathway, and PLC pathway, which are all illustrated in Figure 2 and discussed in the next chapters.

In general, EGF-R network signaling controls cell fate during the development and adulthood of an organism. These include cellular events such as survival, proliferation, stress sensitivity, apoptosis, cell motility, gene expression, transformation, and differentiation. They also play a major role in oncogenesis and angiogenesis by regulating transcription factors with target genes including cell-cycle proteins like CDKs and ECM modulators like MMPs and angiogenetic factors like VEGFs. For example, Erk or PI3-K pathways are deregulated in approximately 30-70% of all human cancers. It is, therefore, extremely important to tightly control the ErbB signaling network at all levels.

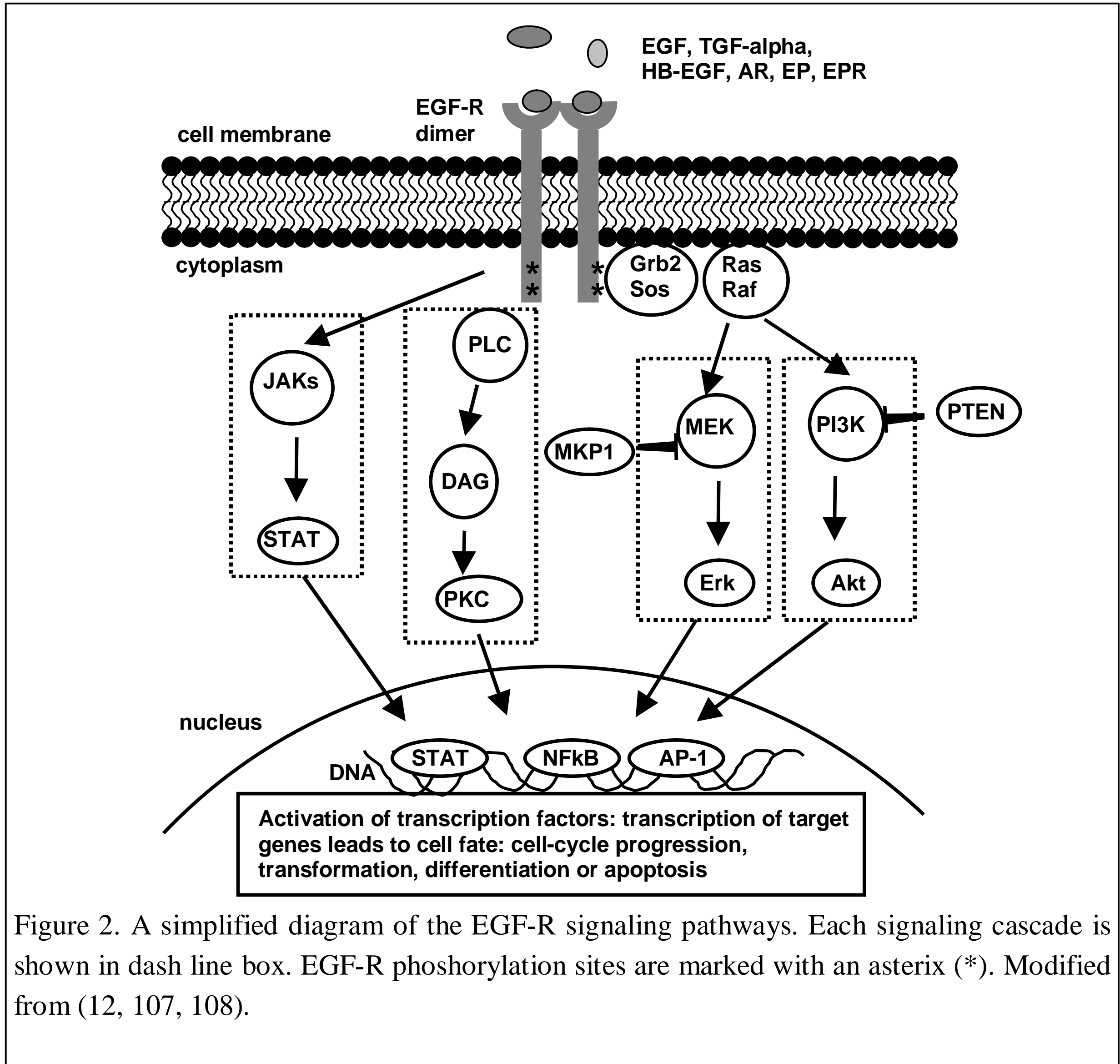

Figure 2. A simplified diagram of the EGF-R signaling pathways. Each signaling cascade is shown in dash line box. EGF-R phoshorylation sites are marked with an asterix (*). Modified from (12, 107, 108).

4.5.1 ErbB receptor activation

In the absence of ligand binding, the extracellular regions of monomeric ErbB receptors (EGF-R, ErbB-3, and ErbB-4) exist in equilibrium between the closed (inactive) and open (active) conformations, of which over 95% are in the closed conformation. Ligand binding to ErbB receptors stabilizes the ErbB extracellular region in the open conformation and induces the formation of receptor homo- and heterodimers. This dimerization also activates the monomer kinase domains to form an asymmetric dimer resulting in phosphorylation on specific tyrosine residues within the cytoplasmic tail (Figure 1) (109, 110). The phosphorylated residues in the cytoplasmic tail and kinase domain serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signaling pathways.

Notably, there are specific differences between the ErbB receptors. The EGF-R has seven known ligands and can form a homodimer or a heterodimer with all the other ErbB family members. On the contrary, ErbB-2 is an orphan receptor but it is transactivated through heterodimerization with other ErbBs (111). ErbB-3 binds several types of neuregulins, but its tyrosine kinase domain is catalytically inactive (112). Therefore, the action of ErbB-3 and ErbB-2 are dependent upon combinatorial interactions with other members of the ErbB family and ErbB-2 is regarded as amplifier of ErbB signaling. ErbB-2 is the preferred heterodimerization partner for all other ErbBs (113).

Ligand binding to EGF-R induces either homo- or heterodimerization, which then autophosphorylates many tyrosine residues within the C-terminus such as Tyr992, Tyr1045, Tyr1068, Tyr1086, Tyr1148, and Tyr1173 (Figure 1A). Alternatively also the Src non-receptor kinase can phosphorylate Tyr845 and Tyr1101. Tyr845 phosphorylation stabilizes the activation loop, maintains the enzyme in an active state, and regulates signal transducer and activator of transcription 5 (STAT5) activity (114). Phospholipase C (PLC)-mediated signaling is stimulated by PLC-binding to a phosphorylated Tyr992 site. The phosphorylation of Tyr1045 creates a docking site for the ubiquitin ligase Cbl, which enables receptor ubiquitination and degradation (115). The phosphorylation of Tyr1068 and Tyr1086 facilitates the binding of the SH2 domain of growth factor receptor-bound protein 2 (Grb2). This binding results in mitogen activated protein kinase (MAPK) activation through the Ras-signaling pathway (116). SHP1 phosphatase can bind to the phosphorylated Tyr1173 domain, which leads to EGF-R dephosphorylation (117).

4.5.1.1 Regulation of the ErbB receptors

Receptor-mediated endocytosis is considered the major desensitization process of EGF-like growth factors, because it robustly removes ligands from the extracellular space and simultaneously targets cell surface receptors to intracellular degradation [reviewed in (118)]. Another suppressor is the ubiquitin ligase Cbl, which interacts with EGF-R directly and

indirectly through Grb2, promoting ubiquitination and degradation of EGF-R (119). Inhibitory signals promoted by crosstalk between RTKs (120), by protein phosphatases (121), and by negative feedback loops may affect signal specificity and biological outcome (122).

4.5.2 MAPK signaling pathway

Mammalian MAPKs can be activated by wide variety of stimuli, which include hormones (e.g., insulin), growth factors [e.g., PDGF, EGF and fibroblast growth factor (FGF)], inflammatory cytokines of tumor necrosis factor (TNF) family and environmental stresses such as radiation, osmotic shock, and ischemic injury (123, 124). These stimuli may act through different receptor families that are coupled to MAPK pathways (RTKs, G protein-coupled receptors (GPCRs), cytokine receptors and Ser/Thr kinase receptors). Here only EGF-R related signaling is discussed.

To date, six distinct groups of MAPKs have been characterized in mammals: extracellular regulated kinases (ERK1/2), Jun NH2 terminal kinases (JNK1/2/3), p38 (p38 α/β/δ/γ), ERK7/8, ERK3/4, and ERK5. Major MAPK pathways are shown in Figure 3. Although each MAPK is unique, they share some common features, and have thus been grouped together in to one family. The central three-staged signaling module is standard for these pathways. It consists of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. The MAPKKKs, also called MEKKs, are Ser/Thr kinases that are activated via phosphorylation and/or their interaction with small GTP proteins of Ras/Rho family in response to extracellular stimuli such as the activation of the EGF-R. MAPKKK activation leads to phosphorylation and activation of downstream MAPKKs, which are dual specificity kinases and can phosphorylate MAPKs on both threonine (Thr) and tyrosine (Tyr) residues on a conserved Thr-X-Tyr (X= any amino acid) motif. Once activated, MAPKs phosphorylate the target substrates on serine (Ser) or threonine (Thr) residues only if a proline (Pro) residue follows these amino acid residues [reviewed in (125)].

4.5.2.1 Erk signaling pathway

The Erk cascade is activated by a large number of extracellular and intracellular stimuli. These include growth factors, serum, and phorbol esters by ligands of GPCRs, cytokines, osmotic stress, and microtubule disorganization (126). Erk1 and Erk2 are ubiquituosly expressed in all tissues.

Erk1$^{-/-}$ mice have defective thymocyte maturation but are otherwise viable and fertile (127). In contrast, Erk2$^{-/-}$ mice embryos die before day E8.5 because of defects in trophoblast and placental development and in mesoderm differentiation (128-130). This suggests that Erk1 is nonessential and can be compensated by Erk2.

The Erk pathway is activated after activation of EGF-R through the recruitment of Grb2 at the SH2-domain (Figure 3). The signal is transduced to small G proteins (e.g., Ras) (131) by phosphorylation that is mediated by son of sevenless (SOS). Activated Ras binds to MAPKKK such as Raf at the plasma membrane and Raf in turn triggers the phosphorylation of MAPKK called MEK1/2 (mitogen-activated protein kinase kinase 1/2) and Erk1/2. Most of the phosphorylated Erk1/2 translocates into the nucleus and activates various substrates. These include transcription factors such as c-Fos, kinases such as p90 ribosomal S6 kinases and MAPK-activated protein kinases (MAPKAPs), and cytoskeletal proteins such as neurofilaments and paxillin (132).

4.5.2.2 JNK signaling pathway

The JNK family was initially identified as ultraviolet (UV)-responsive group of protein kinases involved in the activation of transcription factor AP-1 component c-Jun by phosphorylating N-terminal Ser63 and Ser73 residues (133). Subsequently, it was shown that the JNKs are strongly activated in response to various types of stress, cytokines, growth factor deprivation, DNA damaging agents and, to lesser extent, by stimulation of some GPCRs, serum, and growth factors (Figure 3) (134-136). While JNK1 and 2 are ubiquitously expressed, JNK3 expression is restricted to the brain, heart, and testis.

$JNK1^{-/-}$ and $JNK2^{-/-}$ mice are viable and fertile but have defective T cell differentiation (137, 138). However, $JNK1^{-/-}JNK2^{-/-}$ double knockout (KO) mice have neural tube defects resulting in embryonic lethality (139). $JNK3^{-/-}$ mice are also viable but KO-studies have revealed that JNK3 is a critical component of stress induced JNK signaling in brain and neuronal apoptosis (140, 141).

It is not entirely clear how growth factor receptors, for example, EGF-R, activate the JNK family. One mechanism may be via Ras proto-oncogene and GTP-binding proteins of the Rho family, in particular CDC42 and RAC1. They activate at least 10 different MAPKKKs (i.e., MEKK1–4, MLK2 and -3, Tpl-2, DLK, TAO1 and 2, TAK1, and ASK1/2 (124)). They are specific for different stimuli, which allow the downstream activator MAPKKs called MKK4 and MKK7 to respond to diverse range of external stimuli. TAK1 has been shown to be critical for JNK activation in response to inflammatory cytokines (IL-1, TNF-a, TGF-b, and lymphotoxin-b) and activation by Toll-like receptors (TLR-3, -4, and -9) (142, 143). MEKK3 appears to be critical in response to activation by TLR-8 (144).

Like ERK1/2, the JNKs may relocalize from the cytoplasm to the nucleus following stimulation (145) but not in as significant proportions as Erk. A wide range of nuclear proteins, predominantly transcription factors and nuclear hormone receptors, has been demonstrated to be substrates of JNK (124). JNK has been observed to have a central role in both extrinsic and intrinsic apoptotic pathways and depending on the cellular context it can be either anti- or pro-apoptotic. Many anti- and pro-apoptotic mitochondrial proteins, such as the

Bcl-2 family proteins (Bcl-2, Bcl-xl, Bad, Bim, and Bax) have also shown to be targets of JNK (146).

4.5.2.3 p38 signaling pathway

The same stimuli that activate JNKs also activate p38s. There are four known isoforms of p38, (i.e., $\alpha/\beta/\delta/\gamma$). Among these p38$\alpha$ has been extensively studied.

The p38$\alpha^{-/-}$ mice die at E10.5 due to impaired placental development (147). However, studies using mice with conditional alleles of p38 have revealed an essential role for p38 in the lung and fetal hematopoietic development (148). Moreover, the p38$\alpha^{-/-}$ mice are prone to cancer development in carcinogen- (148) or oncogene-induced cancer models (149), suggesting a tumor suppression function for p38α (150).

Similarly to JNK pathway, Rho family GTPases appears to play an important role as upstream activators of the p38 MAPK pathway (Figure 3.). Several MKKKs have been reported to cause p38 activation; most of them are same with the JNK pathway. Furthermore, the p38 group kinases are activated by MKK3 and MKK6, but also share some upstream kinases with JNK, namely MKK4 and MKK7 (151).

The role of p38 MAPK signaling in cellular responses is diverse, depending on the cell type and stimulus. For example, p38 signaling can negatively regulate cell proliferation by modulating expression of EGF-R (149) or activating p53 (152), thereby activating apoptosis and acting as a tumor suppressor. This type of regulation also involves JNK and c-Jun, as their activity is upregulated in p38$\alpha^{-/-}$ cells and their inactivation can cause suppression of increased proliferation (148, 149). The ability of ionizing radiation to regulate p38 MAPK activity appears to be highly variable (153-155).

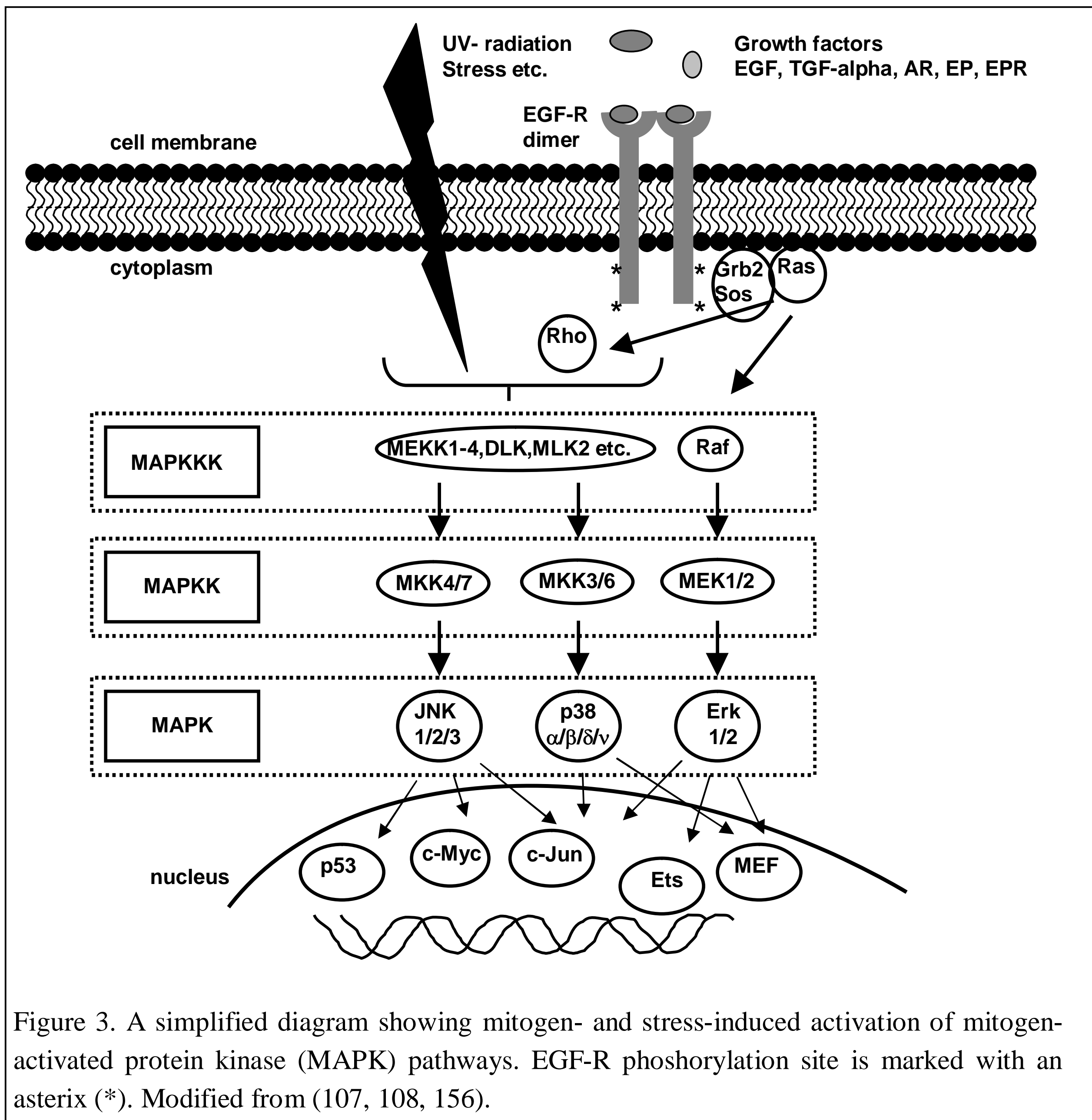

Figure 3. A simplified diagram showing mitogen- and stress-induced activation of mitogen-activated protein kinase (MAPK) pathways. EGF-R phoshorylation site is marked with an asterix (*). Modified from (107, 108, 156).

4.5.3 PI3K-Akt signaling pathway

Like the MAPKs, the PI3K-Akt signaling module is also evolutionarily conserved. As a target of PI3K (157), Akt (also known as protein kinase B) regulates a wide range of biological responses that include cell motility, growth, proliferation, and survival, as well as transcription, protein synthesis, and nutrient metabolism (158, 159). In mammals, three independent genes encode three isoforms of Akt (Akt1/2/3), of which the tissue distribution of Akt3 mRNA is more limited than that of either Akt1 or Akt2 (160).

$Akt1^{-/-}$ mice are small in size (161), indicating that Akt1 is involved in the control of growth and proliferation. $Akt2^{-/-}$ mice in turn have a diabetes-like syndrome (162), indicating that Akt2 regulates cellular metabolism. Similarly to Akt1, Akt3 is also involved in growth control, but considering its limited expression pattern, $Akt3^{-/-}$ mice manifest only a decreased brain size (163).

EGF-R itself is a weak direct activator of PI3K, but can activate PI3-K by via the adaptor protein Grb2 and docking protein Gab1, or by heterodimerization with ErbB3 (164, 165) (Figure 2). PI3K phosphorylates PI 4,5-biphosphate (PIP2) to form PI 3,4,5-triphosphate (PIP3) in a reaction that can be reversed by the PIP3 phosphatase PTEN. PIP3, phosphoinositide-dependent kinase-1 (PDK1), and Akt form a complex at the plasma membrane and PDK1 phosphorylates Akt at its pleckstrin homology (PH) domain. With over 100 substrates, phosphorylated Akt has multiple effects both in the cytoplasm and in the nucleus. These include the inhibition of pro-apoptotic factors, such as BAD (BCL2 antagonist of cell death), procaspase-9, and the Forkhead (FKHR) family of transcription factors (FOXO). Akt-mediated activation of mammalian target of rapamycin complex 1 (mTORC1) is also important in stimulating cell proliferation, and vascular endothelial growth factor (VEGF) and hypoxia inducible factor-α (HIF-α) are important in angiogenesis (166, 167).

4.5.4 STAT signaling pathway

The signal transducer and activator of transcription (STAT) family is comprised of seven proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). EGF-R phosphorylation can activate STAT1, STAT3, and STAT5s (Figure 2). EGF-R signals to STATs directly by binding to its SH2 domain but also through EGF-R-mediated activation of Src, upstream of STATs (168).

The activated STAT proteins translocate into the nucleus and regulate gene expression crucial for cell survival, proliferation, transformation, and oncogenesis (169).

4.5.5 PLC-PKC signaling pathway

PLC (phospholipase C) binds through its SH2 domain to phosphorylated EGF-R tyrosine kinase to become active (Figure 2). Once activated, PLC hydrolyses phosphatidylinositol 4,5-biphosphate to diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 mediates calcium release from intracellular stores, affecting a large number of Ca2+-dependent enzymes, whereas DAG is a cofactor for the activation of the serine/threonine kinase protein kinase-C (PKC). PKC has extensive homology to PKB (Akt1/2/3) within their kinase domains and are, therefore, members of the AGC kinase family (other kinases are PKA and PKG). The activation of PKC results in cell-cycle progression, transformation, differentiation, or apoptosis depending on the cell context (170).

4.5.6 Signaling pathway regulation, specificity, and crosstalk

Protein phosphorylation by kinases is regulated by reverse actions of the phosphatases (dephosphorylation), providing an important means of controlling protein activity. In general, because MAPK kinases are involved in cancer development as tumor promoters, their phosphatases can be considered as tumor suppressors. For example in a recent study, mitogen-activated protein kinase phosphatase-1 (MKP-1) is shown to inhibit invasion in human glioma cells (171). Likewise, inactiving mutation of the PTEN phosphatase on the PI3K-Akt signaling pathway is associated with multiple cancers such as glioblastoma and prostate. For example, PTEN is mutated in 50% of metastatic prostate cancers (172).

Another mechanism that contributes to the specificity of MAPK cascades is the formation of multiprotein complexes via multidomain proteins called as scaffold proteins. These proteins bring together the components of a single pathway, and insulate the module from activation by irrelevant stimuli and negative regulators like phosphatases. They can also determine the localization of the cascade components and provide better stability to some components of the cascade. By doing so, scaffold proteins induce faster kinetics of activation, modify signaling duration and intensity, secure better interaction between distinct components, and modify the cross-talk with other pathways (173).

An example of a negative-feedback loop modulator is the Sprouty2 protein in the MAPK cascade. Its expression is induced by activated Erk. Subsequentially, Sprouty2 binds through its conserved cysteine-rich domain to Ras or Raf resulting in inhibition of the phosphorylation and activation of Raf, and following activation of Erk. Recent study shows that this inhibitory effect is also regulated by another protein called Tesk1, which interacts with Sprouty2 to abrogate its inhibition of Erk phosphorylation (174).

The crosstalk between distict pathways can be either inhibitory or stimulatory. For example, Akt can also block Erk signaling through inhibition of c-Raf [Raf1; (175)], but this crosstalk between Akt and Erk signaling is not ubiquitous and appears to occur only in specific settings. In addition to Erk, JNK and p38 have also been shown to be inhibited by Akt signaling. Akt can directly phosphorylate apoptosis signal-regulated kinase 1 (ASK1, also known as MAPKKK5), which is an upstream activating kinase within the JNK and p38 pathways (176). This creates one possible balance switch between PI3K-Akt survival signaling and JNK/p38 apoptotic signaling.

Other regulatory mechanisms include subcellular localization of proteins and post-translational modifications such as acetylation, sumoylation, and ubiquitination, which seem to play an important role in transducing the signal by altering the protein stability, duration of activation, localization, or protein-protein association (125).

4.5.7 EGF-R transactivation

RTK transactivation refers to a mechanism by which a ligand indirectly activates a RTK for which it does not serve as a direct ligand. Multiple RTKs, such as EGF-R, platelet-derived growth factor receptor (PDGF-R), and c-Met, are potential targets of receptor transactivation by diverse ligand/receptor families (177-179). GPCRs and their ligands (such as thombin, angiotensin, lysophosphoatidic acid, and endothelin) represent one example of a receptor family that participates in EGF-R family transactivation (177, 180). For example, GPCR stimulation can lead to metalloproteinase dependent processing of EGF-like ligands, which in turn activate EGF-R (181). Janus tyrosine kinase pathway agonists, such as leptin, growth hormone, and prolactin, and the Frizzled receptor ligand WNT have also been found to activate EGF-R-dependent cell signaling (182, 183). The binding of WNT ligand to its receptor Frizzled transactivates EGF-R. The mechanism seems to be similar to that described for GPCRs, as it is rapid and blocked by metalloproteinase inhibitors; however, the target ligand has not been identified. WNT- Frizzled-mediated transactivation has been observed in normal mammary cells (183) and in breast cancer cells.

Cytoplasmic mechanisms of transactivation include a direct phosphorylation of EGF-R tyrosines by Jak2 or by non-RTKs, such as Src (184, 185). Direct transphosphorylation of kinase domain tyrosines can result from RTK heterodimerization such as PDGFR/EGF-R, c-Met/EGF-R (178, 186).

Radiation can also induce EGF-R activation. In this special type of activation the EGF-R is internalised into the nucleus and binds to the catalytic subunit of the DNA-PK, a key enzyme of DNA double strand break repair. Bound to the DNA, the EGF-R can act as a transcription factor or a co-factor of DNA repair (187-189).

4.6 EGF-R in cancer

Abnormalities in the expression and signaling pathways downstream of EGF-R family contribute to malignant transformation in human cancers, especially including those of the epithelial and neuronal origins. EGF-R is frequently overexpressed in the majority of human carcinomas (13, 190) but the frequency of this phenomenon varies among the different tumor types and tumor stage, overexpression being more common in the metastatic stage.

Due to the high frequency of expression of individual ErbB receptor types in human carcinomas, co-expression of different receptors occurs in the majority of tumors. This phenomenon might be important for tumor pathogenesis, as some of the signaling pathways activated by these receptors differ, resulting in additive or synergistic effects (191). Particularly, overexpression of ErbB2 leads to EGF-R-ErbB2 heterodimerization with amplified signaling and increased proliferation, migration, and resistance to apoptosis (192). The redundancy of expression in human carcinomas is not limited to the ErbB receptors. In

fact, a number of studies have demonstrated that co-expression of different EGF-like peptides occurs in a majority of human carcinomas [reviewed in (193)]. Gene amplification, activating mutations as well as up-regulated autocrine loops by increased release of ligands (194), makes the EGF-R system a significant component potentially associated in all six hallmarks of cancer (4).

4.6.1 EGF-R mutations

Two main categories of *EGF-R* mutations have been identified: deletion of the extracellular domain and somatic mutations in the tyrosine kinase domain, both leading to increased signaling activity. For example, in non small cell lung cancer (NSCLC) almost 90% of all somatic mutations occur at two mutuational "hot spots" in the tyrosine kinase domain as either delEx19 and/or L858R (195). Other mutations include T790M and insertion mutations in exon 20 (196)**.** An EGF-R deletion mutant called *EGF-RvIII* lacking a portion of the extracellular ligand binding domain (exons 2-7) is the most prevalent naturally occurring form of *EGF-R* mutation, and is found in most glioblastomas and medulloblastomas. In addition, expression of *EGF-RvIII* has been described to occur in breast, ovarian, and lung carcinomas (197).

4.6.2 EGF-R expression in cancer

Gene amplification of EGF-R has been demonstrated to occur in different tumor types and it is usually associated with overexpression of EGF-R protein. However, overexpression of EGF-R in the absence of gene amplification has also been described (13, 190). In glioblastoma multiforme (GBM), *EGF-R* gene amplification has been found in 37% to 58% of the tumors (198). The mutant *EGF-RvIII* accounts for more than 50% of the genomic alteration of EGF-R observed in GBM (199). On average, 50% to 70% of lung, colon, and breast carcinomas have been found to express EGF-R or ErbB-3. In contrast, ErbB-2 expression is generally more restricted, with approximately 15% of human primary breast carcinomas expressing this receptor. The expression of ErbB-4 has been mainly investigated in breast carcinoma, where this receptor is overexpressed in approximately 50% of the tumors [reviewed in (190, 200)] but it has been recently demonstrated to occur in 22% of human primary colon carcinomas (201). In the clinic EGF-R has been associated with chemoresistance, disease progression, and poor survival (202).

One example of cancers, in which EGF-R is likely to be clinically important, is prostate cancer. Prostate cancer is the most common cancer in men in Europe, with about 190 000 new cases (203) and about 80 000 deaths annually (204). The progression of advanced, metastatic androgen-independent prostate cancer is the final stage of this disease and constitutes the majority of mortality.

It is known that EGF-R expression increases during the progression of prostate cancer (205). Correlation of disease progression and hormone-refractory disease suggests that EGF-R-targeted drugs could be of therapeutic relevance in prostate cancer. Thus far, however the prognostic significance of EGF-R expression remains unclear, as reports on this issue are contradictory [reviewed in (206)]. In a recent study, EGF-R expression in prostate cancer was not found to be an independent prognostic variable according to univariate analysis (207).

4.6.3 EGF-R downstream signaling

Key downstream effectors of EGF-R include Ras and the MAPKKK Raf proto-oncogenes and protein tyrosine kinases like c-Src. They can all be mutationally activated and/or overexpressed in a wide variety of human cancers. As described in chapter 4.5, they are important signal transduction elements in many growth factor receptor signals for proliferation and transformation and if mutated and thereby activated they can significantly interfere with EGF-R targeted therapies (1, 108).

4.6.4 EGF-R targeted therapies

Since EGF-R pathways are commonly deregulated in human epithelial tumors, therapeutic agents directed against the EGF-R represent a promising and important group of biologically based treatment strategies. Next these EGF-R-directed therapies are discussed.

First of all, the EGF-R targeted anticancer drugs are not curative in solid human tumors. However, when used alone, they can provide palliation and in combination with chemo- or radiotherapy they can significantly improve patient´s outcome. To date, EGF-R-directed therapies are approved for the treatment of colon, lung, head and neck, and pancreatic cancer (208).

Two classes of well identified groups of EGF-R inhibitors are in clinical use: Monoclonal antibodies (cetuximab (Erbitux®) and panitumumab (Vectibix®)), which bind at the extra cellular part of the receptor and prevent binding of the natural ligands, and small molecular tyrosine kinase inhibitors (TKIs) (gefitinib (ZD1839, Iressa®); erlotinib (OSI 774, Tarceva®)), which inhibit phosphorylation of the intracellular tyrosine kinase by blocking the ATP binding site (Figure 4). Preclinical data indicate considerable heterogeneity in the tumor responses between TKIs and antibodies (209-211). The phase III trial with radiotherapy and simultaneous treatment with cetuximab in patients with squamous-cell carcinoma of the head and neck (HNSCC) showed an improvement of local tumor control and survival (212). Likewise, cetuximab-chemotherapy (irinotecan) combination was shown to be significantly better than gefinitib or erlotinib alone in improving response rates and progression free survival of colon carcinoma patients (213). Furthermore, erlotinib in combination of chemotherapy improved response rates and survival in comparison to chemotherapy alone in pancreatic cancer (214). In contrast, in lung cancer patients, a phase III trial failed to show

that gefinitib would be effective in improving survival (215). Likewise, gefitinib as monotherapy in patients with non-metastatic hormone refractory prostate cancer (HRPC) in a phase II trial showed no significant activity (216). Moreover, in a recent phase III study, the addition of cetuximab to chemotherapy and bevacizumab (antibody against VEGF) treatment in metastatic colorectal cancer resulted in a significantly shorter progression-free survival (217). In prostate cancer treatment radiotherapy is important treatment modality, however so far no results of the clinical trials on EGF-R TKIs in combination with radiotherapy are available.

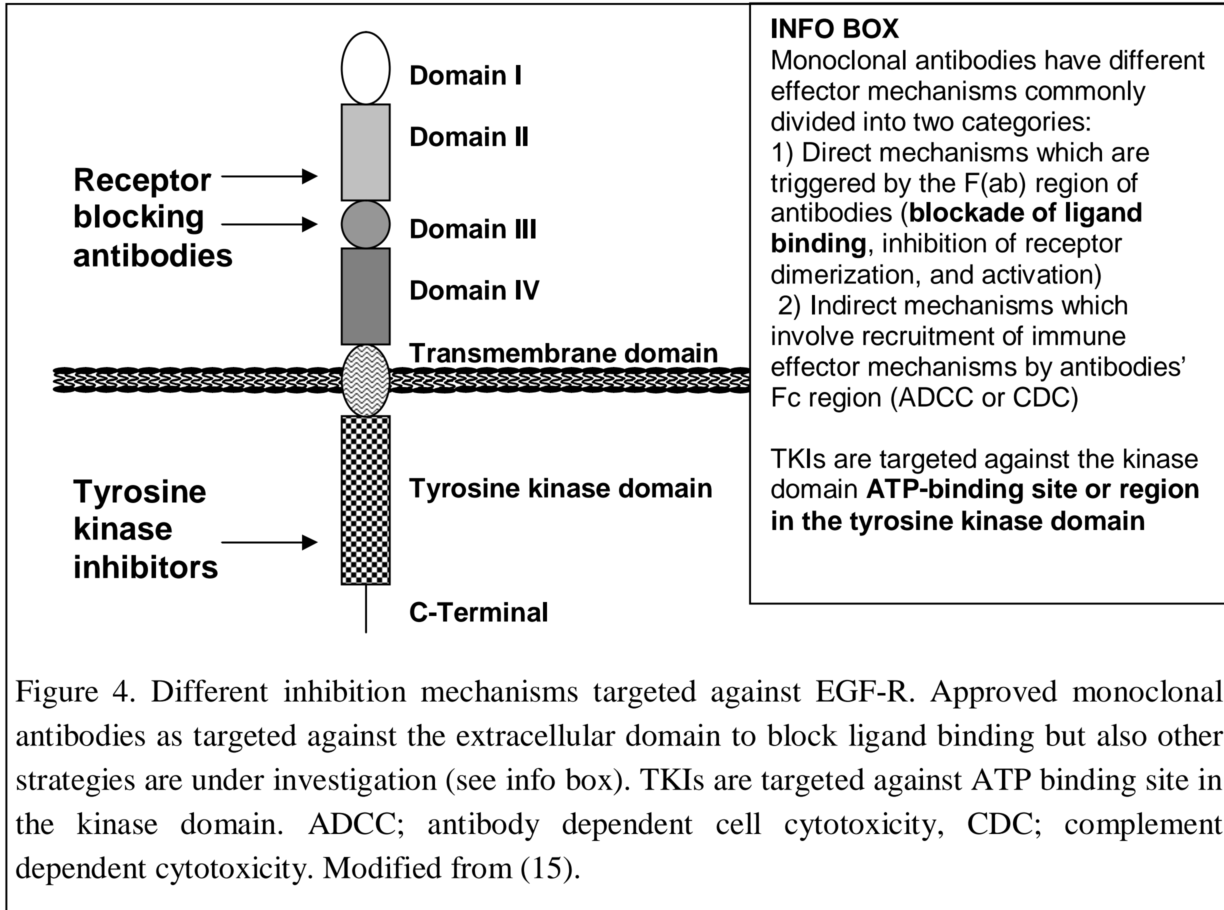

Figure 4. Different inhibition mechanisms targeted against EGF-R. Approved monoclonal antibodies as targeted against the extracellular domain to block ligand binding but also other strategies are under investigation (see info box). TKIs are targeted against ATP binding site in the kinase domain. ADCC; antibody dependent cell cytotoxicity, CDC; complement dependent cytotoxicity. Modified from (15).

4.6.4.1 Mechanisms of resistance to anti-ErbB therapeutics

There are several molecular explanations for the mechanisms of resistance to anti-ErbB therapeutics. One resistance mechanism is the activation of alternative tyrosine kinase pathways. One of the pathways contributing to the resistance of anti-EGF-R therapies is the Akt survival pathway, which is known to be activated by ErbB-3-dependent mechanism resulting from Met amplification (218). Another EGF-R-independent pathway is the IGFI-R pathway, which activates Akt after decreased expression of IGF binding protein-3 (IGFBP3) (219) .

The ligand independent activation of the pathways downstream of EGF-R is another resistance mechanism. It has been demonstrated for the most commonly expressed mutation variant, EGF-RvIII (220). In addition, EGF-RvIII cannot bind the EGF-R-targeted monoclonal antibody cetuximab, and has been reported to be resistant to gefitinib (221). Mutations in the ATP-binding region in the kinase domain can also lead to impaired (222) or to a total blockade (T790M mutation) of TKI binding in NSCLC (223).

Furthermore, the efficacy of EGF-R-therapies depends on additional mutations in the EGF-R-dependent pathways. For example, tumor cells overexpressing EGF-R but having wild type K-Ras were effectively radiosensitized by the EGF-R-TK inhibitor BIB1382BS, whereas tumor cells presenting mutated K-Ras were not (210, 224). K-Ras mutated tumor cells were demonstrated to overproduce EGF-R ligands TGFα and amphiregulin, which in an autocrine manner selectively stimulated EGF-R-PI3K-Akt survival signaling (225). However, no correlation has been found between activated Akt and survival time in NSCLC patients treated with gefitinib in a randomised phase III trial (226). Other important resistance mechanisms of EGF-R targeted therapies are mutations in the PTEN phosphatase. Loss or mutation of PTEN might cause tumor-cell resistance to EGF-R therapeutics, because in cells with low PTEN levels activation of the PI3K-Akt pathway becomes independent of EGF-R activation. The occurrence of PTEN mutation increases as prostate cancer develops towards the metastatic type (172). Loss of PTEN also enhances JNK activation and Akt and JNK activation are highly colocalized in human prostate cancer (227).

4.7 AP-1 transcription factor

When these above-mentioned signaling pathways are activated by ErbB receptors, their downstream targets are various transcription factors such as c-myc, STAT, and nuclear factor kB (NFkB).

One major target of the EGF-R-MAPK cascades is transcription factor activator protein-1 (AP-1). It is composed of dimers of various combinations of the Fos and Jun proteins (228) or closely related ATF and CREB proteins. The Fos family consists of four genes (c-*Fos, FosB, Fra-1,* and *Fra-2*), whereas the Jun family has three members (c-*Jun, JunB,* and *JunD*). Fos and Jun proteins can form heterodimers with Jun family proteins and Jun proteins can also form homodimers (229, 230).

4.7.1 Structure of AP-1

A common feature of all AP-1 proteins is the bZIP domain, which is a basic DNA-binding domain combined with a leucine zipper region. The leucine zipper is responsible for dimerization, which is a requirement for DNA binding mediated by the basic domain (Figure 5). The basic domain is mediating binding to a specific DNA sequence, TGAC/GTCA known as the TRE (TPA-responsive element) or AP-1 site (231), which is found in the promoter

region of many genes including those involved in cell growth and cell cycle control. Different AP-1 dimer combinations and the surrounding DNA sequence determine the affinity for a given TRE (229, 232, 233).

The transactivation domain is responsible for mediating transcriptional activity. Within the transactivation domain c-Jun has two serines (Ser63 and 73) and two threonines (Thr91 and 93), which are essential for its transcriptional activity (See chapter 4.7.3). Likewise, the c-Fos has two treonines (Thr325 and 331) and two serines (Ser362 and 374) (234). The individual Jun and Fos proteins have significantly different transactivation potentials. Jun, Fos, and FosB are considered strong transactivators; JunB, JunD, and Fra-2 have only weak transactivation potential and Fra-1 lacks the transactivation domain totally. The AP-1 proteins also have domains which act as docking sites for several kinases. These include DEF domain for Erk in c-Fos and delta domain for JNK in c-Jun.

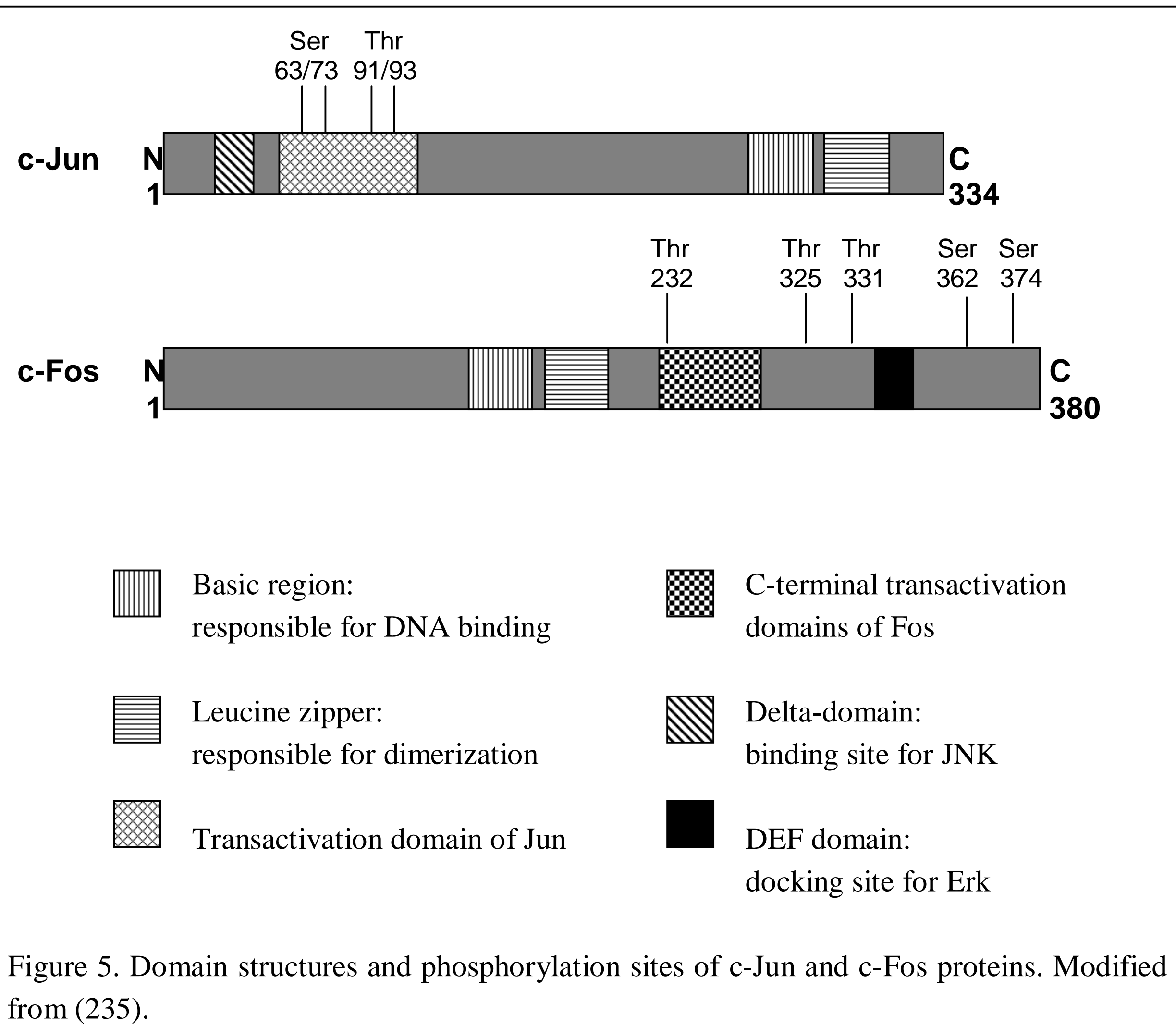

Figure 5. Domain structures and phosphorylation sites of c-Jun and c-Fos proteins. Modified from (235).

4.7.2 AP-1 expression

Each of the AP-1 proteins is differentially expressed and regulated, which means that every cell type has a complex mixture of AP-1 dimers. The proximal promoters of the AP-1 genes are conserved between different species and AP-1 can positively or negatively regulate its own transcription. Even if most of the AP-1 genes are inducible, this is not reflected at the protein level as such, because both posttranscriptional and posttranslational modifications can regulate AP-1 expression (228, 236).

In adult mice c-Jun is expressed in many different cell types at low levels. It´s expression is enhanced in response to many stimuli including growth factors (for example EGF, NGF and FGF), UV irradiation, or cytokines. JunD in turn has high basal expression in many cell types (237). JunB expression is restricted to differentiating epidermal cells and endodermal gut epithelium (238) and it is inducible by growth factors.

4.7.3 Transcriptional activity of AP-1

Transcriptional activity and protein stability of the most studied AP-1 family member, c-Jun, is increased by JNK or Erk mediated phosphorylation of serines 63 and 73 (133, 239), whereas phosphorylation of threonines 91 and 93 may enhance c-Jun DNA binding ability (240). JNKs are the only MAPKs which can phosphorylate all four sites [reviewed in (241)]. Similarly, the stabilization and activation of c-Fos is mediated by the activity of Erks phosphorylating two c-Fos c-terminal sites (Ser362 and Ser374) (234). In addition, the Erks are involved in the induction of c-Fos expression via phosphorylating the transcription factor Elk-1/TCF, which activates the *c-fos* promoter (242).

Similarly, other Fos, Jun and ATF proteins are regulated by phosphorylation giving rise to dimers with different transactivation properties [reviewed in (236, 243)]. Transcription cofactors (coactivators and corepressors) can also interact with AP-1 proteins and other transcription factors and the basal transcriptional machinery (including RNA polymerase II and basal transcription factors) (Figure 6). For example, c-Jun phosphorylation may potentiate its transcriptional activity through recruitment of CREB binding protein (CBP), which is proposed to connect the phosphorylated activation domains of coactivator CREB or c-Jun to the basal transcriptional machinery (244). Another transcriptional coactivator, DNA topoisomerase I (Topo I), was recently shown to interact with c-Jun affecting c-Jun mediated EGF-R expression and proliferation (245). Other cofactors include histone acetyltransferase (HAT) and deacetylases (HDAC) that control gene transcription through modification of chromatin structure. For example HDAC SRT1 binds to c-Jun and inhibits it´s transciptional activity (246). Furthermore, AP-1 activity can be down-regulated by sumoylation (247) and ubiquitin-dependent protein degradation (248).

Altogether, the regulation of net AP-1 activity can be achieved through changes in the transcription of genes encoding AP-1 subunits, control of the stability of their mRNAs, post-translational processing and turnover of pre-existing or newly synthesized AP-1 subunits, and specific interactions between AP-1 proteins and other transcription factors such as ETS (249). The abundance of different AP-1 members within a given cell, as well as the cell lineage, differentiation stage, microenvironment, and type of stimulus have an impact on how AP-1 modulates the decision of a cell to proliferate, differentiate, or die by apoptosis.

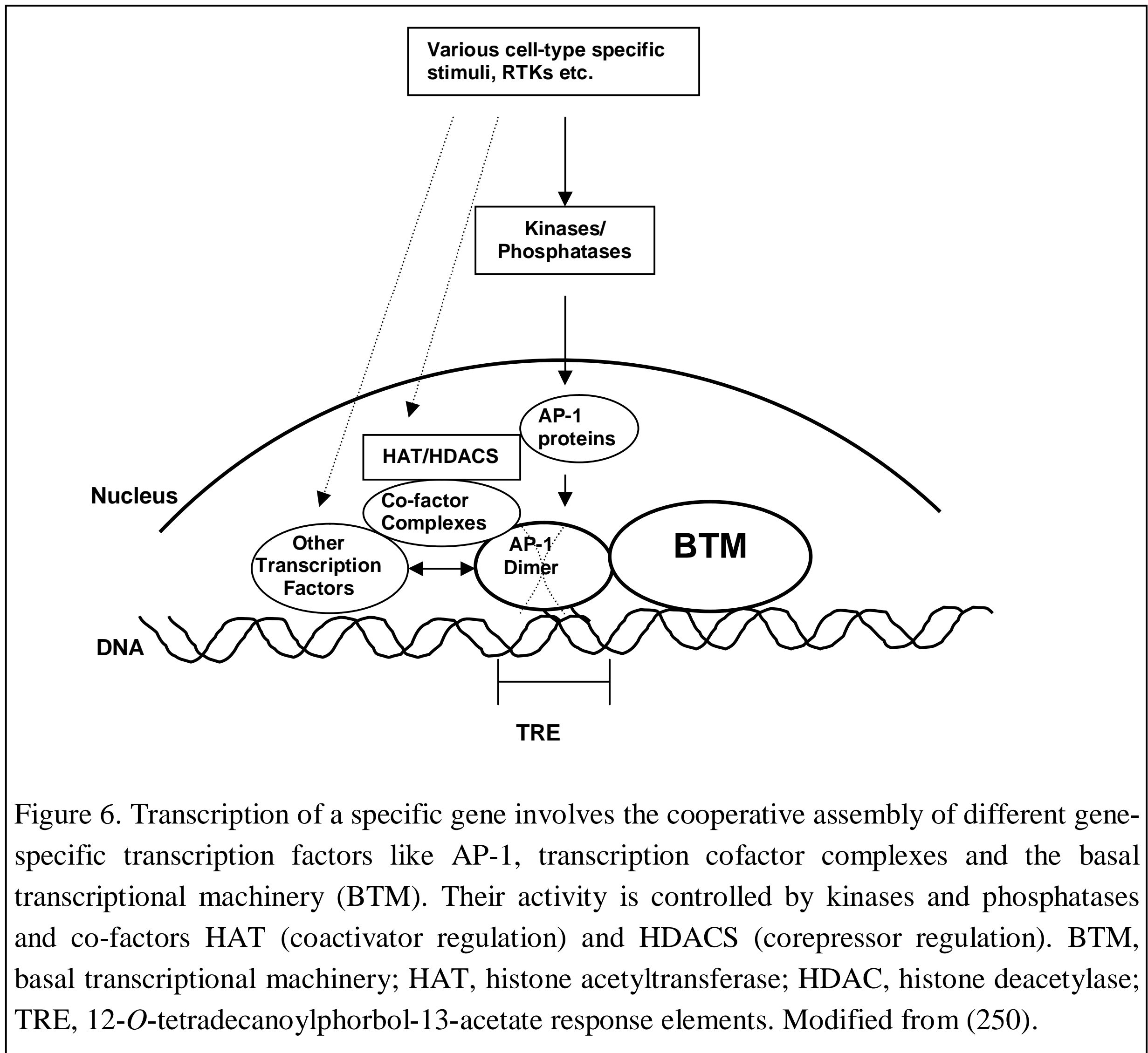

Figure 6. Transcription of a specific gene involves the cooperative assembly of different gene-specific transcription factors like AP-1, transcription cofactor complexes and the basal transcriptional machinery (BTM). Their activity is controlled by kinases and phosphatases and co-factors HAT (coactivator regulation) and HDACS (corepressor regulation). BTM, basal transcriptional machinery; HAT, histone acetyltransferase; HDAC, histone deacetylase; TRE, 12-*O*-tetradecanoylphorbol-13-acetate response elements. Modified from (250).

4.8 AP-1 in development

Studies in mice deficient in Jun or Fos expression have provided valuable information on their role in development and disease.

The phenotypes of AP-1 knock-out (KO) mice are described in the following chapter. Interestingly, only the absence of JunB and Fra-1 gives rise to a similar phenotype whereas

inactivation of the other AP-1 members leads to distinct phenotypes, indicating specific and unique functions for each AP-1 subunit. However, c-Jun KO mice can be rescued with JunD.

AP-1 members can also compensate each others function *in vivo*. For example during the development and differentiation of the immune system none of the c-Fos or c-Jun KO mice show altered B or T cell production or function regardless of *in vitro* data that have implicated AP-1 as a critical transcription factor of this system [reviewed by (251)].

4.8.1 Jun family knockout-mice

Loss of c-Jun leads to embryonic lethality due to defects and abnormalities at the molecular level in the heart, aorta, and liver (252, 253). Loss of JunB also leads to embryonic lethality, because yolk sac vascularization is impaired (254). Mice lacking JunD are viable but mutant males show impaired spermatogenesis (237).

4.8.2 Fos family knockout-mice

Mice lacking c-Fos are viable and fertile but lack osteoclasts resulting in an osteopetrotic phenotype (255). In contrast, mice lacking FosB develop normally (256). Loss of Fra-1 leads to embryonic lethality due to defects in the placenta and the yolk sac (257), which has similarities compared to JunB$^{-/-}$ mice. The phenotype of mice lacking Fra-2 has not yet been reported. It is however known that Fra-2 controls osteoclast survival and size (258).

4.9 AP-1 controlling cellular growth and apoptosis

4.9.1 Proliferation

The above mentioned studies using cells or mice either lacking or overexpressing single or multiple AP-1 components have demonstrated that AP-1 proteins have versatile functions in the regulation of cell proliferation, and have identified some AP-1 target genes, which directly link AP-1 expression to the cell cycle machinery. Control of cell proliferation by AP-1 seems to be mainly mediated by its ability to regulate the expression and function of cell-cycle regulators including cyclinD1 (259) and CDK inhibitory proteins such as p21$^{Waf1/Cip1}$. Particularly, c-Jun is a negative regulator of p53 tumor suppressor and its target gene, the CDK inhibitor p21$^{Waf1/Cip1}$, thus making c-Jun a positive regulator of cell proliferation, which is important in cancer development (260, 261).

However, the effects of AP-1 are highly dependent on the cellular subtypes. For example, the tissue-specific inactivation of c-Jun in mice has highlighted that c-Jun is essential for regulation of cell cycle in fibroblasts, hepatocytes, and keratinocytes (262-264). In contrast, JunB and JunD are often considered to be negative regulators of cell proliferation. Other members of the AP-1 family c-Fos, FosB, and Fra-1 appear to be dispensable for cell cycle

progression, since fibroblasts and embryonic stem cells lacking these components have no proliferation defect (256, 257, 265-267).

4.9.2 Apoptosis

JNK phosphorylates c-Jun, which results in enhanced transcription of target genes involved in stress-induced apoptosis. Among the pro-apoptotic targets of c-Jun are the genes that encode FasL and TNF-α. c-Fos might negatively regulate *FasL* expression, since a transcriptional repressor element within the *FasL* promoter constitutively binds c-Fos but not c-Jun (268). Enhanced apoptosis in the absence of c-Jun is observed in keratinocytes and notochordal cells (264, 269). In contrast, in differentiated neuronal cells, c-Jun mediates JNK induced apoptosis, whereas in non-differentiated neuronal cells ATF-2, not c-Jun, is mediating the apoptotic signals (270). These studies reveal a critical, but cell type specific roles for c-Jun in survival signaling. Also other AP-1 members play important roles in regulation of pro-apoptotic and anti-apoptotic target genes. For example, JunB downregulates anti-apoptotic Bcl-family proteins leading to increased apoptosis of myeloid cells (271).

4.10 AP-1 in cancer

AP-1 proteins are significant modulators of tumorigenesis; as they regulate the expression of critical target genes in all steps of tumorigenesis [oncogenic transformation, proliferation (EGF-R, cell cycle proteins), apoptosis (Bcl proteins), invasive growth (MMPs), and angiogenesis (VEGF) (reviewed in (272)]. In addition, AP-1 activity is maintained by mutated signaling proteins, such as EGF-R, Ras, and Raf or by various growth factors, whose expression are deregulated in cancer development. No mutations in *jun* or *fos* genes have been identified so far but a recent study showed *jun* oncogene amplification in highly aggressive sarcomas (273). In general, the expression of several AP-1 components is frequently upregulated in many cancers.

The role of AP-1 in tumorigenesis is subunit and tissue dependent. For example, c-Jun is required for cellular transformation by activated Ras in fibroblasts *in vitro* since transformation is suppressed in fibroblasts lacking c-Jun or expressing a truncated, dominant-negative form of c-Jun (274). c-Jun is also a positive regulator of proliferation (See also 4.9.1 Proliferation), regulating cell cycle genes and p53 (261). Moreover, c-Jun and c-Fos can induce transition of tumor cells from an epithelial to a mesenchymal morphology known as the epithelial–mesenchymal transition (EMT) (275, 276). In contrast, ectopic expression of JunB or JunD in immortalized fibroblasts inhibits Ras-induced oncogenic transformation suggesting that these Jun proteins can act as anti-oncogenes (tumor suppressors) (277).

At present, the role of AP-1 in malignant tumors *in vivo* is widely studied. In general, increased expression of AP-1 subunits has been observed during growth of malignant tumors but there is no regular pattern of AP-1 complexes that would serve as a marker for increased

invasion or malignancy. However, over-expression of c-Fos can contribute to development of osteosarcomas (278) and splenic marginal zone lymphomas (279). Also elevated Fra-1 mRNA and protein have been detected in multiple tumors as well as transformed cell lines, including breast, colon, and lung (280) indicating that Fra-1 may have a role in cancerous transformation.

AP-1 function in prostate cancer is still poorly understood. However, the high expression of IL-6, which functions as a growth and differentiation factor in hormone-refractory prostate cancer, is due to enhanced promoter activity resulting from combined activation of NFkB, JunD, and Fra-1 (281). A recent study reveals a role for AP-1 in prostate cancer progression, especially activated c-Jun as a marker of high-risk prostate cancer (282). Another study shows that c-Jun in stromal fibroblasts regulates production and paracrine signals of IGF-1 and stimulate epithelial proliferation in benign prostatic hyperplasia (283).

4.11 Matrix metalloproteinases as AP-1 target genes

Interaction of the cells with extracellular matrix (ECM) is critical for the normal development and function of organisms. Zinc-dependent endopeptidases called matrix metalloproteases (MMPs) are a major group of enzymes that regulate this cell-matrix composition and tissue modeling. Although, they are nowadays also considered as important regulators of signaling networks by modifing the activity of signaling molecules on the cell membrane or ECM [reviewed in (284)]. One example of such activity is discussed in chapter 4.11.2.

In general, MMPs are synthesized by most cells but particularly fibroblasts and immediately secreted into the ECM as inactive precursors. Insufficient control of MMPs prevents normal cell migration, while excessive degradation results in loss of cell attachment to the ECM, as well as pathologic destruction of connective tissue, resulting in diseases such as arthritis, atherosclerosis, periodontitis, and cancer. Interestingly, MMP deficient mice have only mild phenotypes suggesting redundancy and many overlapping substrates [reviewed in (285)] and, moreover, that MMPs are required mainly for postnatal tissue development and tissue remodelling. Indeed, most MMPs are expressed at low levels under normal conditions in tissues but their expression is induced when remodeling of ECM is required. These situations normally include wound healing and inflammation [reviewed in (286)].

4.11.1 Regulation of MMPs

MMP expression is primarily regulated at the transcriptional level. Transcription factor AP-1 is one of the major regulators of growth factor-inducible MMP expression. These MMPs include for example MMP1 (collagenase-1), -3 (stromelysin-1), and -9 (gelatinase B). Inducible MMP genes can be stimulated or repressed by growth factors and cytokines, which in many cases results in 20–50 fold changes in mRNA and protein levels. In contrast, the constitutively synthesized MMP2 (gelatinase A) is widely expressed by most cell types but

the MMP2 promoter does not have an AP-1 binding site (287).

Interaction between AP-1 and the members of different transcription factors like ETS provide fine-tuning of the transcriptional regulation of MMP promoter activity. The ETS proteins usually do not dimerize and bind to DNA alone, but co-operate with AP-1, for which they function as co-activators (249, 288). This type of a functional interplay between AP-1 and ETS factors in the regulation of MMP gene expression may modulate the response of MMP promoters. The activity of the transcription factors that control growth factor-responsive MMPs is phosphorylation-dependent and mediated by PKC and MAPK pathways (Erk, Jnk and p38), but cyclic adenosine monophosphate (cAMP) also plays a role in this regulation. (289-291).

In addition to transcriptional control other regulatory levels are also involved. These include activation and inhibition of MMP activity, compartmentalization to specific membranes, the cysteine-switch mechanism and protein degradation (292). The important physiological activators of inactive precursor MMPs are plasmin and urokinase type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (293). All active forms of matrix metalloproteinases are inhibited by tissue inhibitors of metalloproteinases, TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) (294) but also inhibitors of metalloproteinases (IMPs).

4.11.2 EGF-R and MMP interactions

Although the identity of all metalloprotease(s) involved in ectodomain shedding of pro-ErbB ligands stimulated by various reagents is not known, both MMP and ADAM family of metalloproteases are implicated to have a role in it (See 4.3 EGF-R ligands). The ADAM family, particularly ADAM10 and ADAM17, has been shown to mediate extracellular cleavage of several EGF-R/ErbB ligand precursors, whereas MMP-3 and MMP-7 cleave and produce an active form of HB-EGF *in vitro* (295). Other signaling molecules, such as VEGF, IGF and FGF, which are located extracellularly in the ECM, are also processed by MMPs leading to their activation and to possible EGF-R transactivation by their respective receptors. These and numerous other *in vitro* and *in vivo* studies have established that MMPs participate in the development of tumors as well as in invasion, metastasis and angiogenesis [reviewed in (296)]. For example, in primary breast tumors, there is a correlation between high EGF-R activity and high ADAM17 levels (297). Similar observation has been hade in gastrointestinal stromal tumors (GIST), in which ADAM17 is upregulated and coexpressed with EGF-R and EGF-R ligands HB-EGF and amphiregulin (298).

5. AIMS OF THE STUDY

The purpose of this study was to investigate the function of EGF-R and its downstream signaling during development and pathological events in normal and cancer cell-line models and in fetal intestine, especially focusing on the MAPK/AP-1 signaling pathway but also covering the PI3K/Akt pathway. The functions of these EGF-R regulated pathways needed to be understood in further detail to confirm and clarify their roles in events mentioned in the following specific aims. Despite preciding studies, the exact mechanisms of these signaling pathways in these settings have remained unclear, therefore, the specific aims were:

1) To identify whether EGF-R and AP-1 pathways interact in the fibroblasts

2) To determine the signaling network of EGF-R in the fetal enterocytes

3) To explore the role of AP-1 signaling in prostate cancer cell proliferation and radioresistance

6. MATERIALS AND METHODS

6.1 Materials

The materials used in this study are summarized below.

Antibodies	Manufacturer/Provider/Reference	Study
Actin	Santa Cruz	III
Alpha-Tubulin	Sigma	II
Akt	Cell Signaling Technologies	II, III
c-Fos	Santa Cruz	I, III
c-Jun	Santa Cruz	I, III
COX-2	Cayman Chemical	II
Cyclin B	NeoMarkers	II
Cyclin D1	NeoMarkers	II
EGF-R	Sigma	I, II, III
ERK1/2	Santa Cruz	I, II, III
Fra1	Santa Cruz	III
Fra2	Santa Cruz	III
JNK	Santa Cruz	I
JunB	Santa Cruz	III
JunD	Santa Cruz	III
p21	Santa Cruz	II
p38	Cell Signaling Technologies	I, II, III
phospho Akt (ser476)	Cell Signaling Technologies	II, III
phospho EGF-R	Biosource International	I, II, III
phospho c-Jun (ser73)	Cell Signaling Technologies	I
phospho Erk	Promega	I, II, III
phospho JNK	Promega	I, II
phospho p38	Cell Signaling Technologies	I, II
Conjugates:		
HRP-goat anti-rabbit	Jackson Laboratories	I, II, III
HRP-rabbit anti-mouse	Jackson Laboratories	I, II, III
TexasRed-mouse anti-rabbit	Jackson Laboratories	II

Expression vectors	Manufacturer/Provider/Reference	Study
adMEKca	(299) (Foschi M, et al.)	I
adTAM67	(300) (Auer KL, et al.)	I
AP-1 Luc	Clontech	III
Col Luc	(301) (Treier M, et al.)	I
Col Luc mut	(301) (Treier M, et al.)	I

6.2 Methods

The methods used in this study are summarized below.

6.2.1 Cell culture and treatments

All cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

DU145 (III)

DU145 (HTB-81, prostate carcinoma cell-line) was purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/mL streptomycin.

FHs74Int cells (II)

FHs74Int (CCL-241, Fetal human small intestine cell-line) was purchased from the ATCC and cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% FCS, 2mM L-glutamine, 100 units/ml penicillin, 100 µg/mL streptomycin, nonessential amino acids, 0.5 mM sodium pyruvate, 1 mM oxaloacetic acid, and 0.2 units/ml insulin.

MEF cells (I)

Mouse embryonal fibroblasts were derived from E15 and E17 wild type (wt) and EGF-R null mice embryos. Cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/mL streptomycin.

PC-3 cells (III)

PC-3 (CRL-1435, prostate adenocarcinoma cell-line) was purchased from the ATCC and cultured in Hank´s F-12 medium supplemented with 7% FCS, 2mM L-glutamine, 100 units/ml penicillin, and 100 µg/mL streptomycin.

Inhibitor treatments (I,II,III)

EGF-R-inhibitor ZD1839 (10 µM; AstraZeneca, London, UK), Mek-inhibitor PD98059 (20 µM; Calbiochem, San Diego, CA), JNK-inhibitor SP (20 µM; Calbiochem), SB (20 µM; Calbiochem), PI3 K-inhibitor LY294002 (25 µM; Calbiochem), and COX-inhibitor Ind (50 µM; Dumex-Alpharma, Copenhagen, Denmark) were added to the cultures 1 h before additional treatments including serum, EGF, PGE_2, and TPA.

6.2.2 Expression analyses

Adenovirus infection (I)

Wild type or EGF-R null MEFs were cultured on 100-mm diameter plates in DMEM containing 1% FBS and infected with recombinant adenoviruses for dominant negative c-Jun [AdTAM67 (300)] or catalytically active MEK1 [AdMEK1ca (299)] in a total volume of 3 ml and at a multiplicity of infection of 1500 (virus/cell ratio). After overnight infection, the cells were washed with PBS and cultured in serum free DMEM for additional 6 h with or without 20 ng/ml EGF.

Transfections and reporter gene analysis (I,III)

For transactivation studies in *(I)*, duplicates of 60-mm diameter plates containing 50 000 cells were transfected with the luciferase reporter constructs for collagenase 1 and mutated collagenase 1 promoters [AP1+ Ets+; intact AP-1 and ETS-binding sites, and AP1- Ets+; mutated AP-1 site but functional Ets-binding site (301)] using the Fugene-6 reagent (Roche). After 18 h the cells were stimulated with 20 ng/ml EGF or 10 nM TPA for additional 6 h. The activity of collagenase reporter was normalized to the protein concentration. Luciferase assays were performed according to the manufacturer's instructions (Promega).

For transactivation studies in *(III)*, duplicates of 60-mm diameter plates containing 50 000 cells were transfected with the luciferase reporter constructs for AP-1 binding site TRE (TPA response element) using the Lipofectamine 2000 reagent (Invitrogen). The activity of collagenase reporter was normalized to the protein concentration. Luciferase assays were performed according to the manufacturer's instructions (Promega).

For transfections of siRNA oligonucleotides in *(III)*, triplicates of 24-well plate wells containing 50 000 cells were transfected with Fra1, JunD (Dharmacon), c-Jun, and Fra2 (Sigma-Proligo) siRNAs using the Lipofectamine 2000 reagent (Invitrogen).

6.2.3 RNA analyses

Northern analysis (I)

Total RNA was isolated from MEFs by the single step method using Trizol (Invitrogen). For Northern blot analysis, 10 μg of RNA was separated on a 1% agarose-formaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham). Filters were hybridized with [γ-^{32}P]dCTP-labeled cDNAs coding for c-Jun (302), c-Fos (303), MMP-2 (304), MMP-3 (304), MMP-14 (305), and GAPDH (306) cDNAs. Hybridizations and washing conditions were performed according to the instructions of the manufacturer.

Real time quantitative PCR (II)

Total RNA was converted to cDNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamers. Real-time PCR reactions were performed with the Gene Amp 5700 Sequence detection system (Applied Biosystems, Foster City, CA). Human 18S rRNA served as an endogenous control. Each sample was measured in triplicates, and data analyzed by the delta-delta method for comparing relative expression results (ratio, $2^{-[[DELTA]CP\ sample - [DELTA]CP\ control]}$).

6.2.4 Electrophoretic mobility shift assay (EMSA)

The cells were harvested, centrifuged, and quick-frozen in liquid nitrogen. Cell pellets were homogenized in lysis buffer. Equal amounts of soluble protein were measured using BCA Protein Assay Kit (Pierce).

To assay AP-1 DNA-binding activity, cell extracts were incubated for 20 min at room temperature in reaction buffer containing [γ-^{32}P]ATP-labeled oligonucleotide probe. Protein–DNA complexes were resolved on a 4% non-denaturating polyacrylamide gel containing 0.5X TBE and visualized by autoradiography.

6.2.5 Protein analyses

Chromatin immunoprecipitation assay and PCR analysis (I)

Chromatin immunoprecipitation (ChIP) was performed as previously described (307). Briefly, wild type MEFs were fixed with formaldehyde and sonicated. Lysates were preincubated with protein A Sepharose and subjected to immunoprecipitation overnight at +4°C with rabbit IgG or antibodies against c-Jun and c-Fos (Cell Signaling Technology and Santa Cruz, respectively). Precipitates were washed several times and eluated from beads with elution buffer. Crosslinking was reverted by adding NaCl and heating at +67°C for 4h. The samples were precipitated with ethanol overnight at -20°C. After centrifugation, DNA was suspended

to TE buffer, recovered using NucleoSpin Extract II purification system (Macherey-Nagel), and analysed for AP-1 promoter sequence in *MMP-3* gene using PCR.

PCR was performed on ChIP products for 30 cycles using DyNAzyme II polymerase (Finnzymes). Control reactions with mouse genomic DNA were always carried out along the immunoprecipitated samples. The following primers for *MMP-3* gene fragment were used: (-189/+97) 5´-TGCCCCAGTTTTCTCTTTTG-3 and 5´-CGGAAGACCCTTCATTTTCA-3´. The PCR products were fractionated on agarose gels, stained with ethidium bromide, and analysed using AlphaImager™ 2200 Documentation and Analysis System (Alpha Innotech Corp.).

Enzyme Linked Immuno-Sorbent Assay (ELISA) (II)

PGE_2 concentration in the conditioned culture medium was analyzed using enzyme immunoassay according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI).

Immunofluorescence staining (II)

FHs74Int cells were fixed in 4% paraformaldehyde (PFA). EGF-R-phospho-specific antibodies (Biosource International, Camarillo, CA) and Texas-Red-conjugated secondary antibodies (Jackson Laboratories, Bar Harbor, ME) were used. Actin filaments were stained with TRITC-conjugated phalloidin (Sigma Chemical Co.).

Immunohistochemistry (II)

Paraffin-sections (5 µm) were deparaffinized. For antigen unmasking, the slides were microwaved in 10 mM citrate-buffer (for COX-2 detection) or Proteinase K (Ready-to-use Proteinase K; Dako Cytomation, Glostrup, Denmark) treated (for EGF-R detection). Immunostaining was performed with COX-2 or EGF-R antibodies (#sc-03; Santa Cruz Biochemicals) and biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized by avidin-biotin peroxidase complex solution (Vectastain ABComplex, Vector Laboratories) and 3-amino-9-ethylcarbazole (Lab Vision Corp., Fremont, CA). Counterstaining was performed with Mayer's hemalum (Merck, Darmstadt, Germany). Nonimmune rabbit serum served as a negative control.

Immunoprecipitation (II)

Cells were lysed in RIPA lysis buffer. Supernatants were incubated with protein A Sepharose beads (Amersham, Little Chalfont, UK) and with appropriate antibodies for overnight at 4°C. After washing in RIPA buffer, the absorbed complexes were removed from the beads by heating it in SDS sample buffer and subjected to Western analysis.

In vitro kinase assay (I)

Cells were washed with PBS and solubilized in lysis buffer. JNK was immunoprecipitated using polyclonal JNK antibody (Santa Cruz) for 1 h at 4°C. Immunocomplexes were coupled to protein-A-Sepharose beads for 1 h and washed several times with dilution buffer. Kinase reactions were performed in kinase buffer for 20 min at 30°C using GST-c-Jun protein (amino acids 5–105) as a substrate. The phosphorylated c-Jun proteins were analyzed on a 10% SDS-PAGE and immunoblotting using an antibody against c-Jun phosphorylated on serine 73 (Cell Signaling Technology).

Western analysis (I, II, III)

Cells were lysed in SDS sample buffer and sonicated. An equal amount (50 µg/lane) of protein was separated in a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA) by electroblotting. Immunoblotting was performed using specific primary antibodies, horseradish peroxidase–conjugated secondary antibodies (Jackson Laboratories) and enhanced chemiluminescence protocol (Super Signal, Pierce, Rockford, IL).

6.2.6 Functional assays

Collagen contraction assay (I)

Collagen gels were prepared using Collagen Type 1 (BD Biosciences). Seven volumes of collagen were mixed with two volumes of five-fold concentrated DMEM and one volume of 0.2M HEPES (pH7.4), and kept on ice. Cells were mixed gently into neutralized collagen solution before transferred into 24-well plates (50 000 cells/well). Collagen polymerization was initiated by incubating the plates at 37°C for 30 min. Gels were detached from the well walls and cell culture media containing 10% FBS and appropriate MAPK and EGF-R inhibitors were added into the wells. Contraction process was observed daily. The statistical significance of differences seen in contraction assays was analyzed using student’s t-test. All p-values were two tailed.

FACS analysis (III)

For apoptosis and cell cycle assays the cells were harvested onto hypotonic Propidium Iodine solution and analyzed using FACS (CellCalibur; Becton Dickinson, Bedford, MA) and CellQuest (Apoptosis) as well as ModFit (cell cycle) softwares.

Irridation and colony forming assay (III)

Cells were counted, plated and cultured for two days, and transfected one day prior receiving a dose of 4 Gy [unit of absorbed dose (J/kg)] at the Department of Oncology, Helsinki University Hospital, Helsinki, Finland. The irradiation was performed with the Varian Clinac 600C/D linear accelerator (Varian Medical Systems Inc.; Palo Alto, CA) using a 6-MV photon beam. The dose rate was 4 Gy/min.

For colony forming assay cells were counted six hours after irradiation and one thousand cells were plated on a 6-well plate. After eight days the cells were fixed with 2% PFA and stained with 20% crystal violet stain, washed, and colonies ≥20 cells were counted under a microscope.

Zymogram assay (I)

Analyses for gelatinase activity was carried out as previously described (308). Conditioned cell culture media was applied to 10% PAGE gels containing 2mg/ml gelatin (Sigma) in non-reducing 4x Laemmli sample buffer. After electrophoresis, the gels were rinsed in washing buffer and reaction buffer and incubated in reaction buffer overnight at +37°C. The gels were stained with Coomassie brilliant blue and destained with destaining solution.

7. RESULTS AND DISCUSSION

7.1 EGF-R, MAPK, and AP-1 regulate MMP function in fibroblasts (I)

EGF-R in fibroblasts

The EGF-R growth factor receptor is implicated to be a regulator of many cellular events such as proliferation, migration, survival, and apoptosis. Activated EGF-R conducts these effects via downstream signaling pathways MAPK, PI3K, STAT, and PLC-PKC, which transmit the signal to the nucleus and activate transcription factors such as AP-1. The EGF-R signaling is essential for many cell types during development and its deregulation or overexpression might lead to uncontrolled growth and tumorigenesis (12). During development or tumorigenesis, stromal cells, such as EGF-R expressing fibroblasts, can influence surrounding cells and tissues by expressing growth factors and MMPs which alter cell proliferation, angiogenesis, migration, and invasion capacities (28, 93, 105, 309). In addition, EGF-R downstream effector AP-1, which has a role in regulating the above mentioned expression of growh factors and MMPs, is frequently overexpressed in various human tumors and cancer cells. It has been recently shown that AP-1 can significantly promote growth, motility, and invasion of human pulmonary epithelial cells via activating MMPs and EGF-R (310). The connection between the expression of AP-1 subunits and EGF-R induction has been revealed also in fibroblasts (311). However, normally MMPs are required during development for tissue-remodelling events such as bone and vascular remodelling, as well as mammary development (284), but they are also required to maintain homeostasis in adult tissue and take part in wound healing and infection (286).

In this study, we investigated the role of EGF-R signaling, culminating to the activated AP-1, during fibroblast proliferation and matrix remodeling. Wild type (wt) and EGF-R$^{-/-}$ mouse embryonal fibroblasts (MEFs) were used as experimental model.

First, the cellular characteristics such as proliferation rate, saturation density, apoptosis, morphology, and stress fiber formation of both wt and EGF-R$^{-/-}$ MEFs were studied. Wt and EGF-R$^{-/-}$ MEFs were cultured and their growth properties compared. In contrast to the wt MEFs, the proliferation rates and saturation densities of the EGF-R$^{-/-}$ MEFs at early passage numbers were reduced. However, after spontaneous immortalization, which occured approximately at passage number ten, no significant differences in the proliferation rates between wt and EGF-R$^{-/-}$ fibroblasts were detected. Interestingly, c-Jun$^{-/-}$ MEFs had a similar proliferation defect and a prolonged crisis before spontaneous immortalization (261). In addition, the reduced proliferation potential of JNK1$^{-/-}$ MEFs is similar to c-Jun$^{-/-}$ MEFs showing that the JNK1 also regulates fibroblast proliferation (312). To conclude, EGF-R and its downstream signaling pathways were involved in proliferation events in fibroblasts. However, EGF-R$^{-/-}$ fibroblasts showed no differences in other cellular characterstics such as

saturation density, apoptosis, morphology or stress fiber formation as compared with their wt counterparts.

EGF-R and MMP interplay in fibroblasts

The role of EGF-R in regulating MMP transcription and activity during cancer progression is well established (313, 314). However, the data on the role of EGF-R in fibroblasts during ECM remodelling is scarce. It is known that fibroblasts are involved in ECM remodelling such as wound contraction, which is a fundamental event in wound healing. Interestingly, wound healing is impaired in mice with a targeted deletion in the *MMP-3* (stromelysin-1) gene (100).

Therefore, we tested whether EGF-R depletion results in changes in ECM composition. Especially, we asked if the expression and activity of MMPs was impaired in EGF-R deficient fibroblasts.

To analyze MMP activity in cell culture, we performed a collagen contraction assay, which mimics wound contraction *in vitro*. In this assay the ECM surrounding cells is composed of type I collagen and the reduction in gel diameter correlates to MMP activity (315). Although the contraction of collagen gels is known to induce collagenase (MMP-1) and stromelysin-1 (MMP-3) activity (316), the ability of fibroblasts to contract collagen gels *in vitro* also depends on cytokines and cell adhesion receptors (such as integrins) interacting with type I collagen and laminin (317).

The collagen contraction assay demonstrated a major difference in the contraction efficiency between wt and EGF-R$^{-/-}$ MEFs. While the wt MEFs contracted the gels by 66% from the original diameter, the EGF-R$^{-/-}$ cells showed contraction of only 24%. Since this assay can be also used to study ECM reorganization in a simplified manner in wound healing, we performed additional experiments using EGF-R inhibitor. These analyses showed that EGF-R inhibitor significantly prevent contraction of wt MEFs. The data confirmed that collagen contraction of fibroblasts is primarily EGF-R-dependent. As MMP (pan)inhibitor Batimastat prevented collagen contraction of the wt MEFs, the results also indicate that the capacity of fibroblasts to contract collagen requires MMP activity.

To further determine which MMPs or ECM molecules are abnormally expressed in EGF-R$^{-/-}$ MEFs, we compared RNA expression of wt and EGF-R$^{-/-}$ MEFs using ECM and adhesion pathways-focused microarrays. The EGF-R-deficient cells showed significant downregulation in the mRNA levels of several MMPs in comparison to wt cells. In particular, the expression of MMP-2, -11, -13, and -17 mRNAs was suppressed. However, other ECM molecules were also shown to be diffentially expressed between wt and EGF-R$^{-/-}$ MEFs. These included tissue inhibitor of metalloproteinases-1 (TIMP-1) and TIMP-2. Furthermore, the wt MEFs expressed MMP-2, -3, and -14 mRNA levels constitutively and the MMP-3 mRNA levels

were further increased using EGF stimulus in contrast to undetectable MMP-3 and low MMP-2 and -14 mRNA levels in EGF-R$^{-/-}$ MEFs.

The activity of MMP-2 was measured using a zymogram assay. As a result, MMP2 activity was found to be significantly reduced in the EGF-R$^{-/-}$ MEFs in comparison to wt MEFs. The finding correlates with the low MMP-2 mRNA expression. Basal MMP-9 expression levels and activities in turn were under detection limit in both cell lines.

Our data show that EGF-R plays a crucial role in ECM modulation by controlling the expression and function of ECM components such as MMPs. This is in line with other studies. For example, matrix metalloproteinase driven EGF-R activation promotes human lung epithelial cell motility and invasion (318) and phenotype of ADAM$^{-/-}$ mice resembles that of EGF-R$^{-/-}$ mice (319). As ADAM17 is essential for the activation (ligand release) of EGF-R ligands *in vivo*, it may regulate autocrine signaling through the EGF-R creating a feedback mechanism (320).

AP-1 regulates MMP expression in fibroblasts

It has been shown that the activation of AP-1 plays an important role in TPA- and EGF-induced tumor promotion in both *in vitro* and *in vivo* conditions (280). As the transcription factor AP-1 has also been characterized as a direct regulator of MMP expression, we studied whether AP-1 activity is regulated by EGF-R dependent mechanism. It is known that AP-1 protein Fra-1 markedly stimulates MMP-2 and MMP-9 mRNA expression (318) but also other regulatory pathways such as NFκB (321), intracellular calcium (322), and nitric oxide (323) have been shown to regulate the expression of MMP-2 and MMP-9.

To study basal and EGF-induced mRNA levels of *c-jun* and *c-fos* in wt and EGF-R$^{-/-}$ MEFs we used Northern blotting technique. Exposure of cells to EGF caused a rapid induction of *c-jun* and *c-fos* mRNAs only in wt MEFs. Consistently, an induction of c-Fos and c-Jun protein levels was seen only in the wt MEFs.

These data demonstrate the importance of EGF-R in the regulation of AP-1 and MMP expression. Since it is known that MAP kinase pathways have a role in EGF induced AP-1 activation (240), we compared the activities of major pathways Erk1/2, Jnk1/2, and p38 in wt and EGF-R$^{-/-}$ MEFs. Our studies revealed that in wt MEFs, EGF rapidly phosphorylated EGF-R, Erks, and Jnks, whereas no p38 activity was detected. The kinetics of the activity of Erk and Jnk were consistent with the induction of MMP-3 expression. In contrast, induction of Erk and Jnk activities was impaired in response to EGF in the EGF-R$^{-/-}$ MEFs. Despite the differences in their responses to EGF, both wt and EGF-R$^{-/-}$ MEF cells had similar TPA or UV responses indicating that the MAPK kinase activities in the EGF-R$^{-/-}$ MEFs are intact. Furthermore, no changes in the expression levels of Erk1/2, Jnk1/2, or p38 were detected in these cell types.

Previous studies of MAPK cascades have revealed that the Erk subgroup of MAP kinases is activated most potently by mitogenic stimuli, such as EGF, whereas Jnk and p38 kinases are activated by other type of stimuli. Therefore, it is not surprising that EGF-R$^{-/-}$ MEFs have impaired Erk activity but no other substancial kinase defects reflected in UV stimulus responses.

EGF-R, MAPK, and AP-1 signaling regulate MMP expression and activity

In order to study the role of Erk, Jnk, and p38 pathways in the EGF-R-dependent regulation of MMP expression in MEFs, we used specific kinase inhibitors to block basal or EGF stimulated kinase activity [ZD1839 (Iressa®) for EGF-R, PD98059 for Mek (upstream kinase of Erk), SB203580 for p38 and SP600125 for Jnk] and analyzed the expression of MMP-3, -14, and -2 mRNAs with Northern blot technique. Again, when mRNA expressions of different MMPs were analyzed in wt and EGF-R$^{-/-}$ MEFs, striking differences were discovered between these cell lines. In wt MEFs, MMP-3 mRNA expression was downregulated in EGF-R inhibitor treated cells with or without EGF. EGF-induction of MMPs was mediated through Erk pathway, whereas Jnk inhibitor could only suppress the basal MMP-3 mRNA levels. Basal MMP-14 expression was dependent on EGF-R and Erk signaling, but p38 kinase inhibitor did not have any effect on the expression of MMPs. None of the inhibitors influenced the MMP-2 mRNA levels or MMP-2 activity. Basis for the impaired MMP-2 expression in the EGF-R$^{-/-}$ MEFs remain unclear. However, it is known that MMPs can activate each other by proteolysis. For example, MT1-MMP (MMP-14) has been shown to activate pro-MMP-2 and release the active MMP-2 in the extracellular space (324). This could be a plausible explanation to reduced MMP-2 activity detected in the zymogram assay. To summarize, the results show that the basal and inducible expression of MMP-3 and -14 are regulated by Erk- and Jnk-pathways, but the activities of these pathways or EGF-R are not required for the expression of MMP-2 in MEFs.

Subsequently, we studied the involment of these pathways in modulating the ECM. For this we used collagen contraction assay with the Erk, Jnk, and p38 inhibitors in order to study whether these inhibitors can prevent the collagen contraction of wt or EGF-R$^{-/-}$ MEFs. The addition of Mek and Jnk inhibitors resulted in partial inhibition of gel contraction, whereas p38αinhibitor did not have a major influence. However, this effect of Jnk inhibitor on gel contraction might partly result also from its negative affect on cell proliferation as this was observed in a proliferation assay. No suppression on proliferation of wt MEFs was observed with Erk inhibitor. Moreover, the MAPK inhibitors did not enhance the contraction defect in EGF-R$^{-/-}$ MEFs but Jnk inhibitor had a negative effect on proliferation. Together with the previous results obtained with EGF-R inhibitor, these results show that EGF-R-dependent activation of Erk pathway is required for collagen contraction in MEFs, whereas Jnk also regulates proliferation independently of EGF-R.

Next we analyzed the effect of EGF-R, Mek, p38, and Jnk inhibitors on the AP-1 subunits during EGF stimulus in wt MEFs. In line with previous data showing that c-Jun and c-Fos expression was downregulated in EGF-$R^{-/-}$ MEFs, EGF-R inhibitor prevented the induction of both *c-jun* and *c-fos* mRNA levels in response to EGF in wt MEFs. In addition, the Mek inhibitor decreased *c-fos*, but not *c-jun*, mRNA levels, whereas p38 and Jnk inhibitors did not have an effect. This type of Erk regulated c-Fos expression has been previously reported to be mediated by Elk-1 (242). However, the downregulation of AP-1 mRNA levels is not reflected at the protein level directly because also posttranscriptional and posttranslational modifications can regulate AP-1 expression (228, 236). The most important posttranslational modification, which increases protein stability of c-Jun or c-Fos, is JNK or Erk mediated phosphorylation. In detail, the Erks phosphorylate c-Fos serines 362 and 374 (234) and JNKs or Erks phosphorylate c-Jun serines 63 and 73 (133, 239). Indeed, when the protein levels were studied, both Mek and Jnk inhibitors decreased c-Jun protein levels as well as phosphorylation (Ser73), suggesting that c-Jun protein stability requires both Erk and Jnk activities. Interestingly, p38 kinase inhibitor did not inhibit but rather stimulated the expression of c-Fos and c-Jun. This might result from a compensating effect by Jnk pathway, because $p38^{-/-}$ MEFs are reported to show upregulation of the JNK-c-Jun pathway (148).

To further analyze the significance of AP-1 in regulating EGF-R-dependent (325) MMP expression in MEFs, we infected MEFs with an adenovirus construct producing TAM67, which a truncated, transactivation deficient form of c-Jun resulting in defective AP-1 activity. The expression of MMP-3 mRNA levels in the TAM67-infected wt MEFs showed a major supperession compared to control MEFs. Similar suppression was detected after EGF treatment. Moreover, we also analyzed the role of Mek/Erk in regulating MMP-3 expression using an adenovirus construct producing constitutively activated Mek infected into EGF-$R^{-/-}$ MEFs. This resulted in upregulation of MMP-3 expression in EGF-$R^{-/-}$ MEFs; however the expression level was still lower compared to wt MEFs. This is in line with with previous data showing that activated Mek1 stimulates MMP-3 expression in fibroblasts (325).

In summary, this study shows that EGF-R signaling is involved in regulating proliferation and MMP expression in MEFs and that the effect is mediated by MAPK and AP-1 activities. The results support previous studies related to MMP mediated ECM modulation during development, highlighting the importance of stromal derived EGF-R signaling (93). Indeed, our result show that the expression of distinct MMPs is dependent on EGF-R signaling but interstingly also proliferation shows EGF-R dependence, as the proliferation defect of MEFs at early passages suggests. However this proliferation defect can be compensated by other mechanisms at later passages, resulting in EGF-R independent growth. Similar results have been acquired in an *in vivo* study using EGF-$R^{-/-}$ mice in a wound healing assay (326). This demonstrates that EGF-R is required in both important steps in wound healing; proliferation of epithelial keratinocytes in addition to would contraction mediated by MMP activities, which indicate stromal-epithelial interaction. Furthermore, the expression of many MMPs

involved in ECM remodelling is AP-1 regulated suggesting that AP-1 has a functional role in this process [reviewed in (327)]. In our studies EGF-induced and AP-1 regulated MMP-3 showed to be an important regulator of collagen contraction. Consistent with this, MMP-3$^{-/-}$ MEFs show impaired collagen contraction (315) suggesting a connection between EGF-R signaling and MMP expression in fibroblasts.

To elucidate the potential effects of EGF-R activation in MEFs, we illustrated the consequential signaling events in Figure 7. In this figure, based on our data, EGF activates particularly Erk kinases but also Jnk kinases in wt MEFs, resulting in transcriptional activation of AP-1 and increased proliferation of MEFs and MMP upregulation. MMP expression correlates to AP-1 activity and, furthermore, the expression and activity of specific MMPs lead to collagen contraction. The lack of EGF-R in MEFs manifests itself also in reduced basal expression and activity levels of MMPs in addition to decreased rate of proliferation.

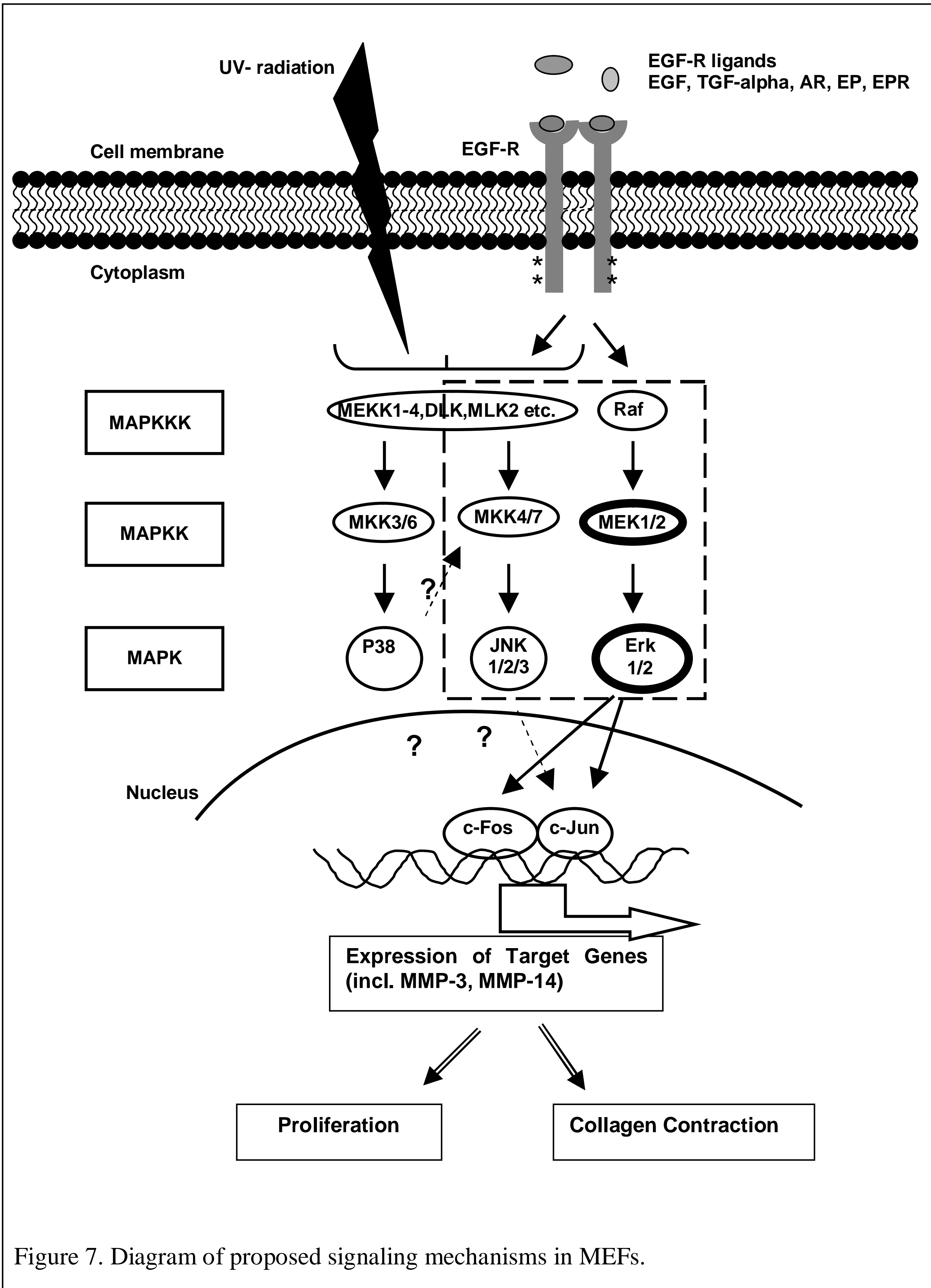

Figure 7. Diagram of proposed signaling mechanisms in MEFs.

7.2 Hydrocortisone and indomethacin negatively modulate EGF-R signaling in human fetal intestine (II)

EGF-R signaling in FHs74Int cells

EGF-R and its ligands are important for the maturation of the embryonic gut and also postnatally in maintaining mucosal integrity (39, 328-330). Also hydrocortisone (HC) has been shown to accelerate maturation of the intestinal tract, mainly the appearance of the brush-border membrane hydrolases and lipoprotein synthesis (331-333). On the otherhand, concomitant use of HC and indomethasine (Ind) on very-birth weight infants has been associated with increased risk of intestinal perforations (334-336). Since the mechanisms behind this adverse event are unknown, we asked whether they could include modulation of EGF-R signaling.

As a model system we used an immortalized, EGF-R expressing fetal intestinal cell line FHs74Int and various different kinase specific inhibitors. First, we analysed the patterns of EGF-R signaling in FHs74Int cells treated with EGF. Stimulation with EGF led to the activation of EGF-R, Erk, and Akt kinases as expected and the specific EGF-R inhibitor ZD1839 blocked EGF-R phosphorylation and down-regulated both the MEK and PI-3 kinase signaling pathways to basal levels. Jnk and p38 pathways were not affected by EGF.

Enterocyte proliferation

Next we sought to analyse the effects of EGF and HC on fetal enterocyte proliferation. In addition, the effects of Ind and prostaglandin PGE_2 were studied; Ind is a nonspecific inhibitor of COX, which catalyses the production of prostaglandin PGE_2. Following nine days of culture, the number of cells, treated with EGF, HC, PGE_2 or Ind, was calculated. EGF increased cell number by 80% in contrast to HC, Ind, or PGE_2, which had no significant impact on proliferation. EGF-induced cell proliferation was prevented by kinase specific inhibitors for EGF-R (ZD1839), Mek (PD98059), and Jnk (SP600125). We also analysed the effects of combination treatments. Interestingly, when HC was used with EGF, cell proliferation was increased by 162 ± 17% (d 9). This synergistic effect was blocked using ZD1839. Similar observation has been reported also previously suggesting that the proliferative effect of HC is based on increased expression of Erk (337) or membrane receptors and enhanced EGF-EGF-R binding [reviewed in (338)] leading to modified EGF-induction. However, HC alone or in combination with EGF had no affect on EGF-R, Erk, or Akt activities in the FHs74Int cells. Furthermore, we also analyzed whether the cell cycle regulators p21 and cyclin D1 were behind the proliferative synergism between EGF and HC. However, only EGF stimulus led to up-regulation of both p21 and cyclin D1 expression. This was in line with previous studies, in which activation of Erk is needed for the expression of cyclin D1 and p21 leading to cell cycle re-entry in CCL39 fibroblasts (339) or enterocytes (340).

In addition, our analysis comparing apoptosis between treated (EGF, PGE_2, HC or Ind) or untreated cells revealed distinct differences in the level of apoptosis only in the cells treated with the high concentration of Ind (100 μM).

Our results demonstrated that EGF signaling was important for the proliferation of intestinal epithelial cells. A similar result was obtained in a recent study in which another EGF-R specific inhibitor AG1478 significantly reduced the proliferation of cells within the epithelial cell layer of cultured gut explants (341). Indeed, this type of novel *in vivo* 3D models of the intestine are very useful to study the roles of EGF-R signaling and PGE_2 because the homeostasis of proliferation and apoptosis is created by interaction between different cell-types in the epithelium and stroma (77, 342). This is reflected by the known difficulties in intestinal epithelial cell growth and differentiation in *in vitro* models (343).

EGF-R signaling and COX-2 interplay in vivo

It is known that EGF-R signaling and cyclo-oxygenase-2 (COX-2) have also roles in the development and progression of intestinal polyps and cancers. Direct cross-talk between these signaling pathways has been reported: in LS-174T colorectal carcinoma cells PGE_2 induces the transactivation of EGF-R via Src kinase with a subsequent increase in migration and invasion (344). Furthermore, EGF enhances the expression of COX-2 in tracheal cell line EGV6 (345).

Therefore, we studied whether EGF-R and COX-2 co-localize also in the fetal intestine *in vivo*. To study this, we immunostained human fetal intestine with COX-2 or EGF-R antibodies. A strong COX-2 immunoreactivity was detected in the crypts in duodenum and jejunum and also weakly in ileum. Similar results were obtained from EGF-R immunostaining. To further analyze the EGF-R and COX-2 interplay, we compared EGF-R and COX-2 immunostaining between EGF-$R^{-/-}$ and wt mice fetal intestines because EGF-$R^{-/-}$ mice have similar perforations in their intestine as the indomethasine treated infants (39). Interestingly, COX-2 expression was mainly detected in the crypts of the wt mouse jejunum similar to the location of immunoreactive EGF-R. In contrast, COX-2 expression was greatly reduced in the EGF-$R^{-/-}$ mouse jejunum. These results were confirmed by Western blot analysis. According the analysis, EGF-$R^{-/-}$ fetal intestine contained less COX-2 protein than the wt intestine.

EGF-R signaling and COX-2 interplay in vitro

Next we studied by qt-PCR whether EGF upregulated COX-2 levels in FHs74Int cells and was this regulation mediated by the Erk and PI3K-Akt pathways. Indeed, EGF induced COX-2 mRNA levels by 25- to 30-fold ($p < 0.01$) and this effect was inhibited by EGF-R, Erk, and PI3-K inhibitors but also by HC ($p < 0.01$), which alone had no significant effect on COX-2 transcription.

These results were confirmed by ELISA, in which PGE_2 amounts were measured from cell culture media. Indeed, EGF increased PGE_2 production by 175% ($p < 0.01$) correlating with the increased COX-2 mRNA expression, and HC downregulated this by 40% ($p < 0.05$). Ind, EGF-R, MAPK, and PI3-K inhibitors suppressed the PGE_2 production to baseline as could be expected from the mRNA data.

Furthermore, even if PGE_2 had no significant effect on the growth of the FHsInt74 cells, it induced the activation of EGF-R, Erk, and Akt, all of which were inhibited by ZD1839. This verifies that transactivation of EGF-R by PGE_2 takes place also in human fetal intestine cells *in vitro*. These results suggest a dual role for EGF-R signaling in regulating COX-2 expression and on the other hand, mediating the PGE_2 stimulatory effect.

The effect of EGF and HC on migration of FHs74Int cells

Intestinal homeostasis is a balance between proliferation, apoptosis, and migration of enterocytes and defects in these events might lead to intestinal perforation. Several mitogenic stimuli have been reported to be involved in intestinal migration. These include PGE_2 (344) as well as EGF-R ligands EGF (329) and TGFα (346). Therefore we analyzed their role in FHs74Int cell migration using a Transwell migration assay. Since HC and Ind have been clinically associated with intestinal perforations; also they were included in the assay. The stimulation of FHs74Int cells with EGF, HC, and PGE_2 consistently ($p < 0.05$) increased migration. Furthermore, EGF and HC had a synergetic effect by doubling cell migration ($p < 0.05$). However, this increase in migration was not due to proliferation, because of the short time-line of the migration assay (36h) compared with the proliferation assay (9 days). These migratory stimuli were completely abolished by ZD1839 ($p < 0.05$) but also a slight inhibition of 15% by Ind was measured ($p < 0.05$). The increase in migration by EGF also correlated to EGF-induced transformation of FHs74Int cells to a fibroblast-like appearance. Interestingly, also the phenotypic change was most significant when EGF was combined with HC.

In conclusion, our data demonstrated the involvement of the EGF-R not only in the EGF-induced but also in the PGE_2-induced migration of fetal intestinal cells. Therefore, interrupting PGE_2 production by the COX inhibitor, Indomethacine, might imbalance the functional role of EGF-R in the intestinal homeostasis which could lead to intestinal perforations.

In response to mucosal injury, enterocytes migrate from the crypts to villus tip. This is followed by epithelial proliferation with new enterocytes arising from stem cells in the crypt region of the intestine. After intestinal damage, basolateral EGF-R becomes exposed to the luminal environment and may be accessed by endogenous luminal EGF-R ligands such as EGF (347). In fact, there are several cues that EGF-R signaling has a crucial role in regulating intestinal injury responses. Firstly, EGF-R null mice showed features of necrotizing enterocolitis (NEC) (39) and secondly, low salivary EGF levels have been shown to correlate

with the development of NEC (348). Furthermore, EGF is also needed for goblet cell proliferation (80), which protects the enterocytes from the luminal contact by secreting mucin. These results indicate a potential positive effect for EGF administration for preterm infants with the risk of intestinal perforations. Indeed, EGF has been shown to have a cytoprotective role in the intestine reducing apoptosis (79) and improving intestinal barrier function (80). In addition, in a recent prospective trial, recombinant human EGF was used to treat neonates suffering from NEC with a positive proliferative effect (349). However, the potential side-effects need to be dealt with i.e. overexpression of EGF has been reported to result in growth retardation (29) and, moreover, both EGF-R and its ligands have been associated with carcinogenesis. This is particularily true for carcinoma of the colon where also COX2 overexpression has been reported (350). Indeed, a non-selective COX inhibitor used with an EGF-R inhibitor reduced polyp formation in $APC^{Min/+}$ mice more effectively than either agent alone demonstrating EGF-R and COX-2 interplay (351).

Another interesting observation was that the addition of HC with EGF had a potentiating effect on both proliferation and migration, which indicate that HC might operate using EGF-regulated signaling pathways in FHs74Int cells. Similar observations have been made with ovarian epithelial cells (352, 353), skin fibroblasts, and keratinocytes (354). However, the effect of HC in cultured cells is not well defined. In preterm infants, however, glucocorticoids such as dexamethasone but not HC have also severe long-term side-effects affecting the brain and the heart (355). At the molecular level this difference might result from different affinities to mineralocorticoid receptor and glucocorticoid receptor as well as dexamethasone´s higher potency and longer duration of action. To conclude, both HC and Ind have capabilities to negatively interphere with the cellular behaviour of enterocytes in fetal intestine. Therefore, their concomitant admistration should always be critically evaluated.

Finally, a more complete understanding of how these elaborate cellular processes during a given stimulus lead to a desired response is needed. The diagram in Figure 8 illustrates signaling events, such as Erk and Akt pathways, which according to our studies; regulate cellular events such as proliferation, migration, transformation, and apoptosis in enterocytes. In addition, they regulate COX-2 expression, which consiquentially effects PGE_2 levels. PGE_2 has also the ability to transactivate EGF-R. HC stimulus enhances EGF-induced proliferation, migration, and transformation via yet an unknown mechanism.

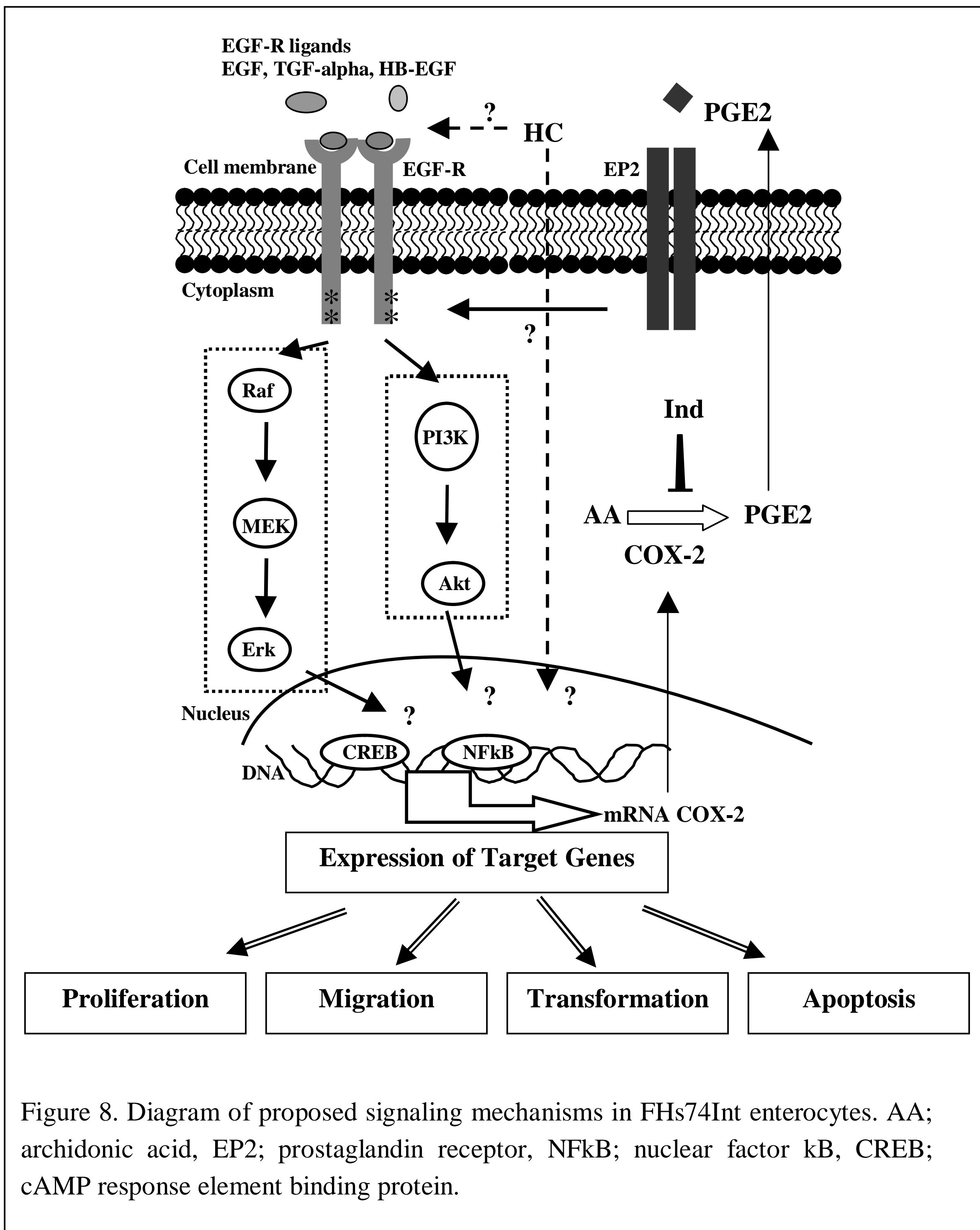

Figure 8. Diagram of proposed signaling mechanisms in FHs74Int enterocytes. AA; archidonic acid, EP2; prostaglandin receptor, NFkB; nuclear factor kB, CREB; cAMP response element binding protein.

7.3 Transcription factor AP-1 promotes cell growth and radioresistance in PC-3 prostate cancer cells (III)

Prostate carcinoma (PC) cells in the advanced state of disease can proliferate in the absence of androgen. One possible cause for this could be the upregulation of EGF-R expression during tumor progression (205), which might compensate for the loss of androgen mediated proliferation. This induction of EGF-R expression might be mediated by JKTBP1, a novel nuclear ribonucleoprotein (356). There exist several levels of crosstalk between androgen and EGF-R in cancer cells, which could take place during different stages of PC: EGF can induce the activation of the androgen receptor (357), androgen can mediate EGF-R up-regulation (358), and androgen receptor can interact with the EGF-R (359), all of which are associated with the MAPK and/or PI3K activities. However, the data covering EGF-R mediated MAPK and PI3K signaling in advanced androgen-independent PC is scarce. Because only few effective treatments exist for this stage of disease and the treatment efficacy should be improved, it is important to gather more information about the behaviour of PC and its dependence of EGF-R.

Therefore, our purpose was to determine the roles of EGF-R downstream pathways, focusing on MAPK and PI3K-Akt signaling and transcription factor AP-1 in cell growth and radiation response. As an experimental model we used PC-3 androgen independent prostate cancer cell line.

As indicated in earlier reports, EGF-R signaling pathways MAPK and PI3-K show increased activity in many cancers including PC, which affect cellular processes like growth and resistance to chemotherapy (360, 361). In addition, in advanced PC the tumor-suppressor gene *PTEN* that downregulates Akt activity (362) is frequently mutated and produce a non-functional form of PTEN. Moreover, the oncogene *ras* has the capacity to transform the prostate cancer cells towards androgen independent growth (363), emphasizing the importance of these pathways in PC.

In our studies the expression levels and activity (phosphorylation state) of EGF-R, MAPK, and PI3-K in PC-3 cells were assessed by immunoblot analysis. The results showed that the treatment of the cells with EGF-R specific inhibitor (ZD1839) not only abrogated baseline phosphorylation of EGF-R but also partially suppressed of Erk and Akt activities. MEK inhibitor (PD) in turn specifically suppressed Erk activity, whereas PI3K inhibitor (LY) not only fully prevented Akt phosphorylation but was also able to suppress EGF-R expression and phosphorylation, as well as Erk phosphorylation.

It is known that high number of EGF-R is associated with decreased responses to radiation therapy. In addition, inhibition of EGF-R enhances the efficiency of radiation therapy in murine carcinomas expressing high levels of EGF-R (364). We, therefore, performed a

clonogenic survival assay for PC-3 cells by treating them with increasing doses of ionizing radiation (0, 2, 4, and 8 Gy) with or without a 12h-pretreatment with kinase specific inhibitors. The treatments with EGF-R or MEK inhibitors showed no impact on the survival of PC-3 cells when incubation times were short (6 hours). In contrast, PI3K inhibitor sensitized PC-3 cells to radiation in a dose dependent manner. When the long term effect of inhibitors was analyzed, EGF-R inhibitor resulted in 60% decrease in colony formation in nonradiated control cells. A decrease in cell proliferation in the presence of PI3K inhibitor was also observed.

The results demonstrate the dependence of EGF-R and PI3-K signaling for proliferation of PC-3 cells. In addition, PI3K is involved in mediating resistance to radiation. In comparison, MEK/Erk activities were not essential for either cellular event. This is in line with *in vivo* studies done with prostate cancer xenografts, in which EGF-R inhibition caused a decrease in proliferation (365) but had no radiosensitizing effect (366). However, prostate cancer xenografts with high EGF-R expression were sensitized to radiation when dual EGF-R/VEGF-R inhibitor, AEE788 was used (367). The radiation response appears to be cell specific, since inhibition of EGF-R in gliomas radiosensitizes cells to apoptosis (368).

Cell cycle progression and apoptosis

In order to analyze the role of EGF-R signaling in proliferation and radiation responses of PC-3 cells, we next investigated the effect of radiation treatment on the cell cycle distribution and apoptosis. In the study, PI staining measuring DNA content and FACS were used. The treatment of the cells with PI3K inhibitor without radiation led to increased amount of the cells in the G1 phase and a concomitant decrease of the cells in the G2 and S phases. EGF-R or MEK inhibitors did not affect cell cycle distribution. Radiation of PC-3 cells in turn resulted in a significant increase of the amount of cells in the G2 phase. Treatment with PI3K inhibitor in combination with radiation further enhanced this effect.

The effect of kinase inhibitors and/or radiation on apoptotic rate of PC-3 cells was measured using Annexin V/PI staining. Treatments with EGF-R or PI3K inhibitors alone or in combination with radiation led to an increase in apoptosis. However, increased apoptosis after combination treatment was not synergistic. Radiation alone or the inhibition of MEK had no effect on apoptosis.

These results together with clonogenic survival assay demostrate that PI3K-Akt pathway plays a critical role in the radioresistance of PC-3 cells. The molecular effect of Akt on the cell cycle might be associated with Akt´s ability to inhibit cell cycle regulator $p27^{Kip1}$ directly or to downregulate its expression (369).

AP-1 is regulated by EGF-R in PC-3 cells

It has been shown that EGF-R can activate transcription factor AP-1 leading to the expression of AP-1 target genes followed by changes in cellular behaviour (311, 318). Therefore, we studied the roles of EGF-R mediated MAPK and PI3K cascades in PC-3 cells in order to pinpoint the contribution of each cascade in regulating AP-1.

To study possible regulatory function of AP-1 in prostate cancer cell growth and radiosensitivity, we examined expression levels of AP-1 subunits in PC-3 cells using immunoblotting. The expression of three Jun (c-Jun, JunB, JunD) and three Fos (c-Fos, Fra1, Fra2) subunits were assessed after 24h treatment with inhibitors. After exposure of cells to EGF-R inhibitor, the expression of c-Jun, Fra-1, and Fra-2 was slighly decreased in comparison to nontreated control cells. MEK inhibitor suppressed c-Fos levels strongly and Fra-2 levels only sightly. PI3K inhibition in turn led to a decrease in the expression of all the other AP-1 subunits except JunB, indicating that PI3K pathway activity is critical for the expression of AP-1 subunits. In contrast, the regulatory role of EGF-R and MEK might be less important.

Next, we performed a gel mobility shift assay to assess the AP-1 DNA-binding activity of the PC-3 cells. In untreated cells, a high constitutive AP-1 DNA-binding activity was detected. The EGF-R and PI3K inhibitors suppressed AP-1 DNA binding activity, while the MEK inhibitor had only a slight attenuating effect on DNA-binding. We further investigated the composition of active AP-1 DNA-binding complex using antibody perturbation assay. Based on these experiments, the DNA-binding complex was found to compose of all Jun proteins, Fra-1 and Fra-2, whereas c-Fos, FosB, or ATF-2 were not detected in the DNA binding complex.

To further study EGF-R, MAPK, and/or PI3K dependent regulation of AP-1, we examined the transactivation potential of AP-1 by quantifying AP-1 luciferase reporter activities in the inhibitor treated PC-3 cells. This analysis showed that all inhibitors were able to reduce the AP-1 promoter activity. Again, the most prominent inhibition was seen with the PI3K blockade. The results clearly demonstrate that a constitutively active AP-1 in PC-3 cells is composed of Fra-1, Fra-2, and JunD subunits and regulated by PI3K.

AP-1 subunits Fra-1, Fra-2, and JunD are required for proliferation and resistance to radiation

In order to analyze the role of AP-1 in PC-3 cell proliferation and radiation responses, the expression of Fra-1, Fra-2, and JunD was knocked down with siRNAs. The cells were transfected with *c-jun, junD, fra-1,* or *fra-2* siRNAs and the suppression of corresponding protein expression was analyzed by immunoblotting. *SiCONTROL* siRNA served as a

negative control. The analysis verified a marked downregulation of the AP-1 subunits in comparison to PC-3 cells transfected only with the control siRNA.

Next a colony forming assay was performed. Irridation (4Gy) of PC-3 cells led to approximately 25% cell survival. Downregulation of JunD, Fra1, or Fra2 expression with siRNAs sensitized PC-3 cells to irradiation by reducing the colony formation to 36%, 58%, and 78%, respectively, as compared to si*CONTROL* and *c-jun* siRNAs. Combination of *c-jun* siRNA with *fra-1* or *fra-2* siRNAs did not further enhance this effect, in comparison to each siRNAs alone. In contrast, combination of *junD* siRNA with *fra-1* or *fra-2* siRNAs resulted in a greater reduction in colony formation than either siRNA alone. These results clearly imply that JunD, Fra-1, and Fra-2 are needed for the survival of PC-3 cells.

The proliferation rate of PC-3 cells was also reduced after the suppression of JunD, Fra-1, and Fra-2 expression. In contrast, suppression of c-Jun had no significant effect on the proliferation in comparison to control cells. Therefore, these results together with our previous studies indicate that JunD, Fra-1, and Fra-2 are essential for both the radioresistance and proliferation of PC-3 cells.

We also determined the effect of a downregulation of AP-1 subunit on the cell-cycle distribution. The suppression of JunD, Fra-1, and Fra-2 by siRNAs alone or the combination of *junD* with *fra-1* or *fra-2* siRNAs led to the increased fraction of cells in G2 phase in comparison to cells transfected with si*CONTROL* and *c-jun* siRNAs. The results demonstrate that JunD, Fra-1, and Fra-2, but not c-Jun mediate radioresistance of the PC-3 cells.

Notably AP-1 has a unique role in prostate cancer, since it is a downstream effector of EGF-R and it can also regulate transcriptional activity of the androgen receptor (370). Indeed, in a recent *in vivo* study using *Nkx3.1;Pten* mice and gene expression profiling, AP-1 transcription factor subunits c-Jun and c-Fos were demonstrated to be downstream effectors of EGF-R and MAPK signaling in cancer progression. Moreover, c-Jun subunit was associated with disease recurrence (282). C-Jun has also been indicated to have a definite role in androgen-independent prostate cancer development (371).

In other respects, this type of the use of gene expression profiling has not only provided new insights into prostate cancer, but has also confirmed and deepened the knowledge of the known signaling mechanisms, such as the function of AP-1 in prostate cancer. Nonetheless, it must be kept in mind that the cell context and microenviroment signaling might also influence AP-1 subunit expression [reviewed in (372)]. This should to take into consideration when the *in vivo* and *in vitro* studies are interpreted.

In summary, our *in vitro* study provides cues to the role of AP-1 and its upstream effectors in growth and radiationresponse of PC-3 prostate cancer cells. The results suggest that the inhibitors of particular AP-1 subunits or Akt may be useful in designing new treatments

against advanced prostate cancer. Also EGF-R inhibition is advantageous if the expression levels are high, as might be the case in the metastatic stage of PC. In addition, the assessment of EGFR downstream signaling, such as the PTEN and Ras functionality, should also be considered.

In Figure 9 we summarize both the upstream signals that modulate AP-1 activity and the AP-1 components that mediate specific cellular resposes. EGF-induced EGF-R activation leads to Erk and Akt pathway activation, which are involved in regulating AP-1 expression and activity. Particularly Akt activity has a dramatic effect on proliferation, cell-cycle progression, and survival of PC-3 cells. AP-1 components JunD, Fra1, and Fra2 are the main downstream effectors of Erk and Akt pathways in PC-3 cells and their downregulation leads to sensitization of cells to irradiation, reduced proliferation, and changes in cell-cycle distribution.

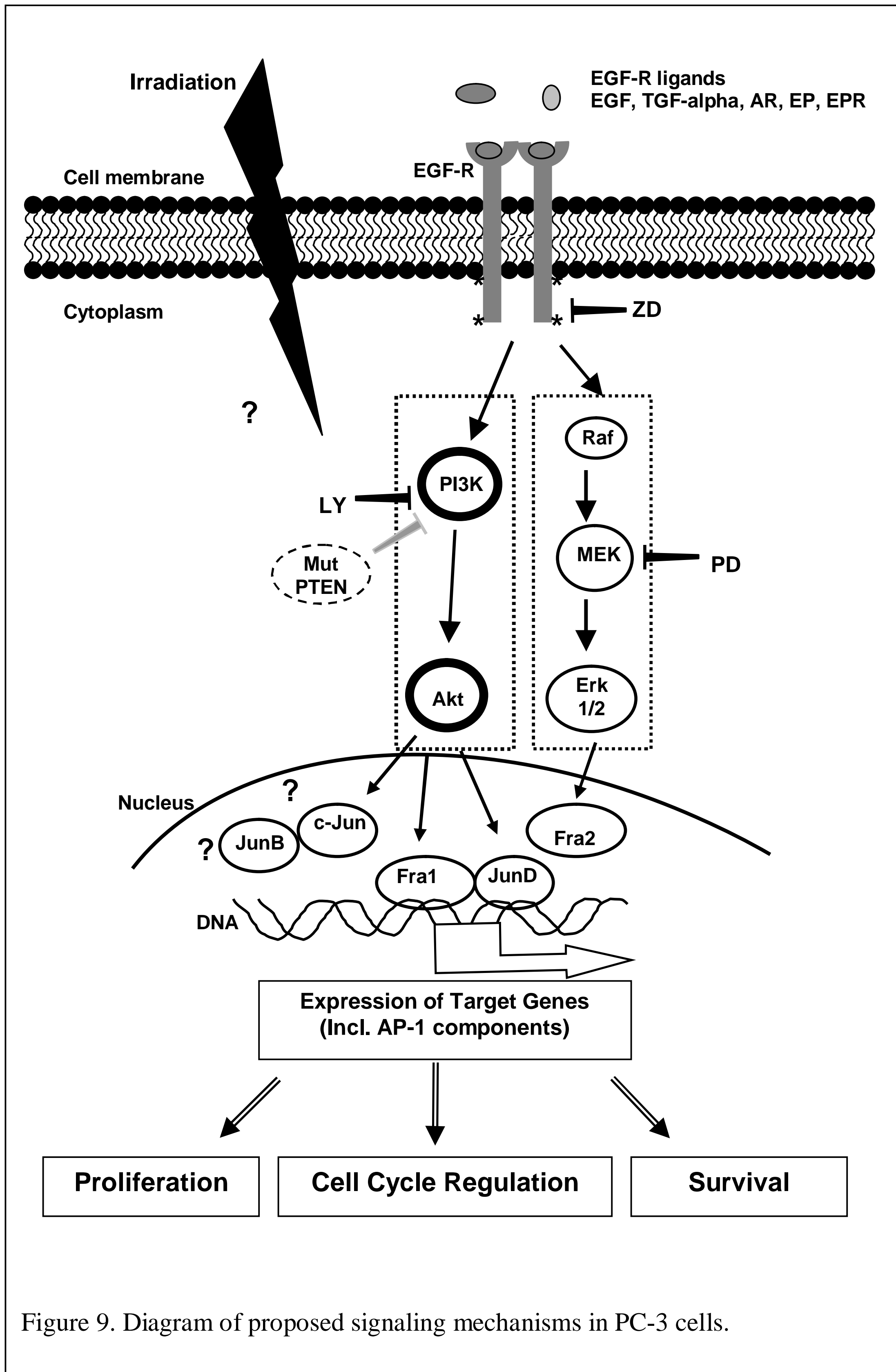

Figure 9. Diagram of proposed signaling mechanisms in PC-3 cells.

8. CONCLUDING REMARKS

The role of the EGF-R as a relay station between various inputs from the environment, cellular responses and other RTKs underlines the significance of this signal-transducing receptor. The inputs are relayed by specific messengers, the kinase pathways, to the nucleus, where the signal activates transcription factors leading to a change in a transcription of a desired target gene. The end product of this activity is therefore a specific protein, which carries out the desired cellular response.

There is substancial amount of evidence that the EGF-R regulated pathways are essential in normal cellular responses like proliferation, apoptosis and migration during development and adult life. However, when these pathways are deregulated they contribute to abnormal growth and tumorigenesis by accelerating proliferation, inhibiting apoptosis, sustaining angiogenesis, and invasion.

Our studies investigated the interplay between EGF-R network and AP-1 transcription factor. As a model system we used cell cultures of mouse embryonal fibroblasts (MEFs), human fetal enterocytes (FHs74Int) and human hormone-refractory prostate cancer cells (PC-3). They provided a mesenchymal, an epithelial and a malignant cell niche that partially differed in their response to the active EGF-R and AP-1 pathways. This resulted in changes in ECM remodelling, intestinal maturation, and resistance against radiotherapy.

The main conlusions made from these studies were:

1. Mesenchymal cells (fibroblasts) show coordinated EGF-R and AP-1 dependence in MMP expression and cell proliferation. Interestingly, Erk- and Jnk-pathways are required not only for EGF-induced expression but also for basal expression of MMP-3 and -14, which are AP-1 target genes.

2. Intestinal epithelial cells show EGF-R dependence in cell proliferation and an EGF-HC synergistic effect. COX inhibition by Ind also results in negative impact on enterocyte proliferation.

3. Prostate cancer cells are dependent on Akt pathway and AP-1 subunits JunD, Fra1, and Fra2 in their resistance against radiotherapy. EGF-R in turn is mainly required for cell proliferation.

Although, these cells express different levels of EGF-R on their surfaces, striking importance of this RTK is revealed. The results help us to understand the mechanisms of EGF-R and AP-1 signaling. In general, the proteins studied here are kinases or transcription factors, which function by activating other proteins or regulating gene transcription. The cellular outcome is dependent on this complicated network of the pathways, which converge at the transciption

factors. This should be considered in the design of future cancer therapies since deregulated activation of numerous signaling pathways is a key element of cancer. Therefore, this study along with those of others is beginning to define a new rational combinational inhibitor treatment strategy for cancer.

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10. REFERENCES

1. Khavari, T. A., and Rinn, J. (2007) *Cell. Cycle* 6, 2928-2931
2. de Larco, J. E., and Todaro, G. J. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 4001-4005
3. Sporn, M. B., and Roberts, A. B. (1985) *Nature* 313, 745-747
4. Hanahan, D., and Weinberg, R. A. (2000) *Cell* 100, 57-70
5. Carpenter, G., King, L.,Jr, and Cohen, S. (1979) *J. Biol. Chem.* 254, 4884-4891
6. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M. D. (1984) *Nature* 307, 521-527
7. Boerner, J. L., Danielsen, A., and Maihle, N. J. (2003) *Exp. Cell Res.* 284, 111-121
8. Zandi, R., Larsen, A. B., Andersen, P., Stockhausen, M. T., and Poulsen, H. S. (2007) *Cell. Signal.* 19, 2013-2023
9. Schneider, M. R., Werner, S., Paus, R., and Wolf, E. (2008) *Am. J. Pathol.* 173, 14-24
10. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) *EMBO J.* 19, 3159-3167
11. Yarden, Y. (2001) *Eur. J. Cancer* 37 Suppl 4, S3-8
12. Yarden, Y., and Sliwkowski, M. X. (2001) *Nat. Rev. Mol. Cell Biol.* 2, 127-137
13. Salomon, D. S., Brandt, R., Ciardiello, F., and Normanno, N. (1995) *Crit. Rev. Oncol. Hematol.* 19, 183-232
14. Normanno, N., De Luca, A., Maiello, M. R., Campiglio, M., Napolitano, M., Mancino, M., Carotenuto, A., Viglietto, G., and Menard, S. (2006) *J. Cell. Physiol.* 207, 420-427
15. Kumar, A., Petri, E. T., Halmos, B., and Boggon, T. J. (2008) *J. Clin. Oncol.* 26, 1742-1751
16. Beerli, R. R., and Hynes, N. E. (1996) *J. Biol. Chem.* 271, 6071-6076
17. French, A. R., Tadaki, D. K., Niyogi, S. K., and Lauffenburger, D. A. (1995) *J. Biol. Chem.* 270, 4334-4340
18. Waterman, H., Sabanai, I., Geiger, B., and Yarden, Y. (1998) *J. Biol. Chem.* 273, 13819-13827
19. Lee, D. C., Fenton, S. E., Berkowitz, E. A., and Hissong, M. A. (1995) *Pharmacol. Rev.* 47, 51-85
20. COHEN, S. (1962) *J. Biol. Chem.* 237, 1555-1562
21. Massague, J., and Pandiella, A. (1993) *Annu. Rev. Biochem.* 62, 515-541
22. Sahin, U., and Blobel, C. P. (2007) *FEBS Lett.* 581, 41-44
23. Sahin, U., Weskamp, G., Kelly, K., Zhou, H. M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P., and Blobel, C. P. (2004) *J. Cell Biol.* 164, 769-779
24. Carraway, K. L.,3rd, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M., and Lai, C. (1997) *Nature* 387, 512-516
25. Strachan, L., Murison, J. G., Prestidge, R. L., Sleeman, M. A., Watson, J. D., and Kumble, K. D. (2001) *J. Biol. Chem.* 276, 18265-18271
26. Elenius, K., Corfas, G., Paul, S., Choi, C. J., Rio, C., Plowman, G. D., and Klagsbrun, M. (1997) *J. Biol. Chem.* 272, 26761-26768
27. Brachmann, R., Lindquist, P. B., Nagashima, M., Kohr, W., Lipari, T., Napier, M., and Derynck, R. (1989) *Cell* 56, 691-700
28. De Luca, A., Carotenuto, A., Rachiglio, A., Gallo, M., Maiello, M. R., Aldinucci, D., Pinto, A., and Normanno, N. (2008) *J. Cell. Physiol.* 214, 559-567
29. Chan, S. Y., and Wong, R. W. (2000) *J. Biol. Chem.* 275, 38693-38698
30. Provenzano, A. P., Besner, G. E., James, P. F., and Harding, P. A. (2005) *Growth Factors* 23, 19-31
31. Schneider, M. R., Dahlhoff, M., Herbach, N., Renner-Mueller, I., Dalke, C., Puk, O., Graw, J., Wanke, R., and Wolf, E. (2005) *Endocrinology* 146, 5237-5246
32. Chernausek, S. D., Dickson, B. A., Smith, E. P., and Hoath, S. B. (1991) *Am. J. Physiol.* 260, E416-21
33. Serrero, G., and Mills, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3912-3916
34. Chien, H. H., Lin, W. L., and Cho, M. I. (2000) *Calcif. Tissue Int.* 67, 141-150
35. Troyer, K. L., Luetteke, N. C., Saxon, M. L., Qiu, T. H., Xian, C. J., and Lee, D. C. (2001) *Gastroenterology* 121, 68-78

36. Raab, G., and Klagsbrun, M. (1997) *Biochim. Biophys. Acta* 1333, F179-99
37. Paria, B. C., Das, S. K., Andrews, G. K., and Dey, S. K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 55-59
38. Miettinen, P. J., Huotari, M., Koivisto, T., Ustinov, J., Palgi, J., Rasilainen, S., Lehtonen, E., Keski-Oja, J., and Otonkoski, T. (2000) *Development* 127, 2617-2627
39. Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z., and Derynck, R. (1995) *Nature* 376, 337-341
40. Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., and Harris, R. C. (1995) *Science* 269, 230-234
41. Sibilia, M., and Wagner, E. F. (1995) *Science* 269, 234-238
42. Miettinen, P. J., Chin, J. R., Shum, L., Slavkin, H. C., Shuler, C. F., Derynck, R., and Werb, Z. (1999) *Nat. Genet.* 22, 69-73
43. Luetteke, N. C., Phillips, H. K., Qiu, T. H., Copeland, N. G., Earp, H. S., Jenkins, N. A., and Lee, D. C. (1994) *Genes Dev.* 8, 399-413
44. Park, S. K., Miller, R., Krane, I., and Vartanian, T. (2001) *J. Cell Biol.* 154, 1245-1258
45. Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995) *Nature* 378, 394-398
46. Leu, M., Bellmunt, E., Schwander, M., Farinas, I., Brenner, H. R., and Muller, U. (2003) *Development* 130, 2291-2301
47. Erickson, S. L., O'Shea, K. S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L. H., and Moore, M. W. (1997) *Development* 124, 4999-5011
48. Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R., and Birchmeier, C. (1997) *Nature* 389, 725-730
49. Golub, M. S., Germann, S. L., and Lloyd, K. C. (2004) *Behav. Brain Res.* 153, 159-170
50. Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995) *Nature* 378, 390-394
51. Schroeder, J. A., and Lee, D. C. (1998) *Cell Growth Differ.* 9, 451-464
52. Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A., and Lee, D. C. (1999) *Development* 126, 2739-2750
53. Kajikawa, K., Yasui, W., Sumiyoshi, H., Yoshida, K., Nakayama, H., Ayhan, A., Yokozaki, H., Ito, H., and Tahara, E. (1991) *Virchows Arch. A Pathol. Anat. Histopathol.* 418, 27-32
54. Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O., and Lee, D. C. (1993) *Cell* 73, 263-278
55. Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. L., and Dunn, A. R. (1993) *Cell* 73, 249-261
56. Dominey, A. M., Wang, X. J., King, L. E.,Jr, Nanney, L. B., Gagne, T. A., Sellheyer, K., Bundman, D. S., Longley, M. A., Rothnagel, J. A., and Greenhalgh, D. A. (1993) *Cell Growth Differ.* 4, 1071-1082
57. Iwamoto, R., Yamazaki, S., Asakura, M. *et al.* (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 3221-3226
58. Paria, B. C., Elenius, K., Klagsbrun, M., and Dey, S. K. (1999) *Development* 126, 1997-2005
59. Shirakata, Y., Kimura, R., Nanba, D. *et al.* (2005) *J. Cell. Sci.* 118, 2363-2370
60. Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C., and Klagsbrun, M. (1991) *Science* 251, 936-939
61. Naglich, J. G., Metherall, J. E., Russell, D. W., and Eidels, L. (1992) *Cell* 69, 1051-1061
62. Dominey, A. M., Wang, X. J., King, L. E.,Jr, Nanney, L. B., Gagne, T. A., Sellheyer, K., Bundman, D. S., Longley, M. A., Rothnagel, J. A., and Greenhalgh, D. A. (1993) *Cell Growth Differ.* 4, 1071-1082
63. Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C., and Lee, D. C. (2003) *EMBO J.* 22, 2704-2716
64. Torring, N., Jorgensen, P. E., Sorensen, B. S., and Nexo, E. (2000) *Anticancer Res.* 20, 91-95
65. Toyoda, H., Komurasaki, T., Ikeda, Y., Yoshimoto, M., and Morimoto, S. (1995) *FEBS Lett.* 377, 403-407
66. Toyoda, H., Komurasaki, T., Uchida, D., and Morimoto, S. (1997) *Biochem. J.* 326 (Pt 1), 69-75
67. Kochupurakkal, B. S., Harari, D., Di-Segni, A. *et al.* (2005) *J. Biol. Chem.* 280, 8503-8512

68. Shirakata, Y., Komurasaki, T., Toyoda, H., Hanakawa, Y., Yamasaki, K., Tokumaru, S., Sayama, K., and Hashimoto, K. (2000) *J. Biol. Chem.* 275, 5748-5753
69. Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A., and Lee, D. C. (1999) *Development* 126, 2739-2750
70. Hall, P. A., Coates, P. J., Ansari, B., and Hopwood, D. (1994) *J. Cell. Sci.* 107 (Pt 12), 3569-3577
71. Hermiston, M. L., Wong, M. H., and Gordon, J. I. (1996) *Genes Dev.* 10, 985-996
72. Kedinger, M., Duluc, I., Fritsch, C., Lorentz, O., Plateroti, M., and Freund, J. N. (1998) *Ann. N. Y. Acad. Sci.* 859, 1-17
73. Scheving, L. A., Shiurba, R. A., Nguyen, T. D., and Gray, G. M. (1989) *J. Biol. Chem.* 264, 1735-1741
74. Montaner, B., and Perez-Tomas, R. (1999) *Cell Biol. Int.* 23, 475-479
75. Miettinen, P. J., Perheentupa, J., Otonkoski, T., Lahteenmaki, A., and Panula, P. (1989) *Pediatr. Res.* 26, 25-30
76. Troyer, K. L., and Lee, D. C. (2001) *J. Mammary Gland Biol. Neoplasia* 6, 7-21
77. Sukhotnik, I., Shteinberg, D., Ben Lulu, S., Bashenko, Y., Mogilner, J. G., Ure, B. M., Shaoul, R., and Coran, A. G. (2008) *Apoptosis* 13, 1344-1355
78. Shin, C. E., Falcone, R. A.,Jr, Stuart, L., Erwin, C. R., and Warner, B. W. (2000) *J. Pediatr. Surg.* 35, 173-6; discussion 177
79. Clark, J. A., Lane, R. H., Maclennan, N. K., Holubec, H., Dvorakova, K., Halpern, M. D., Williams, C. S., Payne, C. M., and Dvorak, B. (2005) *Am. J. Physiol. Gastrointest. Liver Physiol.* 288, G755-62
80. Clark, J. A., Doelle, S. M., Halpern, M. D., Saunders, T. A., Holubec, H., Dvorak, K., Boitano, S. A., and Dvorak, B. (2006) *Am. J. Physiol. Gastrointest. Liver Physiol.* 291, G938-49
81. Okada, M., Ohmura, E., Kamiya, Y., Murakami, H., Onoda, N., Iwashita, M., Wakai, K., Tsushima, T., and Shizume, K. (1991) *Life Sci.* 48, 1151-1156
82. Wong, B. C., Wang, W. P., So, W. H., Shin, V. Y., Wong, W. M., Fung, F. M., Liu, E. S., Hiu, W. M., Lam, S. K., and Cho, C. H. (2001) *Aliment. Pharmacol. Ther.* 15, 1459-1465
83. Sarosiek, J., Jensen, R. T., Maton, P. N., Peura, D. A., Harlow, D., Feng, T., McCallum, R. W., and Pisegna, J. R. (2000) *Am. J. Gastroenterol.* 95, 1158-1165
84. Wang, L., Lucey, M. R., Fras, A. M., Wilson, E. J., and Del Valle, J. (1993) *J. Pharmacol. Exp. Ther.* 265, 308-313
85. Janssen, K. P., el-Marjou, F., Pinto, D., Sastre, X., Rouillard, D., Fouquet, C., Soussi, T., Louvard, D., and Robine, S. (2002) *Gastroenterology* 123, 492-504
86. Schmelz, E. M., Xu, H., Sengupta, R., Du, J., Banerjee, S., Sarkar, F. H., Rishi, A. K., and Majumdar, A. P. (2007) *Cancer Res.* 67, 5389-5396
87. Cunningham, M. P., Essapen, S., Thomas, H., Green, M., Lovell, D. P., Topham, C., Marks, C., and Modjtahedi, H. (2005) *Int. J. Oncol.* 27, 317-325
88. Burdick, J. S., Chung, E., Tanner, G., Sun, M., Paciga, J. E., Cheng, J. Q., Washington, K., Goldenring, J. R., and Coffey, R. J. (2000) *N. Engl. J. Med.* 343, 1697-1701
89. Lee, D., Pearsall, R. S., Das, S., Dey, S. K., Godfrey, V. L., and Threadgill, D. W. (2004) *Mol. Cell. Biol.* 24, 8907-8916
90. Aszodi, A., Legate, K. R., Nakchbandi, I., and Fassler, R. (2006) *Annu. Rev. Cell Dev. Biol.* 22, 591-621
91. Kheradmand, F., Rishi, K., and Werb, Z. (2002) *J. Cell. Sci.* 115, 839-848
92. Sternlicht, M. D., Sunnarborg, S. W., Kouros-Mehr, H., Yu, Y., Lee, D. C., and Werb, Z. (2005) *Development* 132, 3923-3933
93. Wiesen, J. F., Young, P., Werb, Z., and Cunha, G. R. (1999) *Development* 126, 335-344
94. Hom, Y. K., Young, P., Wiesen, J. F., Miettinen, P. J., Derynck, R., Werb, Z., and Cunha, G. R. (1998) *Endocrinology* 139, 913-921
95. Byrne, R. L., Leung, H., and Neal, D. E. (1996) *Br. J. Urol.* 77, 627-633

96. Malfait, F., Coucke, P., Symoens, S., Loeys, B., Nuytinck, L., and De Paepe, A. (2005) *Hum. Mutat.* 25, 28-37
97. Pepin, M., Schwarze, U., Superti-Furga, A., and Byers, P. H. (2000) *N. Engl. J. Med.* 342, 673-680
98. Viglio, S., Zoppi, N., Sangalli, A., Gallanti, A., Barlati, S., Mottes, M., Colombi, M., and Valli, M. (2008) *J. Invest. Dermatol.* 128, 1915-1919
99. Zoppi, N., Barlati, S., and Colombi, M. (2008) *Biochim. Biophys. Acta* 1783, 1177-1188
100. Bullard, K. M., Lund, L., Mudgett, J. S., Mellin, T. N., Hunt, T. K., Murphy, B., Ronan, J., Werb, Z., and Banda, M. J. (1999) *Ann. Surg.* 230, 260-265
101. Radisky, D., Hagios, C., and Bissell, M. J. (2001) *Semin. Cancer Biol.* 11, 87-95
102. Maffini, M. V., Soto, A. M., Calabro, J. M., Ucci, A. A., and Sonnenschein, C. (2004) *J. Cell. Sci.* 117, 1495-1502
103. Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1980) *Nature* 284, 67-68
104. Liotta, L. A., and Stetler-Stevenson, W. G. (1991) *Cancer Res.* 51, 5054s-5059s
105. Guo, X., Oshima, H., Kitmura, T., Taketo, M. M., and Oshima, M. (2008) *J. Biol. Chem.* 283, 19864-19871
106. Blume-Jensen, P., and Hunter, T. (2001) *Nature* 411, 355-365
107. Sweeney, S. E., and Firestein, G. S. (2007) *Nat. Clin. Pract. Rheumatol.* 3, 651-660
108. Katz, M., Amit, I., and Yarden, Y. (2007) *Biochim. Biophys. Acta* 1773, 1161-1176
109. Dawson, J. P., Berger, M. B., Lin, C. C., Schlessinger, J., Lemmon, M. A., and Ferguson, K. M. (2005) *Mol. Cell. Biol.* 25, 7734-7742
110. Ozcan, F., Klein, P., Lemmon, M. A., Lax, I., and Schlessinger, J. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 5735-5740
111. Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M., and Yarden, Y. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 4995-5000
112. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L.,3rd. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 8132-8136
113. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997) *EMBO J.* 16, 1647-1655
114. Biscardi, J. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. (1999) *J. Biol. Chem.* 274, 8335-8343
115. Grovdal, L. M., Stang, E., Sorkin, A., and Madshus, I. H. (2004) *Exp. Cell Res.* 300, 388-395
116. Buday, L., and Downward, J. (1993) *Cell* 73, 611-620
117. Keilhack, H., Tenev, T., Nyakatura, E., Godovac-Zimmermann, J., Nielsen, L., Seedorf, K., and Bohmer, F. D. (1998) *J. Biol. Chem.* 273, 24839-24846
118. Wiley, H. S., and Burke, P. M. (2001) *Traffic* 2, 12-18
119. Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. (2002) *EMBO J.* 21, 303-313
120. Lax, I., Wong, A., Lamothe, B., Lee, A., Frost, A., Hawes, J., and Schlessinger, J. (2002) *Mol. Cell* 10, 709-719
121. Berset, T. A., Hoier, E. F., and Hajnal, A. (2005) *Genes Dev.* 19, 1328-1340
122. Jordan, J. D., Landau, E. M., and Iyengar, R. (2000) *Cell* 103, 193-200
123. Chang, L., and Karin, M. (2001) *Nature* 410, 37-40
124. Kyriakis, J. M., and Avruch, J. (2001) *Physiol. Rev.* 81, 807-869
125. Whitmarsh, A. J. (2007) *Biochim. Biophys. Acta* 1773, 1285-1298
126. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) *Adv. Cancer Res.* 74, 49-139
127. Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999) *Science* 286, 1374-1377
128. Saba-El-Leil, M. K., Vella, F. D., Vernay, B., Voisin, L., Chen, L., Labrecque, N., Ang, S. L., and Meloche, S. (2003) *EMBO Rep.* 4, 964-968

129. Yao, Y., Li, W., Wu, J., Germann, U. A., Su, M. S., Kuida, K., and Boucher, D. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 12759-12764
130. Hatano, N., Mori, Y., Oh-hora, M., Kosugi, A., Fujikawa, T., Nakai, N., Niwa, H., Miyazaki, J., Hamaoka, T., and Ogata, M. (2003) *Genes Cells* 8, 847-856
131. Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) *Cell* 68, 1041-1050
132. Yoon, S., and Seger, R. (2006) *Growth Factors* 24, 21-44
133. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* 7, 2135-2148
134. Behrens, A., Sibilia, M., and Wagner, E. F. (1999) *Nat. Genet.* 21, 326-329
135. Behrens, A., Jochum, W., Sibilia, M., and Wagner, E. F. (2000) *Oncogene* 19, 2657-2663
136. Leppa, S., and Bohmann, D. (1999) *Oncogene* 18, 6158-6162
137. Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) *Science* 282, 2092-2095
138. Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., Davis, R. J., Rincon, M., and Flavell, R. A. (1998) *Immunity* 9, 575-585
139. Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P., and Flavell, R. A. (1999) *Neuron* 22, 667-676
140. Kuan, C. Y., Whitmarsh, A. J., Yang, D. D. *et al.* (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 15184-15189
141. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) *Nature* 389, 865-870
142. Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. (2005) *Nat. Immunol.* 6, 1087-1095
143. Wan, Y. Y., Chi, H., Xie, M., Schneider, M. D., and Flavell, R. A. (2006) *Nat. Immunol.* 7, 851-858
144. Qin, J., Yao, J., Cui, G. *et al.* (2006) *J. Biol. Chem.* 281, 21013-21021
145. Mizukami, Y., Yoshioka, K., Morimoto, S., and Yoshida, K. (1997) *J. Biol. Chem.* 272, 16657-16662
146. Elmore, S. (2007) *Toxicol. Pathol.* 35, 495-516
147. Mudgett, J. S., Ding, J., Guh-Siesel, L., Chartrain, N. A., Yang, L., Gopal, S., and Shen, M. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 10454-10459
148. Hui, L., Bakiri, L., Mairhorfer, A., Schweifer, N., Haslinger, C., Kenner, L., Komnenovic, V., Scheuch, H., Beug, H., and Wagner, E. F. (2007) *Nat. Genet.* 39, 741-749
149. Ventura, J. J., Tenbaum, S., Perdiguero, E., Huth, M., Guerra, C., Barbacid, M., Pasparakis, M., and Nebreda, A. R. (2007) *Nat. Genet.* 39, 750-758
150. Hui, L., Bakiri, L., Stepniak, E., and Wagner, E. F. (2007) *Cell. Cycle* 6, 2429-2433
151. Hu, M. C., Wang, Y. P., Mikhail, A., Qiu, W. R., and Tan, T. H. (1999) *J. Biol. Chem.* 274, 7095-7102
152. Bulavin, D. V., and Fornace, A. J.,Jr. (2004) *Adv. Cancer Res.* 92, 95-118
153. Roduit, R., and Schorderet, D. F. (2008) *Apoptosis* 13, 343-353
154. Kim, M. J., Byun, J. Y., Yun, C. H., Park, I. C., Lee, K. H., and Lee, S. J. (2008) *Mol. Cancer. Res.* 6, 1872-1880
155. Cao, C., Lu, S., Jiang, Q. *et al.* (2008) *Cell. Signal.* 20, 1830-1838
156. Yang, S. H., Sharrocks, A. D., and Whitmarsh, A. J. (2003) *Gene* 320, 3-21
157. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* 81, 727-736
158. Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A., and Sugimoto, C. (2003) *Oncogene* 22, 8983-8998
159. Manning, B. D., and Cantley, L. C. (2007) *Cell* 129, 1261-1274
160. Yang, Z. Z., Tschopp, O., Hemmings-Mieszczak, M., Feng, J., Brodbeck, D., Perentes, E., and Hemmings, B. A. (2003) *J. Biol. Chem.* 278, 32124-32131
161. Chen, W. S., Xu, P. Z., Gottlob, K. *et al.* (2001) *Genes Dev.* 15, 2203-2208
162. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B.,3rd, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) *Science* 292, 1728-1731

163. Easton, R. M., Cho, H., Roovers, K. *et al.* (2005) *Mol. Cell. Biol.* 25, 1869-1878
164. Engelman, J. A., Janne, P. A., Mermel, C., Pearlberg, J., Mukohara, T., Fleet, C., Cichowski, K., Johnson, B. E., and Cantley, L. C. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 3788-3793
165. Sithanandam, G., Smith, G. T., Fields, J. R., Fornwald, L. W., and Anderson, L. M. (2005) *Am. J. Respir. Cell Mol. Biol.* 33, 490-499
166. Hennessy, B. T., Smith, D. L., Ram, P. T., Lu, Y., and Mills, G. B. (2005) *Nat. Rev. Drug Discov.* 4, 988-1004
167. Wendel, H. G., De Stanchina, E., Fridman, J. S., Malina, A., Ray, S., Kogan, S., Cordon-Cardo, C., Pelletier, J., and Lowe, S. W. (2004) *Nature* 428, 332-337
168. Olayioye, M. A., Beuvink, I., Horsch, K., Daly, J. M., and Hynes, N. E. (1999) *J. Biol. Chem.* 274, 17209-17218
169. Bowman, T., Garcia, R., Turkson, J., and Jove, R. (2000) *Oncogene* 19, 2474-2488
170. Oliva, J. L., Griner, E. M., and Kazanietz, M. G. (2005) *Growth Factors* 23, 245-252
171. Jan, H. J., Lee, C. C., Lin, Y. M., Lai, J. H., Wei, H. W., and Lee, H. M. (2009) *Cancer Lett.*
172. Sansal, I., and Sellers, W. R. (2004) *J. Clin. Oncol.* 22, 2954-2963
173. Whitmarsh, A. J. (2006) *Biochem. Soc. Trans.* 34, 828-832
174. Chandramouli, S., Yu, C. Y., Yusoff, P., Lao, D. H., Leong, H. F., Mizuno, K., and Guy, G. R. (2008) *J. Biol. Chem.* 283, 1679-1691
175. Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D., and Glass, D. J. (1999) *Science* 286, 1738-1741
176. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) *Mol. Cell. Biol.* 21, 893-901
177. Gschwind, A., Prenzel, N., and Ullrich, A. (2002) *Cancer Res.* 62, 6329-6336
178. Jo, M., Stolz, D. B., Esplen, J. E., Dorko, K., Michalopoulos, G. K., and Strom, S. C. (2000) *J. Biol. Chem.* 275, 8806-8811
179. Wang, L., Cummings, R., Zhao, Y., Kazlauskas, A., Sham, J. K., Morris, A., Georas, S., Brindley, D. N., and Natarajan, V. (2003) *J. Biol. Chem.* 278, 39931-39940
180. Carpenter, G. (2000) *Sci. STKE* 2000, PE1
181. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* 402, 884-888
182. Eisenberg, A., Biener, E., Charlier, M., Krishnan, R. V., Djiane, J., Herman, B., and Gertler, A. (2004) *FEBS Lett.* 565, 139-142
183. Civenni, G., Holbro, T., and Hynes, N. E. (2003) *EMBO Rep.* 4, 166-171
184. Slomiany, B. L., and Slomiany, A. (2004) *Inflammopharmacology* 12, 233-245
185. Yamauchi, T., Yamauchi, N., Ueki, K. *et al.* (2000) *J. Biol. Chem.* 275, 33937-33944
186. Saito, Y., Haendeler, J., Hojo, Y., Yamamoto, K., and Berk, B. C. (2001) *Mol. Cell. Biol.* 21, 6387-6394
187. Bandyopadhyay, D., Mandal, M., Adam, L., Mendelsohn, J., and Kumar, R. (1998) *J. Biol. Chem.* 273, 1568-1573
188. Huang, S. M., and Harari, P. M. (2000) *Clin. Cancer Res.* 6, 2166-2174
189. Dittmann, K., Mayer, C., Fehrenbacher, B., Schaller, M., Raju, U., Milas, L., Chen, D. J., Kehlbach, R., and Rodemann, H. P. (2005) *J. Biol. Chem.* 280, 31182-31189
190. Normanno, N., Bianco, C., De Luca, A., Maiello, M. R., and Salomon, D. S. (2003) *Endocr. Relat. Cancer* 10, 1-21
191. DiGiovanna, M. P., Stern, D. F., Edgerton, S. M., Whalen, S. G., Moore, D.,2nd, and Thor, A. D. (2005) *J. Clin. Oncol.* 23, 1152-1160
192. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) *Mol. Cell. Biol.* 16, 5276-5287
193. Normanno, N., Bianco, C., De Luca, A., and Salomon, D. S. (2001) *Front. Biosci.* 6, D685-707
194. Mendelsohn, J., and Baselga, J. (2006) *Semin. Oncol.* 33, 369-385

195. Murray, S., Dahabreh, I. J., Linardou, H., Manoloukos, M., Bafaloukos, D., and Kosmidis, P. (2008) *J. Thorac. Oncol.* 3, 832-839
196. Fukui, T., and Mitsudomi, T. (2008) *Gen. Thorac. Cardiovasc. Surg.* 56, 97-103
197. Pedersen, M. W., Meltorn, M., Damstrup, L., and Poulsen, H. S. (2001) *Ann. Oncol.* 12, 745-760
198. Worthylake, R., Opresko, L. K., and Wiley, H. S. (1999) *J. Biol. Chem.* 274, 8865-8874
199. Kuan, C. T., Wikstrand, C. J., and Bigner, D. D. (2000) *Brain Tumor Pathol.* 17, 71-78
200. Abd El-Rehim, D. M., Pinder, S. E., Paish, C. E., Bell, J. A., Rampaul, R. S., Blamey, R. W., Robertson, J. F., Nicholson, R. I., and Ellis, I. O. (2004) *Br. J. Cancer* 91, 1532-1542
201. Lee, J. C., Wang, S. T., Chow, N. H., and Yang, H. B. (2002) *Eur. J. Cancer* 38, 1065-1071
202. Baselga, J. (2002) *Oncologist* 7 Suppl 4, 2-8
203. Boyle, P., and Ferlay, J. (2005) *Ann. Oncol.* 16, 481-488
204. Parkin, D. M., Bray, F., Ferlay, J., and Pisani, P. (2005) *CA Cancer. J. Clin.* 55, 74-108
205. Di Lorenzo, G., Tortora, G., D'Armiento, F. P. *et al.* (2002) *Clin. Cancer Res.* 8, 3438-3444
206. Nicholson, R. I., Gee, J. M., and Harper, M. E. (2001) *Eur. J. Cancer* 37 Suppl 4, S9-15
207. Schlomm, T., Kirstein, P., Iwers, L. *et al.* (2007) *Clin. Cancer Res.* 13, 6579-6584
208. Ciardiello, F., and Tortora, G. (2008) *N. Engl. J. Med.* 358, 1160-1174
209. Krause, M., Schutze, C., Petersen, C., Pimentel, N., Hessel, F., Harstrick, A., and Baumann, M. (2005) *Radiother. Oncol.* 74, 109-115
210. Toulany, M., Dittmann, K., Baumann, M., and Rodemann, H. P. (2005) *Radiother. Oncol.* 74, 117-129
211. Burdak-Rothkamm, S., Rube, C. E., Nguyen, T. P., Ludwig, D., Feldmann, K., Wiegel, T., and Rube, C. (2005) *Strahlenther. Onkol.* 181, 197-204
212. Bonner, J. A., Harari, P. M., Giralt, J. *et al.* (2006) *N. Engl. J. Med.* 354, 567-578
213. Sobrero, A. F., Maurel, J., Fehrenbacher, L. *et al.* (2008) *J. Clin. Oncol.* 26, 2311-2319
214. Senderowicz, A. M., Johnson, J. R., Sridhara, R., Zimmerman, P., Justice, R., and Pazdur, R. (2007) *Oncology (Williston Park)* 21, 1696-706; discussion 1706-9, 1712, 1715
215. Thatcher, N., Chang, A., Parikh, P. *et al.* (2005) *Lancet* 366, 1527-1537
216. Small, E. J., Fontana, J., Tannir, N., DiPaola, R. S., Wilding, G., Rubin, M., Iacona, R. B., and Kabbinavar, F. F. (2007) *BJU Int.* 100, 765-769
217. Tol, J., Koopman, M., Cats, A. *et al.* (2009) *N. Engl. J. Med.* 360, 563-572
218. Camp, E. R., Summy, J., Bauer, T. W., Liu, W., Gallick, G. E., and Ellis, L. M. (2005) *Clin. Cancer Res.* 11, 397-405
219. Guix, M., Faber, A. C., Wang, S. E. *et al.* (2008) *J. Clin. Invest.* 118, 2609-2619
220. Lammering, G., Valerie, K., Lin, P. S., Hewit, T. H., and Schmidt-Ullrich, R. K. (2004) *Radiother. Oncol.* 72, 267-273
221. Heimberger, A. B., Learn, C. A., Archer, G. E. *et al.* (2002) *Clin. Cancer Res.* 8, 3496-3502
222. Riely, G. J., Politi, K. A., Miller, V. A., and Pao, W. (2006) *Clin. Cancer Res.* 12, 7232-7241
223. Kobayashi, S., Boggon, T. J., Dayaram, T., Janne, P. A., Kocher, O., Meyerson, M., Johnson, B. E., Eck, M. J., Tenen, D. G., and Halmos, B. (2005) *N. Engl. J. Med.* 352, 786-792
224. Toulany, M., Kasten-Pisula, U., Brammer, I., Wang, S., Chen, J., Dittmann, K., Baumann, M., Dikomey, E., and Rodemann, H. P. (2006) *Clin. Cancer Res.* 12, 4119-4126
225. Toulany, M., Dittmann, K., Kruger, M., Baumann, M., and Rodemann, H. P. (2005) *Radiother. Oncol.* 76, 143-150
226. Hirsch, F. R., Varella-Garcia, M., Bunn, P. A.,Jr *et al.* (2006) *J. Clin. Oncol.* 24, 5034-5042
227. Vivanco, I., Palaskas, N., Tran, C. *et al.* (2007) *Cancer. Cell.* 11, 555-569
228. Angel, P., and Karin, M. (1991) *Biochim. Biophys. Acta* 1072, 129-157
229. Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E., and Leder, P. (1988) *Cell* 55, 917-924
230. Hai, T., and Curran, T. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3720-3724

231. Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H. J., and Herrlich, P. (1987) *Mol. Cell. Biol.* 7, 2256-2266
232. De Cesare, D., Vallone, D., Caracciolo, A., Sassone-Corsi, P., Nerlov, C., and Verde, P. (1995) *Oncogene* 11, 365-376
233. Hawker, K. L., Vass, J. K., and Ozanne, B. W. (1996) *Oncogene* 13, 283-292
234. Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C., and Blenis, J. (2002) *Nat. Cell Biol.* 4, 556-564
235. Hess, J., Angel, P., and Schorpp-Kistner, M. (2004) *J. Cell. Sci.* 117, 5965-5973
236. Whitmarsh, A. J., and Davis, R. J. (1996) *J. Mol. Med.* 74, 589-607
237. Thepot, D., Weitzman, J. B., Barra, J., Segretain, D., Stinnakre, M. G., Babinet, C., and Yaniv, M. (2000) *Development* 127, 143-153
238. Wilkinson, D. G., Bhatt, S., Ryseck, R. P., and Bravo, R. (1989) *Development* 106, 465-471
239. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* 76, 1025-1037
240. Minden, A., and Karin, M. (1997) *Biochim. Biophys. Acta* 1333, F85-104
241. Karin, M. (1995) *J. Biol. Chem.* 270, 16483-16486
242. Deng, T., and Karin, M. (1994) *Nature* 371, 171-175
243. Karin, M., and Hunter, T. (1995) *Curr. Biol.* 5, 747-757
244. Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994) *Nature* 370, 226-229
245. Mialon, A., Sankinen, M., Soderstrom, H. *et al.* (2005) *Mol. Cell. Biol.* 25, 5040-5051
246. Gao, Z., and Ye, J. (2008) *Biochem. Biophys. Res. Commun.* 376, 793-796
247. Bossis, G., Malnou, C. E., Farras, R., Andermarcher, E., Hipskind, R., Rodriguez, M., Schmidt, D., Muller, S., Jariel-Encontre, I., and Piechaczyk, M. (2005) *Mol. Cell. Biol.* 25, 6964-6979
248. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) *Cell* 78, 787-798
249. Sharrocks, A. D., Brown, A. L., Ling, Y., and Yates, P. R. (1997) *Int. J. Biochem. Cell Biol.* 29, 1371-1387
250. Karamouzis, M. V., Konstantinopoulos, P. A., and Papavassiliou, A. G. (2007) *Mol. Cancer. Res.* 5, 109-120
251. Wagner, E. F., and Eferl, R. (2005) *Immunol. Rev.* 208, 126-140
252. Hilberg, F., Aguzzi, A., Howells, N., and Wagner, E. F. (1993) *Nature* 365, 179-181
253. Eferl, R., Sibilia, M., Hilberg, F., Fuchsbichler, A., Kufferath, I., Guertl, B., Zenz, R., Wagner, E. F., and Zatloukal, K. (1999) *J. Cell Biol.* 145, 1049-1061
254. Schorpp-Kistner, M., Wang, Z. Q., Angel, P., and Wagner, E. F. (1999) *EMBO J.* 18, 934-948
255. Wang, Z. Q., Ovitt, C., Grigoriadis, A. E., Mohle-Steinlein, U., Ruther, U., and Wagner, E. F. (1992) *Nature* 360, 741-745
256. Gruda, M. C., van Amsterdam, J., Rizzo, C. A., Durham, S. K., Lira, S., and Bravo, R. (1996) *Oncogene* 12, 2177-2185
257. Schreiber, M., Wang, Z. Q., Jochum, W., Fetka, I., Elliott, C., and Wagner, E. F. (2000) *Development* 127, 4937-4948
258. Bozec, A., Bakiri, L., Hoebertz, A. *et al.* (2008) *Nature* 454, 221-225
259. Wisdom, R., Johnson, R. S., and Moore, C. (1999) *EMBO J.* 18, 188-197
260. Johnson, R. S., van Lingen, B., Papaioannou, V. E., and Spiegelman, B. M. (1993) *Genes Dev.* 7, 1309-1317
261. Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Mohle-Steinlein, U., Tian, J., Karin, M., Angel, P., and Wagner, E. F. (1999) *Genes Dev.* 13, 607-619
262. Behrens, A., Sibilia, M., David, J. P., Mohle-Steinlein, U., Tronche, F., Schutz, G., and Wagner, E. F. (2002) *EMBO J.* 21, 1782-1790
263. Li, G., Gustafson-Brown, C., Hanks, S. K., Nason, K., Arbeit, J. M., Pogliano, K., Wisdom, R. M., and Johnson, R. S. (2003) *Dev. Cell.* 4, 865-877

264. Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., Sibilia, M., and Wagner, E. F. (2003) *Dev. Cell.* 4, 879-889
265. Brusselbach, S., Mohle-Steinlein, U., Wang, Z. Q., Schreiber, M., Lucibello, F. C., Muller, R., and Wagner, E. F. (1995) *Oncogene* 10, 79-86
266. Field, S. J., Johnson, R. S., Mortensen, R. M., Papaioannou, V. E., Spiegelman, B. M., and Greenberg, M. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 9306-9310
267. Hu, E., Mueller, E., Oliviero, S., Papaioannou, V. E., Johnson, R., and Spiegelman, B. M. (1994) *EMBO J.* 13, 3094-3103
268. Crist, S. A., Griffith, T. S., and Ratliff, T. L. (2003) *J. Biol. Chem.* 278, 35950-35958
269. Behrens, A., Haigh, J., Mechta-Grigoriou, F., Nagy, A., Yaniv, M., and Wagner, E. F. (2003) *Development* 130, 103-109
270. Leppa, S., Eriksson, M., Saffrich, R., Ansorge, W., and Bohmann, D. (2001) *Mol. Cell. Biol.* 21, 4369-4378
271. Passegue, E., Jochum, W., Schorpp-Kistner, M., Mohle-Steinlein, U., and Wagner, E. F. (2001) *Cell* 104, 21-32
272. Jochum, W., Passegue, E., and Wagner, E. F. (2001) *Oncogene* 20, 2401-2412
273. Mariani, O., Brennetot, C., Coindre, J. M., Gruel, N., Ganem, C., Delattre, O., Stern, M. H., and Aurias, A. (2007) *Cancer. Cell.* 11, 361-374
274. Brown, P. H., Alani, R., Preis, L. H., Szabo, E., and Birrer, M. J. (1993) *Oncogene* 8, 877-886
275. Fialka, I., Schwarz, H., Reichmann, E., Oft, M., Busslinger, M., and Beug, H. (1996) *J. Cell Biol.* 132, 1115-1132
276. Reichmann, E., Schwarz, H., Deiner, E. M., Leitner, I., Eilers, M., Berger, J., Busslinger, M., and Beug, H. (1992) *Cell* 71, 1103-1116
277. Passegue, E., and Wagner, E. F. (2000) *EMBO J.* 19, 2969-2979
278. Grigoriadis, A. E., Schellander, K., Wang, Z. Q., and Wagner, E. F. (1993) *J. Cell Biol.* 122, 685-701
279. Troen, G., Nygaard, V., Jenssen, T. K. *et al.* (2004) *J. Mol. Diagn.* 6, 297-307
280. Young, M. R., and Colburn, N. H. (2006) *Gene* 379, 1-11
281. Zerbini, L. F., Wang, Y., Cho, J. Y., and Libermann, T. A. (2003) *Cancer Res.* 63, 2206-2215
282. Ouyang, X., Jessen, W. J., Al-Ahmadie, H. *et al.* (2008) *Cancer Res.* 68, 2132-2144
283. Li, W., Wu, C. L., Febbo, P. G., and Olumi, A. F. (2007) *Am. J. Pathol.* 171, 1189-1198
284. Page-McCaw, A., Ewald, A. J., and Werb, Z. (2007) *Nat. Rev. Mol. Cell Biol.* 8, 221-233
285. Egeblad, M., and Werb, Z. (2002) *Nat. Rev. Cancer.* 2, 161-174
286. Parks, W. C., Wilson, C. L., and Lopez-Boado, Y. S. (2004) *Nat. Rev. Immunol.* 4, 617-629
287. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) *Cell* 49, 729-739
288. Carrere, S., Verger, A., Flourens, A., Stehelin, D., and Duterque-Coquillaud, M. (1998) *Oncogene* 16, 3261-3268
289. McDonnell, S. E., Kerr, L. D., and Matrisian, L. M. (1990) *Mol. Cell. Biol.* 10, 4284-4293
290. Eberhardt, W., Kilz, T., Akool, e., Muller, R., and Pfeilschifter, J. (2005) *Biochem. Pharmacol.* 70, 433-445
291. Frost, J. A., Geppert, T. D., Cobb, M. H., and Feramisco, J. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 3844-3848
292. Chakraborti, S., Mandal, M., Das, S., Mandal, A., and Chakraborti, T. (2003) *Mol. Cell. Biochem.* 253, 269-285
293. Mignatti, P., and Rifkin, D. B. (1993) *Physiol. Rev.* 73, 161-195
294. Willenbrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J., Cockett, M. I., O'Shea, M., Brocklehurst, K., Phillips, I. R., and Murphy, G. (1993) *Biochemistry* 32, 4330-4337
295. Eguchi, S., Frank, G. D., Mifune, M., and Inagami, T. (2003) *Biochem. Soc. Trans.* 31, 1198-1202
296. Brinckerhoff, C. E., and Matrisian, L. M. (2002) *Nat. Rev. Mol. Cell Biol.* 3, 207-214
297. Borrell-Pages, M., Rojo, F., Albanell, J., Baselga, J., and Arribas, J. (2003) *EMBO J.* 22, 1114-1124

298. Nakagawa, M., Nabeshima, K., Asano, S., Hamasaki, M., Uesugi, N., Tani, H., Yamashita, Y., and Iwasaki, H. (2009) *Cancer. Sci.*
299. Foschi, M., Chari, S., Dunn, M. J., and Sorokin, A. (1997) *EMBO J.* 16, 6439-6451
300. Auer, K. L., Contessa, J., Brenz-Verca, S. *et al.* (1998) *Mol. Biol. Cell* 9, 561-573
301. Treier, M., Bohmann, D., and Mlodzik, M. (1995) *Cell* 83, 753-760
302. Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tjian, R. (1987) *Science* 238, 1386-1392
303. Curran, T., Peters, G., Van Beveren, C., Teich, N. M., and Verma, I. M. (1982) *J. Virol.* 44, 674-682
304. Gack, S., Vallon, R., Schaper, J., Ruther, U., and Angel, P. (1994) *J. Biol. Chem.* 269, 10363-10369
305. Madlener, M., Parks, W. C., and Werner, S. (1998) *Exp. Cell Res.* 242, 201-210
306. Fort, P., Marty, L., Piechaczyk, M., el Sabrouty, S., Dani, C., Jeanteur, P., and Blanchard, J. M. (1985) *Nucleic Acids Res.* 13, 1431-1442
307. Kang, S. H., Vieira, K., and Bungert, J. (2002) *Nucleic Acids Res.* 30, e44
308. Miettinen, P. J., Huotari, M., Koivisto, T., Ustinov, J., Palgi, J., Rasilainen, S., Lehtonen, E., Keski-Oja, J., and Otonkoski, T. (2000) *Development* 127, 2617-2627
309. Konstantinopoulos, P. A., Vandoros, G. P., Karamouzis, M. V., Gkermpesi, M., Sotiropoulou-Bonikou, G., and Papavassiliou, A. G. (2007) *Cell. Oncol.* 29, 477-482
310. Adiseshaiah, P., Vaz, M., Machireddy, N., Kalvakolanu, D. V., and Reddy, S. P. (2008) *J. Cell. Physiol.* 216, 405-412
311. Li, J., Ma, C., Huang, Y., Luo, J., and Huang, C. (2003) *Oncogene* 22, 211-219
312. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* 288, 870-874
313. Cox, G., Jones, J. L., and O'Byrne, K. J. (2000) *Clin. Cancer Res.* 6, 2349-2355
314. Langlois, S., Nyalendo, C., Di Tomasso, G., Labrecque, L., Roghi, C., Murphy, G., Gingras, D., and Beliveau, R. (2007) *Mol. Cancer. Res.* 5, 569-583
315. Bullard, K. M., Mudgett, J., Scheuenstuhl, H., Hunt, T. K., and Banda, M. J. (1999) *J. Surg. Res.* 84, 31-34
316. Unemori, E. N., and Werb, Z. (1986) *J. Cell Biol.* 103, 1021-1031
317. Carver, W., Molano, I., Reaves, T. A., Borg, T. K., and Terracio, L. (1995) *J. Cell. Physiol.* 165, 425-437
318. Adiseshaiah, P., Vaz, M., Machireddy, N., Kalvakolanu, D. V., and Reddy, S. P. (2008) *J. Cell. Physiol.* 216, 405-412
319. Peschon, J. J., Slack, J. L., Reddy, P. *et al.* (1998) *Science* 282, 1281-1284
320. Dong, J., Opresko, L. K., Dempsey, P. J., Lauffenburger, D. A., Coffey, R. J., and Wiley, H. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6235-6240
321. Ogawa, K., Chen, F., Kuang, C., and Chen, Y. (2004) *Biochem. J.* 381, 413-422
322. Lohi, J., and Keski-Oja, J. (1995) *J. Biol. Chem.* 270, 17602-17609
323. Marcet-Palacios, M., Graham, K., Cass, C., Befus, A. D., Mayers, I., and Radomski, M. W. (2003) *J. Pharmacol. Exp. Ther.* 307, 429-436
324. Zhang, D., and Brodt, P. (2003) *Oncogene* 22, 974-982
325. Reunanen, N., Li, S. P., Ahonen, M., Foschi, M., Han, J., and Kahari, V. M. (2002) *J. Biol. Chem.* 277, 32360-32368
326. Repertinger, S. K., Campagnaro, E., Fuhrman, J., El-Abaseri, T., Yuspa, S. H., and Hansen, L. A. (2004) *J. Invest. Dermatol.* 123, 982-989
327. Benbow, U., and Brinckerhoff, C. E. (1997) *Matrix Biol.* 15, 519-526
328. Barnard, J. A., Beauchamp, R. D., Russell, W. E., Dubois, R. N., and Coffey, R. J. (1995) *Gastroenterology* 108, 564-580
329. Dise, R. S., Frey, M. R., Whitehead, R. H., and Polk, D. B. (2008) *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G276-85
330. Fiske, W. H., Threadgill, D., and Coffey, R. J. (2009) *Exp. Cell Res.* 315, 583-601

331. Arsenault, P., and Menard, D. (1985) *J. Pediatr. Gastroenterol. Nutr.* 4, 893-901
332. Loirdighi, N., Menard, D., Delvin, D., and Levy, E. (1997) *J. Cell. Biochem.* 66, 65-76
333. Elnif, J., Buddington, R. K., Hansen, N. E., and Sangild, P. T. (2006) *J. Comp. Physiol. [B].* 176, 233-241
334. Grosfeld, J. L., Chaet, M., Molinari, F., Engle, W., Engum, S. A., West, K. W., Rescorla, F. J., and Scherer, L. R.,3rd. (1996) *Ann. Surg.* 224, 350-5; discussion 355-7
335. Attridge, J. T., Clark, R., Walker, M. W., and Gordon, P. V. (2006) *J. Perinatol.* 26, 93-99
336. Attridge, J. T., Clark, R., Walker, M. W., and Gordon, P. V. (2006) *J. Perinatol.* 26, 185-188
337. Salamanca, C. M., Maines-Bandiera, S. L., Leung, P. C., Hu, Y. L., and Auersperg, N. (2004) *J. Soc. Gynecol. Investig.* 11, 241-251
338. Durant, S., Duval, D., and Homo-Delarche, F. (1986) *Endocr. Rev.* 7, 254-269
339. Balmanno, K., and Cook, S. J. (1999) *Oncogene* 18, 3085-3097
340. Sheng, G., Bernabe, K. Q., Guo, J., and Warner, B. W. (2006) *Gastroenterology* 131, 153-164
341. Abud, H. E., Watson, N., and Heath, J. K. (2005) *Exp. Cell Res.* 303, 252-262
342. Shao, J., Sheng, G. G., Mifflin, R. C., Powell, D. W., and Sheng, H. (2006) *Cancer Res.* 66, 846-855
343. Whitehead, R. H., Demmler, K., Rockman, S. P., and Watson, N. K. (1999) *Gastroenterology* 117, 858-865
344. Buchanan, F. G., Wang, D., Bargiacchi, F., and DuBois, R. N. (2003) *J. Biol. Chem.* 278, 35451-35457
345. Hamasaki, Y., Kitzler, J., Hardman, R., Nettesheim, P., and Eling, T. E. (1993) *Arch. Biochem. Biophys.* 304, 226-234
346. Konturek, S. J., Brzozowski, T., Majka, J., Dembinski, A., Slomiany, A., and Slomiany, B. L. (1992) *Scand. J. Gastroenterol.* 27, 649-655
347. Sinha, A., Nightingale, J., West, K. P., Berlanga-Acosta, J., and Playford, R. J. (2003) *N. Engl. J. Med.* 349, 350-357
348. Warner, B. B., Ryan, A. L., Seeger, K., Leonard, A. C., Erwin, C. R., and Warner, B. W. (2007) *J. Pediatr.* 150, 358-363
349. Sullivan, P. B., Lewindon, P. J., Cheng, C., Lenehan, P. F., Kuo, B. S., Haskins, J. R., Goodlad, R. A., Wright, N. A., and de la Iglesia, F. A. (2007) *J. Pediatr. Surg.* 42, 462-469
350. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) *Gastroenterology* 107, 1183-1188
351. Torrance, C. J., Jackson, P. E., Montgomery, E., Kinzler, K. W., Vogelstein, B., Wissner, A., Nunes, M., Frost, P., and Discafani, C. M. (2000) *Nat. Med.* 6, 1024-1028
352. Siemens, C. H., and Auersperg, N. (1988) *J. Cell. Physiol.* 134, 347-356
353. Ahmed, N., Maines-Bandiera, S., Quinn, M. A., Unger, W. G., Dedhar, S., and Auersperg, N. (2006) *Am. J. Physiol. Cell. Physiol.* 290, C1532-42
354. Ponec, M. (1984) *Int. J. Dermatol.* 23, 11-24
355. Rademaker, K. J., and de Vries, W. B. (2008) *Semin. Fetal. Neonatal Med.*
356. Wu, Y. Y., Li, H., Lv, X. Y., Wei, Q., Li, X., Liu, X. Y., Zhou, Q., and Wei, Y. Q. (2008) *Cell Biochem. Funct.* 26, 467-477
357. Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. (1994) *Cancer Res.* 54, 5474-5478
358. Torring, N., Dagnaes-Hansen, F., Sorensen, B. S., Nexo, E., and Hynes, N. E. (2003) *Prostate* 56, 142-149
359. Bonaccorsi, L., Carloni, V., Muratori, M., Formigli, L., Zecchi, S., Forti, G., and Baldi, E. (2004) *Int. J. Cancer* 112, 78-86
360. Price, D. T., Della Rocca, G., Guo, C., Ballo, M. S., Schwinn, D. A., and Luttrell, L. M. (1999) *J. Urol.* 162, 1537-1542
361. Itoh, N., Semba, S., Ito, M., Takeda, H., Kawata, S., and Yamakawa, M. (2002) *Cancer* 94, 3127-3134
362. Cantley, L. C., and Neel, B. G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 4240-4245
363. Voeller, H. J., Wilding, G., and Gelmann, E. P. (1991) *Mol. Endocrinol.* 5, 209-216
364. Milas, L., Fan, Z., Andratschke, N. H., and Ang, K. K. (2004) *Int. J. Radiat. Oncol. Biol. Phys.* 58, 966-971

365. Angelucci, A., Gravina, G. L., Rucci, N., Millimaggi, D., Festuccia, C., Muzi, P., Teti, A., Vicentini, C., and Bologna, M. (2006) *Endocr. Relat. Cancer* 13, 197-210
366. Sirotnak, F. M., She, Y., Lee, F., Chen, J., and Scher, H. I. (2002) *Clin. Cancer Res.* 8, 3870-3876
367. Huamani, J., Willey, C., Thotala, D. *et al.* (2008) *Int. J. Radiat. Oncol. Biol. Phys.* 71, 237-246
368. Geoerger, B., Gaspar, N., Opolon, P., Morizet, J., Devanz, P., Lecluse, Y., Valent, A., Lacroix, L., Grill, J., and Vassal, G. (2008) *Int. J. Cancer* 123, 209-216
369. Graff, J. R., Konicek, B. W., McNulty, A. M. *et al.* (2000) *J. Biol. Chem.* 275, 24500-24505
370. Chen, S. Y., Cai, C., Fisher, C. J., Zheng, Z., Omwancha, J., Hsieh, C. L., and Shemshedini, L. (2006) *Oncogene* 25, 7212-7223
371. Edwards, J., Krishna, N. S., Mukherjee, R., and Bartlett, J. M. (2004) *J. Pathol.* 204, 153-158
372. Liebermann, D. A., Gregory, B., and Hoffman, B. (1998) *Int. J. Oncol.* 12, 685-700