Chapter 2
PI3K/AKT Pathway and the Epithelial–Mesenchymal Transition

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Cast of Characters

The catalytic subunit of the phosphatidylinositol 3-kinase (*PIK3*; EC 2.7.1.137) is one of the most frequently mutated gene in human cancers, as is its inhibitor *PTEN*. By some estimates, *PIK3CA* carries gain-of-function mutations in 32% of colorectal cancers, 36% of hepatocellular carcinomas, 36% of endometrial carcinomas, 25% of breast carcinomas, 15% of anaplastic oligodendrogliomas, and 5% of medulloblastomas and anaplastic astrocytomas (recently reviewed in Velculescu, 2008). Similarly, spontaneous mutations in *PTEN* are found in 50% of endometrial cancers, 30% of glioblastomas, 10% of prostate, and 5% of breast carcinomas. Moreover, inherited mutations in *PTEN* lead to a variety of conditions, such as Cowden syndrome, which are associated with an increased risk of cancer (recently reviewed in Keniry and Parsons, 2008). In addition, frequent alterations and hyperactivation of AKT kinases have been described in almost every tumor type studied (reviewed in Bellacosa et al., 2005; Brugge et al., 2007). While many of the downstream effectors of the AKT pathway are involved in cell autonomous processes (i.e., cell cycle and apoptosis), the following chapter will focus on the implications of aberrant AKT signaling for epithelial–mesenchymal transition, in particular on the PI3K–AKT–NF-κB–Snail pathways in EMT with emphasis on E-cadherin regulation.

Introduction

Epithelial–mesenchymal transition (EMT) is a major developmental process during which epithelial cells develop mesenchymal, fibroblast-like properties, increased motility, and reduced intercellular adhesion. There is growing evidence that

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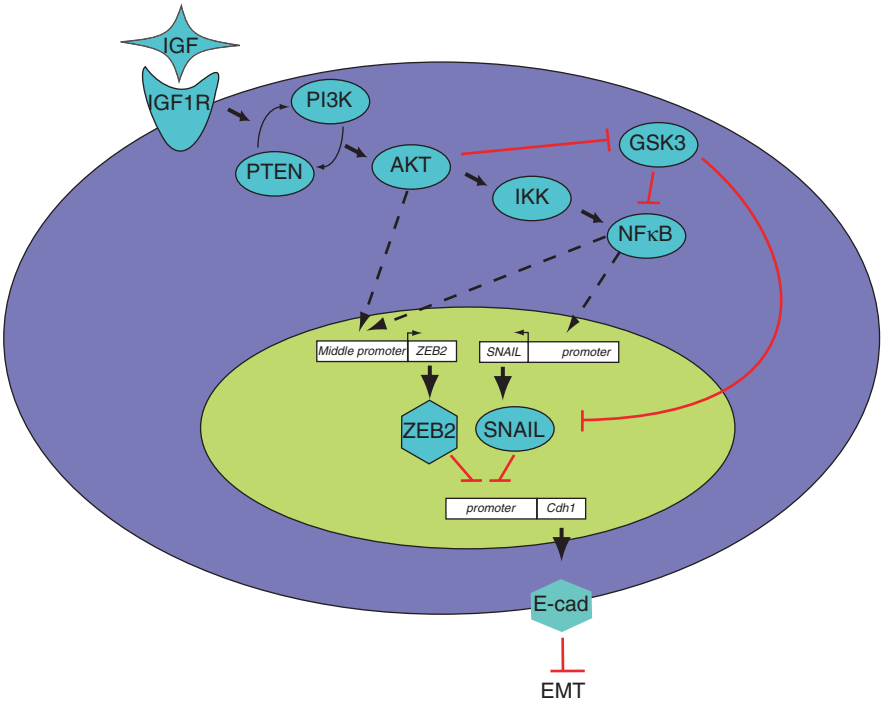

Fig. 2.1 Model of the regulation of E-cadherin transcription by the PI3K/AKT signaling pathway

EMT-like events are central to tumor progression and malignant transformation, endowing the incipient cancer cell with invasive and metastatic properties. Several oncogenic pathways (peptide growth factors, Src, Ras, Ets, integrin, Wnt/β-catenin, and Notch) induce processes characteristic of EMT, such as downregulation of the cell adhesion molecule and obligate epithelial marker E-cadherin. EMT also now appears to involve activation of the IGF/IGF-1R–phosphatidylinositol 3′-kinase (PI3K)/AKT–NF-κB–Snail–E-cadherin axis (Fig. 2.1), which is discussed in the following pages and also Chapter 3.

EMT Definition

EMT was first defined based on morphological features, but currently, morphological, cellular, and molecular factors are also included. Any analysis of EMT requires consideration of the defining features of epithelium and mesenchyme, the start and endpoints of the transition. One fundamental issue is whether the transition involves an abrupt or a gradual passage from one state to the other. Deployment of EMT certainly requires and involves several modifications of the cells, so the transition may

appear to be abrupt or gradual depending on the degree of accuracy or precision that is applied, for technical or other reasons.

Epithelial cellular characteristics as determined in various in vivo or in vitro systems can be classified into five groups: (a) cohesive interactions among cells, allowing the formation of continuous cell layers; (b) presence of three types of membrane domains (apical, lateral, and basal); (c) presence of tight junctions between apical and lateral domains; (d) polarized distribution of the various organelles and components of the cytoskeleton; and (e) near immobility of cells in the local epithelial microenvironment. Based on these properties, epithelia perform three types of function: they form large surfaces for exchange (e.g., the alveolar epithelium in the lung for gaseous exchange), and this includes the creation of cavities by epithelial layer folding (e.g., the intestine and the neural tube); the separation of biological compartments with the selective permeability of the cells, ensuring different ionic compositions of the compartments; and trafficking macromolecules by absorption, transcytosis, and vectorial secretion.

A major property allowing the formation and the maintenance of an epithelium is adhesion: cell–cell adhesion, between the sides of the cells, and cell–matrix adhesion, mostly involving the basal surfaces of the cells. Cell–cell adhesion is a defining characteristic of epithelia, ensuring tissue cohesiveness, whereas other cell types, including mesenchymal cells, may also express cell–matrix adhesion. Epithelial cell–cell adhesion systems are multiple – gap junctions, adherens junctions, desmosomes, and tight junctions – and involve different families of proteins.

Mesenchymal architecture is unlike the supracellular epithelial organization and in fact mesenchymal cells have various characteristics: (a) loose or no interaction between cells, and consequently no continuous cell layer is formed; (b) there are no apical and lateral membranes; (c) the distribution of the cytoskeletal organelles and components is not polarized; and (d) the cells are motile and in some cases invasive. Mesenchymal functions include support and nutrient supply; also mesenchymes may be transitory intermediates during the formation of an epithelial structure from another epithelial structure – mesenchymal to epithelial transition or MET – during development and cancer progression. Nevertheless, mesenchymal architecture can be durable.

In summary, it is the tightness of cell–cell junctions that determines epithelial organization. Cell–cell adhesion is dependent on transmembrane glycoproteins, including E-cadherin, a typical epithelial marker. If cells do not have an epithelial status, they are, by default, mesenchymal.

EMT During Development and Cancer

During early mammalian embryonic development, there are interconversions between epithelium and mesenchyme, the first MET being the formation of the trophectoderm during preimplantation and the first EMT being the formation of the

mesoderm during gastrulation. Mouse mutants have been largely uninformative with regard to PI3K/AKT signaling during early development, but cadherin regulation is clearly involved in early development (Larue et al., 1996). There are further EMT conversions during subsequent embryonic development, some associated with major developmental milestones: the formation of neural crest cells from the neural tube on embryonic day 8 (E8); the formation of the atrial and ventricular mesenchymal septa from the endothelium during heart development on E8; the formation of the sclerotome from somites on E9; the formation of coronary vessel progenitor cells from the epicardium around E10–11; the formation of palate mesenchymal cells from the oral epithelium on E13.5; and the formation of mesenchymal cells during regression of the Mullerian tract on E15. Although regulated differently, these EMT are associated with common events, but the role of PI3K and AKT in these processes remains unclear.

Normal development involves highly regulated spatial and temporal master plans, whereas pathological processes, and in particular transformation, are characterized by stochastic and time-independent sequences of events and some events failing to occur. EMT associated with tumorigenesis may increase the motility and invasiveness of cancer cells, and malignant transformation may involve activation of signaling pathways promoting EMT (Boyer et al., 2000). During tumor progression, there is activation of various processes associated with EMT and resembling those occurring in normal development. Nevertheless, normal EMT and physiopathological EMT differ in important ways. The molecular program leading to EMT during tumor progression is based on amplification of a restricted set of the components of complete developmental EMT. This may be because oncogenic signaling associated with tumorigenesis involves fewer signal transduction pathways.

IGF and EMT

General Functions of IGF

Insulin-like growth factors (IGFs) are peptide ligands that bind to the insulin receptor (IR), the IGF-I receptor (IGF-1R), and the IGF-II receptor (IGF-2R). IR and IGF-1R are both receptor protein tyrosine kinases (RPTK) with intrinsic tyrosine kinase activity and structures similar to that of the classic epidermal growth factor receptor. Structurally related, secreted proteins called IGF-binding proteins (IGFBPs) modulate the biological effects of IGF ligands (but not insulin) and are found in the blood and extracellular spaces. IGFBPs bind to IGFs with affinities similar to those for their receptors. Thus, IGFBPs regulate the bioavailability of IGFs by increasing their longevity, facilitating their transport, and promoting/inhibiting IGF binding to their receptors.

IGFs contribute to various cellular mechanisms including cell growth and cell division, antiapoptotic signaling, invasion, differentiation, migration, and EMT. In particular, phenotypic analysis of genetically engineered mice showed that IGFs are

involved in growth control: the relevant mutant mice display somatic undergrowth. Disruption of the *IGF-II* gene results in a birth weight that is only 60% of that of wild-type mice. Disruption of the *IGF-1R* gene results in a birth weight that is 45% of that of wild-type littermates. The embryo birth weight of *IGF-II* and *IGF-1R* gene double mutants is 30% of that of wild-type littermates. Phenotypic analysis suggests that *IGF-II* signals through an alternative receptor, also an IR.

IGFs have powerful mitogenic effects on many cell types, and this may explain, at least in part, these phenotypes. IGFs stimulate cell growth and cell division of numerous cell types both in vivo and in vitro (granulosa, granulosa-luteal cells, Sertoli, Leydig, prostate epithelial, bladder urothelial, smooth and skeletal muscle cells, and also spermatogonia, astrocytes, and osteoblasts). IGFs also regulate apoptosis and thereby cell number; most IGFs are antiapoptotic and are consequently classified as survival factors. IGFs are thus generally mitogenic and IGF signaling is dysregulated in various cancer cells; these observations have led to the view that inappropriate activation of the IGF pathway may be central to carcinogenesis. IGFs are also implicated in physiological invasion mechanisms during development, such as trophoblast invasion of the endometrium during implantation (Rosenfeld and Roberts, 1999).

Surprisingly, IGF promotes the acquisition or the maintenance of the differentiated state of some cell types; it stimulates differentiation of skeletal muscle cells and Leydig cells, modulates androgen production by Sertoli and Leydig cells, and promotes neuronal differentiation and myelinization of the central and peripheral neural systems. Therefore, although proliferation and differentiation are widely considered to be two mutually exclusive cellular states, both are stimulated by IGF. Note that these activities are not incompatible with induction of EMT by IGFs.

IGFs Induce EMT

Various in vitro models, notably NBT-II, MDCK, and MCF7 cell lines and embryonic stem cells, have been used to study the effects of IGFs on cell–cell adhesion.

If not stimulated by IGFs and insulin, NBT-II, MDCK, and MCF7 cells have standard epithelial cell morphology: polarized and tightly attached to each another. Treatment of NBT-II, MDCK, and MCF7 cells with IGF results in loss of cell–cell contacts, and the cells flatten and spread. Embryonic stem (ES) cells, which are tightly cohesive, undergo the same morphological changes upon IGF treatment, whereas mesenchymal NIH-3T3 cells do not. These morphological modifications appear rapidly after exposure to IGF (typically within 1 h) and are not associated with cell division.

Various molecular events accompany the loss of cell–cell contacts: (i) the rapid internalization of E-cadherin and desmoplakin, leading to the disruption of junctional complexes (adherens junctions, desmosomes, and particularly gap junctions) and (ii) the expression of the mesenchymal-specific marker vimentin after 4 days

of IGF treatment. The cellular and molecular modifications caused by IGF are thus typical of standard EMT. If IGF is removed, NBT-II cells revert to an epithelial morphology within 24 hours, and E-cadherin relocalizes to cell–cell contacts (Morali et al., 2001); in other words, the IGF-induced transition is reversible.

Minimally, EMT is characterized by tightly attached and polarized epithelial cells becoming a set of loosely attached and nonpolarized mesenchymal cells. Full-blown EMT also involves cell motility. IGF can induce migration of some epithelial cells, for example, MCF7 (Guvakova et al. 2002) and melanoma cells (Li et al., 1994), but not others, for example, NBT-II cells (Morali et al., 2001). Whether IGFs induce minimal or complete EMT seems therefore to depend on the cell type.

IGF-1R and EMT

IGF-1R is a RPTK (for reviews, see Ullrich and Schlessinger, 1990; Schlessinger, 2000; Favelyukis et al., 2001) that binds IGF; such binding activates several signaling pathways (see below), and the diversity of the biological effects of IGFs may be a consequence of this multiplicity of signaling pathways. Five families of cytoplasmic proteins interact with IGF-1R and transmit the outside signal to the cytoplasm: (1) the large Grb family of adaptor proteins containing SH2 (Src homology 2) and SH3 domains; (2) the adaptor protein SHC, with SH2 domains and also numerous tyrosines susceptible to phosphorylation by IGF-1R; (3) the Crk family (Crk-I, Crk-II, and Crk-L) of adaptor proteins, containing both SH2 and SH3 domains; (4) the IRS family of adaptor proteins containing a PTB (phosphotyrosine binding) domain, a tyrosine-rich C-terminal region, and a PH domain, but no SH2 domain (note that IRS-1 and IRS-2 are rapidly phosphorylated by activated IGF-1R); and (5) class I PI3K, a heterodimer consisting of a regulatory subunit, p85, and a lipid kinase catalytic subunit, p110.

A constitutively activated IGF-1R (CD8-IGF-1R) has been expressed in MCF10A breast carcinoma cells, and as expected in view of the activities described above, caused EMT (Kim et al., 2007); in wound-healing and trans-well chamber assays, these cells migrated efficiently and, as assessed using a BD Matrigel invasion chamber, were highly invasive.

Downstream of IGFR

PRL-3 and EMT

Tyrosine phosphatase 4a3 (Ptp4a3 or PRL-3), a 22-kDa protein, is expressed in various tissues both during development and in the adult. Its primary function concerns cell growth (Matter et al., 2001). Two lines of evidence have implicated PRL-3 in EMT: its interaction with integrin α1 and its regulation by growth factors and growth factor receptors (Peng et al., 2006). However, induction of IGF-1R

by IGF has not been demonstrated to regulate PRL-3. PRL-3 expression is high in various metastatic cancers (colorectal, breast, ovary, melanoma) and higher in metastatic vs. nonmetastatic tumors of colon, liver, lung, brain, and ovary (Saha et al. 2001; Bardelli et al. 2003). PRL-3 expression promotes cell migration, tumor angiogenesis, invasion, and metastasis in cell culture models, such as the Chinese hamster ovary cancer (CHO), human breast cancer (MCF-7), and murine B16 melanoma (Zeng et al., 2003; Wu et al., 2004). Inversely, the growth of ovarian cancer cells is inhibited by RNA interference-mediated downregulation of PRL-3 (Polato et al., 2005).

Thus, PRL-3 is a metastasis-associated phosphatase that may acts as an upstream regulator of PTEN (Wang et al., 2007a).

PTEN and EMT

Phosphatase and tensin homolog (PTEN) is a lipid and protein phosphatase that inhibits diverse signaling pathways and biological processes via its lipid phosphatase activity on the 3′ phosphate of phosphatidylinositol (PtdIns)(3,4,5)P3 and PtdIns(3,4)P2. PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are elements of the PI3K/AKT signaling pathway and have various cellular functions (see below).

PTEN is involved in proliferation, angiogenesis, and cell survival (Stambolic et al., 1998; Sun et al., 1999; Hamada et al., 2005). Although there is no direct evidence for a major contribution of PTEN to EMT in mammals, it affects cell migration. Indeed, murine embryonic fibroblasts (MEFs) migrate faster in vitro in the absence of PTEN (Liliental et al., 2000). Expression of exogenous PTEN in PTEN-null MEFs, or in aggressive colon or prostate carcinoma cell lines, substantially inhibits migration (Tamura et al. 1998; Liliental et al. 2000; Chu and Tarnawski 2004).

AKT is the best known downstream target of PTEN and may be its main effector in EMT; however, the specific molecular mechanisms connecting PTEN to AKT in mammals have not been investigated. PTEN mutants have been used in an elegant study demonstrating the importance of PTEN and its lipid and protein phosphatase activities in chicken, at the gastrulation stage of development (Leslie et al. 2007). During gastrulation, some ectodermal cells in the primitive streak undergo EMT and migrate away to produce mesodermal cells. These cells then migrate back toward the midline. Cells from the primitive streak can be grafted into a different embryo before outward migration. Consequently, the donor cells can be manipulated genetically after initial and appropriate electroporation of donor chicken embryos and before grafting. This allowed the demonstration that the protein–phosphatase activity, but not the lipid–phosphatase activity, of PTEN in these cells was involved in EMT during early gastrulation. The lipid–phosphatase activity may affect cell polarity and directional cell migration later during gastrulation. If AKT is regulated by the lipid–phosphatase activity, but not the protein–phosphatase activity, of PTEN, it is presumably not involved in early mesodermal EMT in chicken. However, work with other model systems suggests that AKT is involved in EMT.

PI3K and AKT

Biochemical Mechanisms

Direct binding of p85, the regulatory subunit of PI3K, to the tyrosine-phosphorylated forms of IGF-1R and IRS-1 triggers tyrosine phosphorylation and consequently a conformational change of p85, activating p110, the PI3K catalytic subunit. It should be noted that Ras-GTP can activate p110 directly (Kodaki et al. 1994; Rodriguez-Viciana et al. 1996).

Active PI3K phosphorylates the 3′-OH group of the inositol ring of phosphatidylinositol (PtdIns), PtdIns(4)P, and Ptd(4,5)P to produce Ptd(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P3 (also called D3-phosphorylated phosphoinositides), respectively. Amphipathic PtdIns(3,4)P_2 and PtdIns(3,4,5)P3 molecules bind to proteins containing a pleckstrin homology (PH) domain. Serine/threonine kinases, including AKT1, -2, and -3 (also known as PKB alpha, beta, and gamma, respectively), and PDK1 (phosphatidylinositol-dependent kinase 1), translocate to the cell membrane upon binding to these D3-phosphorylated phosphoinositides. AKT is then appropriately localized for phosphorylation of its threonine 308 by PDK1 and serine 473 by PDK2. The identity of PDK2, the kinase(s) responsible for Ser-473/474 phosphorylation, has been the subject of debate (Chan and Tsichlis, 2001). AKT phosphorylated on T308 and S473 is fully active.

AKT kinases phosphorylate diverse molecules at threonine or serine residues with different functional outcomes, stimulatory or inhibitory. For instance, AKT family members regulate the activity of several transcription factors, notably CREB (cAMP-response element-binding protein), members of Forkhead family, and Ets-2. AKT phosphorylation of CREB stimulates CREB-dependent transcription (Du and Montminy, 1998), whereas AKT phosphorylation of FKHR (Forkhead in rhabdomyosarcoma) and FKHRL1 (Forkhead in rhabdomyosarcoma-like 1) inhibits CREB-dependent transcription (Brunet et al. 1999; Tang et al. 1999). Ets-2-dependent transcription is activated when Ets-2 is phosphorylated by JNK-2 in cells in which AKT is also activated (Smith et al. 2000).

Ras-mediated reorganization of the actin cytoskeleton and cell migration also depends on PI3K. Indeed, some membrane lipid targets of PI3K regulate (i) the activity and structure of actin-binding proteins and (ii) the GTPase Rac, thereby controlling membrane folding.

General Functions of AKT

AKT promotes cell cycle progression, cell survival, and tumor cell invasion (Testa and Bellacosa 2001). Interestingly, it also phosphorylates and inhibits GSK-3β, thereby, presumably, linking IGF and Wnt pathways.

Activated AKT kinases phosphorylate numerous substrates associated with cell proliferation, survival, intermediary metabolism, and cell growth. The consensus

sequence for AKT phosphorylation, RXRXXS/T, is found in most but not all these substrates.

AKT stimulates proliferation in various ways. It phosphorylates and inhibits glycogen synthase kinase 3β (GSK3β; the first AKT substrate identified) (Cross et al. 1995) and thereby inhibits the degradation of cyclin D1 (Diehl et al. 1998); it also simultaneously upregulates translation (see below) of the cyclin D1 and D3 mRNAs (Muise-Helmericks et al. 1998). AKT phosphorylates the cell cycle inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ near their nuclear localization signal such that they are retained in the cytoplasm, remaining inactive. In contrast, Mdm2 requires AKT phosphorylation for translocation to the nucleus, where it complexes with p53 promoting its ubiquitin/proteasome-mediated degradation (Testa and Bellacosa 2001). Thus, several tumor suppressors, including $p21^{WAF1}$, $p27^{Kip1}$, and p53, are inhibited by AKT, and this is specular to inhibition of the oncogenic PI3K/AKT axis by the tumor suppressor PTEN. Inhibition of p53 function is particularly relevant to the control of cell cycle checkpoints associated with DNA damage.

AKT also acts through various mechanisms to generate survival signals that prevent programmed cell death (Testa and Bellacosa 2001; Franke et al. 2003). It phosphorylates the proapoptotic factor BAD, and thereby stops cytochrome *c* from being released from mitochondria, and also phosphorylates (pro)caspase 9, thereby inhibiting the consequences of cytochrome *c* release. PED/PEA15, a cytosolic inhibitor of caspase-3, is also phosphorylated and stabilized by AKT (Trencia et al. 2003). AKT kinases deliver antiapoptotic signals involving positive and negative transcriptional mechanisms. AKT phosphorylation restricts nuclear entry of transcription factors of the Forkhead family, as it does for $p21^{WAF1}$ and $p27^{Kip1}$, preventing transcription of proapoptotic genes: *Fas ligand*, *BIM*, *TRAIL*, and *TRADD*. It phosphorylates and activates IκB kinase (IKK), causing degradation of IκB, and consequently stimulates translocation of NF-κB to the nucleus and transcription of *BFL1*, *cIAP1*, *cIAP2*, all antiapoptotic genes. AKT also phosphorylates and inactivates the apoptosis signal-regulating kinase, ASK1.

AKT kinases are also involved in intermediary metabolism, and in particular glucose metabolism. It phosphorylates and inactivates GSK3, resulting in increased glycogen synthesis. Note that GSK3 is also involved in the Wnt/wingless pathway that includes β-catenin and the tumor suppressor APC. Although AKT may thus interact with this pathway, any such interaction is probably indirect and very complex (Grille et al. 2003). Following insulin stimulation, glucose transport is increased by AKT phosphorylation of the glucose transporters GLUT1 and GLUT4, and their translocation to the membrane (Kohn et al. 1996), whereas AKT phosphorylation of phosphofructokinase stimulates glycolysis (Deprez et al. 1997). The relationship between the metabolic consequences of AKT activation and its antiapoptotic functions is complex (Gottlob et al. 2001; Plas et al. 2002), and AKT regulation of cell growth also reveals interplay between different AKT functions.

Work with animal models implicated AKT kinases in the control of cell growth. Cells grow (defined here as an increase in cell size rather than cell number) in response to increased availability of nutrients, energy, and mitogens. mTOR, the mammalian target of rapamycin, is a kinase downstream from PI3K that mediates cell growth pathways by stimulating protein synthesis. mTOR phosphorylates directly or indirectly two targets with immediate effects on translation: p70 ribosomal protein S6 kinase (p70 S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). p70 S6K phosphorylates the ribosomal protein S6, thereby increasing translation of mRNAs containing 5′-terminal oligopolypyrimidine (5′TOP) tracts. In contrast, phosphorylation of 4E-BP1 relieves inhibition of the initiation factor eIF4E such that the efficiency of cap-dependent translation is increased (Ruggero and Pandolfi, 2003).

mTOR is clearly activated downstream from AKT, but the activation mechanism has not been established. Although mTOR is a direct target of AKT in vitro, mTOR activation by AKT in vivo may be very complex. The tuberous sclerosis (TSC) 2 protein is one of the numerous targets of AKT signaling. Tuberous sclerosis complex, a hereditary disorder characterized by the formation of hamartomas in various organs, is a consequence of mutations in either *TSC1* or *TSC2* tumor suppressor genes. The TSC1 and TSC2 proteins form a complex in vivo, and the complex inhibits signaling by mTOR, possibly through TSC2 GTPase-activating protein (GAP) activity toward the Ras family small GTPase Rheb. TSC2 – one of many tumor suppressors antagonized by AKT – is phosphorylated and inhibited by AKT signaling (Inoki et al. 2002; Potter et al. 2002); this destabilizes TSC2 and disrupts its interaction with TSC1, leading to the activation of the mTOR/p70 S6 kinase/eIF4E pathways. Consequently, mTOR is a potential target for chemopreventive or chemotherapeutic treatment of tuberous sclerosis patients.

The mTOR pathway is activated in many human tumors, suggesting that tumorigenesis is associated with abnormal regulation of nutrient availability. However, cell size in tumors is rarely larger than that in normal tissues. Consequently, the abnormal activation of the AKT/mTOR pathway in tumors may be further evidence of interplay between different functions. Indeed, there is recent evidence that the mTOR/eIF4E pathway can provide an antiapoptotic signal, in addition to growth/translation control. It is also plausible that mTOR promotes chromosomal instability which is then selected during tumorigenesis (Aoki et al. 2003).

AKT phosphorylates and activates other targets implicated in cancer, including nitric oxide synthase (that promotes angiogenesis) and the reverse transcriptase subunit of telomerase (that stimulates unlimited replicative potential).

The number of known AKT substrates is growing but it is still not clear whether each of the various members of the AKT family has their own substrates or whether the specificities of AKT1, -2 and -3 are determined by their tissue distribution, temporal expression, or upstream activation. However, work on AKT activation using human cancer and animal models suggests that the family members are not completely redundant and may be differentially activated/inactivated in various physiological and disease states.

AKT and EMT

It has become evident that EMT is one of the many cellular processes subject to AKT kinase regulation. EMT driven by activated AKT (Grille et al. 2003) involves loss of cell–cell adhesion, morphological changes, loss of apico-basolateral cell polarization, induction of cell motility, reduced cell–matrix adhesion, and changes in the production or the distribution of various proteins. For example, desmoplakin, a protein involved in the formation and maintenance of desmosomes, is internalized, and vimentin, an intermediate filament protein found in many mesenchymal cells, is induced. AKT also induces production of metalloproteinases and cell invasion (Kim et al. 2001; Park et al. 2001; Irie et al. 2005).

GSK3 and EMT

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed protein serine kinase. It participates in glycogen metabolism and in both the Wnt/β-catenin and the PI3K/AKT signaling pathways; it has antiapoptotic and proliferative activities (Hoeflich et al. 2000) and is involved in differentiation and morphogenesis (Hoeflich et al. 2000; Tang et al. 2003).

GSK3 is also involved in EMT (Bachelder et al. 2005): treatment with a specific GSK3 inhibitor (SB415286) causes an EMT in human epithelial breast cancer cells (MCF10). Genetic inhibition of GSK3 activates Snail transcription in both MCF10 and human keratinocyte cells (HaCaT) (Zhou et al. 2004; Bachelder et al. 2005); pharmacological inhibition of GSK3 in HaCaT cells leads to the activation of NF-κB through IκB

From AKT to NF-κB

General Functions of NF-κB

The protein NF-κB is a transcription factor that binds to the DNA sequence gggACTTTCC that was originally found in the intronic enhancer of the immunoglobulin κ light chain in B cells. Through its stimulation of the transcription of various genes, including *c-myc*, *Ras*, and *p53*, NF-κB participates in numerous aspects of cell growth, survival, differentiation, and proliferation. However, one of its major known functions is in stress, injury, and especially immune responses. NF-κB is central to tumorigenesis, mainly in solid tumors, in which it is constitutively active and controls the expression and function of a number of pertinent genes (Pacifico and Leonardi, 2006). Active NF-κB switches on the expression of genes that promote proliferation and protect cells from proapoptotic conditions that would otherwise cause them to die. NF-κB can be constitutively active, and this is the consequence of mutations in genes encoding the NF-κB transcription factors

themselves and in genes that control NF-κB activity (such as IκB genes) or is a consequence of constitutive and abnormal secretion of NF-κB-activating factors.

NF-κB and EMT

NF-κB can induce EMT in breast, bladder, and squamous carcinoma cell lines (Huber et al. 2004; Chua et al. 2007; Julien et al. 2007; Wang et al. 2007b), and the induction is either indirect (Wang et al. 2007a) or direct (Chua et al. 2007; Julien et al. 2007). Indirect activation of EMT by NF-κB has been described in estrogen receptor (ERα)-negative breast cancer cells, and in particular MDA-MB-231 cells. In association with c-Jun/Fra-2, p50–p65 NF-κB stimulated the expression of RelB, and this induction of RelB led to the induction of Bcl-2; Bcl-2 then suppressed radiation-induced apoptosis and induced EMT. More direct activation of EMT by NF-κB is found in various cell lines, although the pathways involved, while sharing the same end target – the E-cadherin gene, were not all based on the induction of the same intermediate transcription factor (Snail or ZEB-1/ZEB-2).

Snail and Related Transcription Factors in EMT

Snail is a zinc finger transcription factor and the Snail family also includes Slug (also called Snai2) and Smuc (or Snai3 or Zfp293). Snail is best known as a repressor of transcription and it was identified as being essential for *Drosophila* development and, in particular, correct gastrulation.

Snail and Slug (encoded by Snai1 and Snai2 genes, respectively) together with the transcription factor Twist are involved in mesoderm formation. Snail and E-cadherin expressions are inversely correlated. Abnormal Snail production in numerous cell lines and primary tumors is associated with aggressiveness and loss of E-cadherin expression (Birchmeier and Behrens 1994; De Craene et al. 2005).

Snail or Slug overproduction in vitro induces EMT (Batlle et al. 2000); and repression of Snail RNA production is associated with E-cadherin upregulation and MET. E-cadherin is subject to an interesting positive–negative regulation (Palmer et al. 2004): it is positively regulated by $1,25(OH)_2D3$ via the vitamin D receptor and Snail can repress both E-cadherin and vitamin D receptor, so the balance between vitamin D receptors and Snail may regulate E-cadherin levels (Palmer et al. 2004). Phosphorylation of Snail by the p21-activated kinase PAK1 causes it to be retained in the nucleus and stimulates its repressor activity (Yang et al. 2005). These various observations indicate the complexity of E-cadherin regulation during EMT.

Sip1 (also known as ZFHX1B or SMADIP1) is a member of the delta EF1/Zfh1 family of two-handed zinc finger/homeodomain proteins. It contains a Smad-binding domain through which it interacts with full-length Smad proteins and may therefore modulate EMT induction by the TGFβ signaling pathway. Many patients with mega-colon or Hirschsprung disease carry mutations in the *Sip1* gene (Amiel

and Lyonnet, 2001; Cacheux et al. 2001; Wakamatsu et al. 2001; Yamada et al. 2001; Van de Putte et al. 2003). Mice with targeted inactivation of Sip1 have been obtained. These mice present clinical features of Hirschsprung disease–mental retardation syndrome: they fail to develop postotic vagal neural crest cells – the precursors of the enteric nervous system affected in patients with Hirschsprung disease – and display arrest in the delamination of cranial neural crest cells, which form the skeletal muscle elements of the vertebral head; in the absence of Sip1, the neural crest cells are not correctly delaminated. The delamination of neural crest cells is a good example of EMT requiring cadherin downregulation.

E-cadherin and EMT

E-cadherin is one of the main effectors of EMT, and many details of the regulation of E-cadherin signaling during EMT have been described. E-cadherin participates in both EMT and MET, and cells undergoing EMT necessarily downregulate E-cadherin.

E-cadherin and Epithelial Cells

The detailed overview of this system is provided in Chapter 3. Briefly, this cell–cell adhesion molecule is a calcium-dependent transmembrane glycoprotein. In particular, cadherin molecules associate at the cell surface in a Ca^{2+}-dependent manner through homophilic interactions, thus mediating cell–cell adhesion. It is found in most epithelial cells in both embryonic and adult tissues, and is essential for normal embryonic development and homeostasis. Consequently, there has been substantial interest in its regulation. Generally, both the transcription and the translation of cadherins are regulated, and the mechanisms include changes in subcellular distribution, translational and transcriptional events, and degradation. E-cadherin is classified as a tumor suppressor for two reasons: its gene is silent in various carcinomas, and re-expression of a native form of E-cadherin in carcinomas in vitro reduces the aggressiveness of tumor cells (Vleminckx et al. 1991). Further supporting its classification as a tumor suppressor, germline mutations of the E-cadherin gene (called *CDH1*) are associated with a syndrome of hereditary gastric and colorectal cancer (Guilford et al. 1998; Suriano et al. 2003).

The loss of E-cadherin function observed in some human carcinomas is associated with the production of a defective protein or transcriptional silencing due to promoter hypermethylation. Gene mutations, abnormal post-translational modifications (phosphorylation or glycosylation), and protein degradation (proteolysis) can all lead to the production of a defective E-cadherin protein. Cases of E-cadherin upregulation in tumor progression have also been reported (Kang and Massague 2004; Thiery and Morgan 2004) but only during intravasation and seeding of metastatic cells.

Alternatively, E-cadherin transcriptional repression may result from the activation of the repressors Snail, Slug, Sip1, and Ets. It is still not known how E-cadherin is internalized/sequestered or the E-cadherin gene repressed, but it has been demonstrated that AKT regulates E-cadherin mRNA and protein abundance (Grille et al. 2003). Two main types of consensus-binding sites have been shown to downregulate E-cadherin expression: Ets sites and palindromic E-boxes (E-pal).

Moreover, the loss of expression of E-cadherin during development and transformation is often associated with increased expression of N-cadherin. The molecular mechanisms underlying this widespread switch remain unclear.

E-cadherin Function Is Modulated by IGF

As mentioned above, IGF affects cell–cell adhesion. The cadherin/catenin complex is undoubtedly a critical determinant for cell–cell adhesion and, as a consequence, there has been substantial work on the signaling pathways that may link IGF-1R and the cadherin/catenin complex.

IGF-1R Interacts Indirectly with E-cadherin and β-catenin

As mentioned above, E-cadherin forms the physical link, resulting in cell–cell adhesion by binding adjacent cell surfaces, thus allowing the formation of large cellular networks and tissues. The cytoplasmic domain of cadherin binds to β-catenin, which binds to α-catenin. As a result, the cadherin/catenin complex is linked to the actin-based cytoskeleton. IGF-1R and E-cadherin are coexpressed in most epithelial cells; they also appear to form a membrane-associated complex as assessed by coimmunoprecipitation experiments (Guvakova and Surmacz 1997; Morali et al. 2001). Immunoprecipitation experiments also indicate that the interaction of IGF-1R with E-cadherin, β-catenin, and α-catenin does not impede the binding of cadherins to catenins. Presumably, there is a supra-molecular complex composed of IGF-1R/E-cadherin/β-catenin/α-catenin on the surface of various cells.

Only the cytoplasmic domain of E-cadherin is required to interact with the cytoplasmic domain of the IGF-1Rβ subunit (Morali et al. 2001). However, protein–protein interaction assays reveal that IGF-1R does not interact directly with either E-cadherin or β-catenin (Morali et al. 2001). The molecules linking members of the complex together have not been identified.

IGFs Redistribute Proteins of Adherens Junctions

Most E-cadherin and β-catenin are found at cell–cell contacts in epithelial cells not exposed to IGFs, and IGF-1R is present both at cell–cell contacts and in the cytoplasm. Treatment with IGFs results in the redistribution of E-cadherin and IGF-1R from the membrane to the cytoplasm, and E-cadherin becomes concentrated in a halo around the nucleus. It has been demonstrated that E-cadherin constantly cycles from the cytoplasm to the cell membrane and back (Bauer et al. 1998; Le et al.

1999). If IGFs are extremely abundant, this equilibrium may be perturbed, and E-cadherin is internalized more rapidly than it is readdressed to the membrane.

These various in vitro observations suggest that the expression of each IGF-II, IGF-1R, and E-cadherin is correlated during gastrulation. At this stage, IGF-II is mainly expressed by mesenchymal cells (mesoderm), E-cadherin is generally absent from murine mesodermal cells (Butz and Larue, 1995), and IGF-1R is found at the membrane of epithelial cells (ectoderm and endoderm) and in the cytoplasm of mesodermal cells.

It has also been suggested that E-cadherin degradation, despite not being extensive, is associated with IGF treatment (Morali et al. 2001). Indeed, a subset of E-cadherin molecules partially colocalize with LAMP1 (lysosomal-associated protein 1), an endosomal and lysosomal marker, consistent with E-cadherin being degraded in LAMP1-positive organelles. Thus, it appears that IGFs cause rapid internalization of E-cadherin, leading to some degradation and sequestration in vesicles near nucleus (Morali et al. 2001). This allows a simple model explaining the reversibility of the EMT: as soon as IGFs are removed from the medium, stored E-cadherin is rapidly readdressed to the membrane.

IGFs also determine the distribution of β-catenin. β-Catenin participates in cell–cell adhesion and in signal transduction through the Wnt signaling pathway. In the absence of Wnt, β-catenin is part of a complex containing GSK-3β (glycogen synthase kinase-3β), APC (adenomatous polyposis coli), and axin. GSK-3β phosphorylates β-catenin, which is then ubiquitinated and degraded by proteasomes (Yost et al. 1996; Aberle et al. 1997). Wnt binding to Frizzled, its cell surface receptor, activates the serine–threonine kinase Dishevelled (Dsh) (Yanagawa et al. 1995; Axelrod et al. 1998; Karasawa et al. 2002). Dsh then phosphorylates GSK-3β and thereby inhibits its activity. Unphosphorylated β-catenin (which cannot be degraded) accumulates in the cytoplasm and is translocated into the nucleus (for review, see Novak and Dedhar 1999). In the nucleus, a complex of β-catenin with TCF/LEF (T-cell factors/lymphoid enhancer factors) can induce or repress the expression of numerous genes including *Cyclin D1*, *c-Myc*, *T-Brachyury*, *c-Jun*, *Fra-1*, *Matrix-Metalloprotease-7* (*MMP-7*), *Fibronectin*, *Cyclo-oxygenase-2*, *m-Mitf* (melanocyte-specific microphthalmia transcription factor), receptors *EphB2* and *EphB3*, and *ephrin-B1* (He et al. 1998; Brabletz et al. 1999; Crawford et al. 1999; Gradl et al. 1999; Howe et al. 1999; Mann et al. 1999; Shtutman et al. 1999; Arnold et al. 2000; Takeda et al. 2000; Batlle et al. 2002). Some of these genes are expressed ubiquitously (e.g., *cyclin D1*) and others only in certain cell types (e.g., *m-Mitf* in the melanocyte lineage) (Amae et al. 1998; Fuse et al. 1999). IGF treatment results in the translocation of β-catenin from the cell membrane to the nucleus and of TCF3 from the cytoplasm to the nucleus (Morali et al. 2001). No single gene known to be activated/repressed by β-catenin can induce an EMT. Note that not all gene targets of β-catenin have been identified; some as yet unidentified target gene(s) may be involved in EMT. Also, the IGF signaling pathway may induce the expression of genes involved in EMT.

To conclude, IGFs induce a reversible EMT based on reducing the cell–cell adhesion involving E-cadherin and the redistribution of the proteins associated with it.

Indeed, IGF treatment causes (i) rapid delocalization of E-cadherin from cell–cell contacts, (ii) disruption of the interaction between E-cadherin and β-catenin by phosphorylation, (iii) induction of limited degradation of E-cadherin, (iv) activation of genes via β-catenin/TCF, and, self-evidently (v) induction of the IGF signaling pathway.

Other Regulators of E-cadherin

Ets-Binding Sites in the E-cadherin Promoter

An Ets-binding site has been identified at position -97 in the E-cadherin promoter, and indeed, the expression of c-ets-1 in breast carcinoma cell lines induces EMT, partly due to repression of the E-cadherin gene (Gilles et al. 1997; Rodrigo et al. 1999). Ets binding to this region downregulates E-cadherin promoter activity in keratinocyte cell lines (Rodrigo et al. 1999). Ets factors, in addition to being repressors of E-cadherin transcription, upregulate key mediators of invasiveness, including matrilysin, matrix metalloprotease, collagenase, heparanase, and urokinase (reviewed in Shepherd and Hassell, 2001; Hsu et al. 2004).

E-cadherin E-boxes

E-boxes are widespread in genomic sequences. The human E-cadherin promoter contains three E-box consensus sequences (CANNTG). Two are upstream from the coding sequence and one is in exon 1. Snail, Slug, Sip1/Zeb2, and Zeb1 bind to these E-boxes and repress E-cadherin transcription. Presumably, the E-cadherin gene is tightly regulated by the binding of these various transcription factors to its E-boxes.

Conclusions

There has been substantial work on EMT over the last 20 years, and some of the key molecular and cellular events involved have been identified. Also, the signaling pathways mediating the critical events that make up EMT have been described. Our improved insight into these molecular processes leading to and constituting EMT provides the basis for clinical application. In particular, novel therapies involving inhibition of EMT could be developed to prevent the various manifestations of cancer, and particularly local invasion and metastasis. A detailed understanding of the EMT pathways, and their involvement in cell physiology in general, would undoubtedly be beneficial for rational development of therapies with minimal effects on other cellular functions, and therefore minimal toxicity.

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