CHAPTER 5

Role of Transforming Growth Factor-β

in Human Cancer

Introduction

Compromised MMR function provides the molecular basis for a "mutator phenotype", meaning a dramatic predisposition to somatic mutations. Mutations involving proto-oncogenes, tumor-suppressor genes, and genes responsible for programmed cell death accumulate and subsequently may cause neoplastic transformation. Microsatellite foci in coding regions of certain growth regulatory genes, for instance, *PTEN*, *BAX*, *IGFRII*, *TGFBR2*, *hMSH6*, and *hMSH3* may significantly foster tumor progression.

TGFBR2 is one of the most commonly mutated genes in CRCs with MSI (up to 90% of tumors) [1]. These mutations arise from the insertion or deletion of one or two adenine bases in a ten-base-pair polyadenine stretch within a region that encodes the *TGFBR2* extracellular domain, and result in truncated and inactivated forms of the receptor. The cells in which the mutations take place attain a selective growth advantage allowing them to expand in clonal fashion and to acquire the additional hits necessary for progressive tumor development. However, patients that have lost *TGFBR2* from their tumors have a better prognosis than patients with sporadic colon cancer that retain functional TGF-β receptors [2]. It therefore appears that complete abrogation of TGF-β signaling, although leading to loss of growth control and early tumor onset, paradoxically has a protective effect on tumor progression.

The human TGF-β family comprises more than 30 factors that can be divided into two distinct groups: factors such as activin, nodal, lefty, myostatin, and TGF-β are clustered in one family, and bone morphogenetic proteins (BMPs), anti-muellerian hormone (AMH, also known as MIS), and various growth and differentiation factors (GDFs) are grouped into the other one (Figure 1) [3].

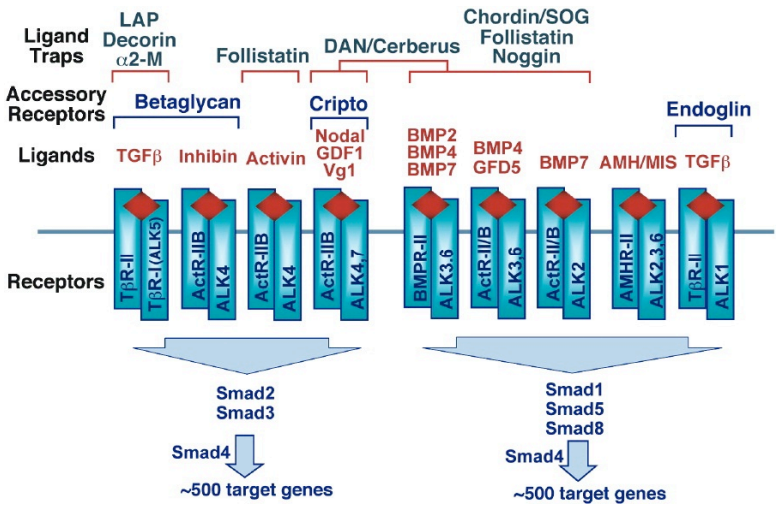

Figure 1. *Human TGF-β family.*

TGF-β binding induces the formation of a serine/threonine kinase complex. The type II receptors phosphorylate and activate the type I receptors that then phosphorylate a family of transcription factors, the Smads (Figure 2). Receptors of the TGF-β family phosphorylate Smads 2 and 3, whereas those of the other branch phosphorylate Smads 1, 5, and 8. Once activated, the receptor substrate Smads (RSmads) shuttle to the nucleus and form a complex with Smad4, a binding partner common to all RSmads [3]. The Smad4-RSmad complexes must associate with additional DNA-binding cofactors to achieve binding with high affinity and selectivity to specific target genes.

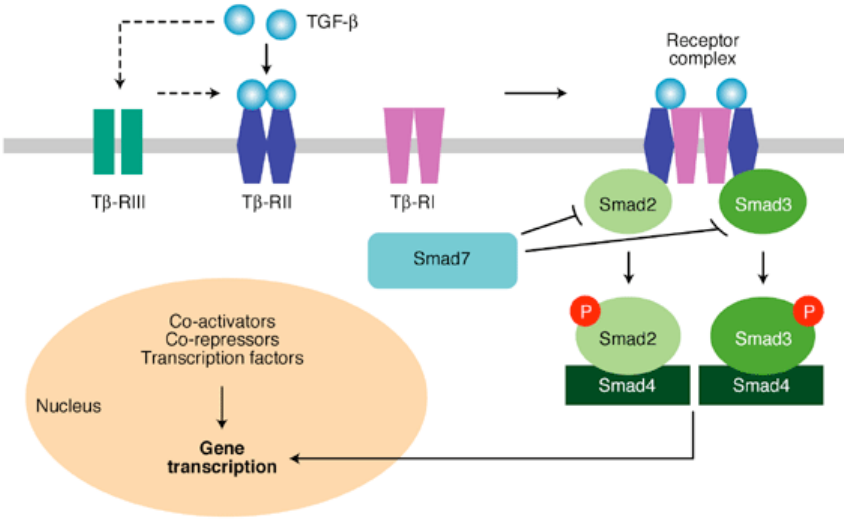

Figure 2. *The transforming growth factor β signaling pathway.*

Although TβRI, TβRII, Smad2, Smad3, and Smad4 comprise the core Smad-dependent TGF-β signaling pathway, Smad-independent signaling through RAS-ERK MAP kinase pathway, p38 MAP kinase and JNK signaling, as well as Rho GTPases signaling and the PI3 kinase/Akt pathway, have been reported (Figure 3) [4,5]. The precise molecular mechanisms by which the TGF-β signaling pathway signals to these pathways have not been established.

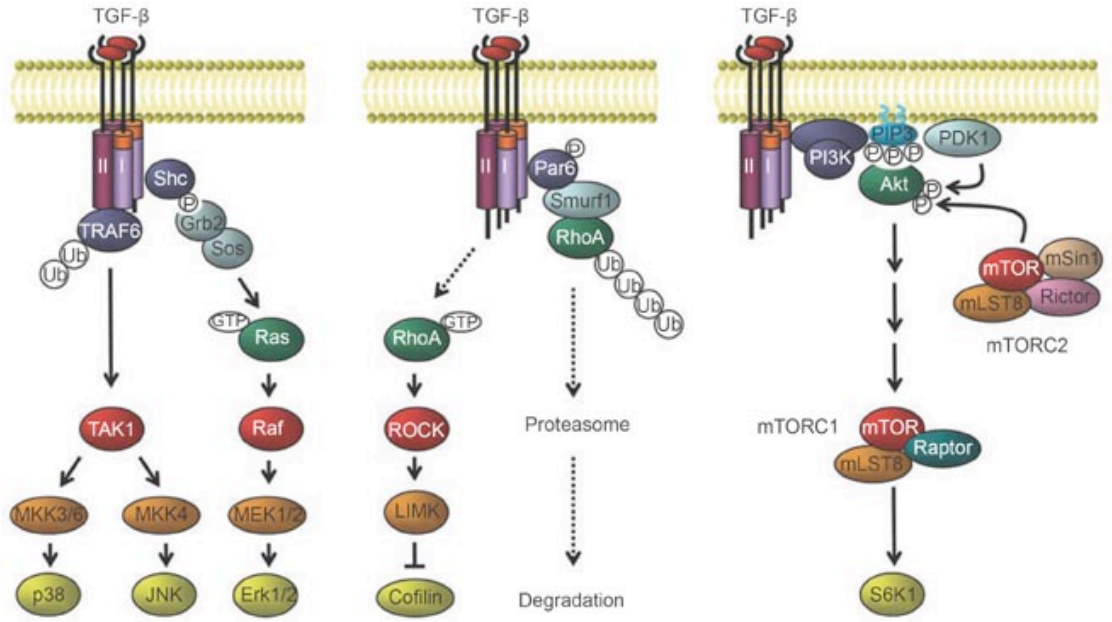

Figure 3. *TGF-β-dependent, Smad-independent signaling.*

TGF-β Signaling in Cancer: a Double-Edged Sword

TGF-β can act as both a tumor suppressor and as a significant stimulator of tumor progression, invasion and metastasis. At early stages of tumorigenesis it acts directly on the cancer cell to suppress tumor outgrowth. However, as the tumor progress TGF-β stimulate tumor progression by its pleiotropic activities on both the cancer cell *per se* and on non-malignant stromal cells types.

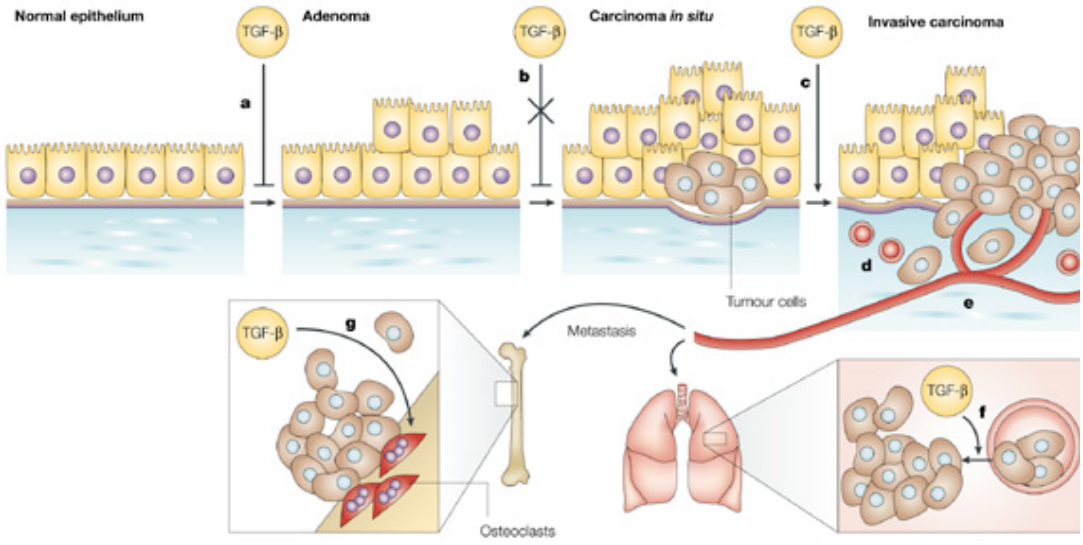

Figure 4. *Role of TGF-β in human malignancy.*

Resistance to Antiproliferative Signals, Independence From Exogenous Growth Signals and Evasion of Apoptosis

TGF-β potently inhibits epithelial, endothelial, and hematopoietic cell proliferation. TGF-β is able to prevent progression through the cell cycle by inducing expression of the cyclin kinase inhibitors $p15^{INK4b}$, $p21^{CIP1}$, and $p27^{KIP1}$, and by directly suppressing c-*myc* expression. However, virtually all epithelial-derived tumors (> 85% of all human cancers) become resistant to the growth-inhibitory effects of TGF-β. Whereas in some cancers,

including colon and pancreatic cancers, mechanisms for resistance are well defined, in most human cancers, including cancers of the breast, lung, and prostate, these mechanisms remain poorly defined. In the same time, TGF-β is able to increase the production of mitogenic growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), TGF-α, and to increase expression of the platelet derived growth factor receptor (PDGFR), and the epithelial growth factor receptor (EGFR). In addition, TGF-β can activate Smad independent pathways, including the RAS/Raf/MAPK pathway, which often mediates the proliferative signal of growth factors.

In addition to regulation of cell proliferation, TGF-β has been shown to induce apoptosis in a cell and context-specific manner. TGF-β-induced apoptosis may occur through both p53-dependent and p53-independent mechanisms, and involves caspase activation, upregulation of proapoptotic factors (ie, Bax), and/or downregulation of antiapoptotic factors (ie, Bcl-2 and Bcl-xL) [6,7]. The TGF-β signaling pathway also interacts with other pathways that regulate apoptosis. For example, TGF-β is able to enhance Fas-induced apoptosis, whereas activation of the PI-3 kinase/Akt pathway is able to inhibit TGF-β-mediated apoptosis [8,9]. Resistance to TGF-β-induced apoptosis may be an essential component of tumorigenesis, particularly for cancers arising from tissues in which TGF-β is a prominent regulator of apoptosis, such as hepatocellular carcinoma and prostate cancer. In addition, the ability of TGF-β to induce apoptosis in lymphocytes may be a critical component for the immunosuppressive effect of TGF-β during tumorigenesis.

Tissues Invasion and Metastasis

TGF-β is a potent regulator of cellular adhesion, motility, and the extracellular matrix. It regulates the adhesive properties of cells by decreasing the expression of the cell

adhesion molecule E-cadherin, by increasing the expression of invasion-associated integrins including $\alpha_{III}\beta_1$, and the expression of integrin-binding proteins including fibulin-5 [10,11]. TGF-β also directly increases the motility of epithelial cells and breast cancer cells [12,13]. TGF-β stimulates the production of the extracellular matrix by directly increasing the production of extracellular matrix proteins, decreasing the production of enzymes that degrade the extracellular matrix, and increasing the production of proteins that inhibit enzymes that degrade the extracellular matrix [14]. However, during tumorigenesis, TGF-β frequently stimulates the proteolytic activity of cancer cells by increasing the expression of matrix-degrading enzymes [15].

Therefore, by decreasing the adhesiveness and increasing the motility and proteolytic activity of cancer cells, increased levels of TGF-β result in more invasive and aggressive phenotype of cancer cells.

Induction of Angiogenesis

TGF-β can function either as a proangiogenic or antiangiogenic factor *in vitro*, whereas the preponderance of evidence supports its proangiogenic role *in vivo* . Several lines of evidence support a prominent role for the TGF-β signaling pathway in stimulating angiogenesis. First, targeted deletion of members of this pathway in mice, including TGF-β1, TβRI, and TβRII, all result in aberrant angiogenesis [16-18]. Second, two endothelial-specific TGF-β receptors, endoglin (a type III receptor in the TGF-β family) and ALK-1 (a type I receptor in the TGF-β family), are essential for angiogenesis as demonstrated by their mutation in the human vascular disorder hereditary hemorrhagic telangiectasia and by the embryonic lethal phenotype due to defects in angiogenesis exhibited by mice in which their expression has been abolished [19,20]. Third, expression of endoglin on endothelial

cells is dramatically increased during tumor-induced angiogenesis [21]. Finally, TGF-β induces the expression of vascular endothelial growth factor, which then directly promotes angiogenesis [22].

Evasion of the Immune System

Cancer cells express tumor-specific antigens that normally would be recognized by the immune system and lead to destruction of the cancer cell; during tumorigenesis, most cancer cells acquire the ability to evade this immunosurveillance. Although there are multiple mechanisms by which cancer cells evade an immune response, a major mechanism is active cancer cell-mediated immunosuppression via secretion of TGF-β, which is a potent immunosuppressive cytokine [23]. The immunosuppressive effects of TGF-β are mediated predominantly through effects on T cells and antigen presenting cells (APCs). TGF-β is produced by T cells and blocks production of interleukin 2 (IL-2) to inhibit IL-2-dependent proliferation of T cells [24]. TGF-β also inhibits the differentiation of T cells, and prevents naïve T cells from acquiring effector (cytotoxic or helper) functions [25]. TGF-β may also mediate some of its immunosuppressive effects on T cells through $CD4^+CD25^+$ regulatory T cells, which both secrete TGF-β1 and express cell surface-bound TGF-β1 [26]. These effects of TGF-β on T cells have been validated in murine models. TGF-β1-deficient mice develop a severe autoimmune phenotype leading to death by 3 weeks, in part, from overactive T cells, and T-cell-specific abrogation of TGF-β signaling in mice results in spontaneous T-cell activation and the development of an autoimmune disease of the lung and colon [27,28]. TGF-β also has potent effects on APCs. Macrophages secrete TGF-β, which inhibits tissue macrophage activation [29]. TGF-β also is required for differentiation of dendritic cells from precursors, primarily by protecting

their viability [30]. *In vivo*, TGF-β-deficient mice have a complete absence of Langerhans cells in the epidermis, although they express functional precursors, suggesting that TGF-β is required for normal Langerhans cell development and/or migration to the epidermis [31].

Induction of Epithelial to Mesenchymal Transition

TGF-β is a potent inducer of epithelial to mesenchymal transition (EMT) in development and pathology [32,33]. In cardiogenesis, TGF-β has been shown to play a key role in the EMT that occurs in the atrioventricular canal and the outflow tract region and, accordingly, the expression of TGF-β1 and TGF-β2 increases at the onset of EMT in the atrioventricular canal endothelium and myocardium, respectively [34]. Severe cardiac abnormalities including defective atrioventricular junction are observed in TGF-β1 and TGF-β2-deficient mice. TGF-β signaling has also been implicated in the EMT that is associated with palatal morphogenesis. TGF-β3 null mice present a cleft palate, resulting from the lack of fusion of the two palatal shelves [35].

Several lines of evidence implicate increased TGF-β signaling as a key effector of EMT in cancer progression and metastasis. Transfection of a dominant-negative TβRII into spindle skin carcinoma cell lines reduced their tumorigenicity *in vivo* and forced the formation of differentiated squamous carcinomas in contrast to the fibroblastoid spindle tumors formed by the parental cell line [36]. Similar observation were also made using a Ras-transformed mammary epithelial cell system and a fibroblastoid colon CT26 carcinoma cell line [37]. In addition to a mesenchymal to epithelial reversion of CT26 cells *in vitro*, transfection of a dominant-negative TβRII mutant into this highly metastatic carcinoma line also resulted in complete suppression of metastasis *in vivo*. Interestingly, the restoration of

TβRII in tumor cells that carry a mutation in this gene made the resultant cells more invasive *in vivo*.

Various studies have explored the roles of TGF-β-activated Smads in EMT. Increased expression of Smad2 or Smad3 with Smad4 induces EMT, or enhances the induction of EMT by the activated form of TβRI, in normal murine mammary gland (NMuMG) cells, whereas expression of dominant negative versions of Smad2 or Smad3 blocks TGF-β-induced EMT in this cell system [4,38]. Similarly, RNA interference-mediated knockdown of Smad4 expression or expression of a dominant negative mutant of Smad4 results in preserved E-cadherin expression, suppression of fibrotic type I collagen synthesis *in vitro*, and decreased bone metastasis *in vivo* [4,39-41]. Furthermore, genetic ablation of Smad4 leads to preservation of epithelial markers and a lower degree of EMT in adenocarcinoma [42].

Several non-Smad signaling have been involved in TGF-β-induced EMT. Between these pathways the activation of ERK MAP kinases signaling contributes to and may even be required for TGF-β-induced EMT. Increased RAS-ERK MAP kinase signaling, e.g. in response to growth factor stimulation or due to expression of mutant *RAS*, enhances TGF-β-induced EMT, as apparent by the morphological changes and downregulation of E-cadherin expression [43-46]. Consistent with this cooperation, blocking the kinase function of MEK1/2 using a chemical inhibitor, thus resulting in inactivation of the ERK1/2 MAP kinases, inhibits TGF-β-induced EMT [47].

The Epithelial to Mesenchymal Transition

The epithelial to mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components (Figure 5) [48].

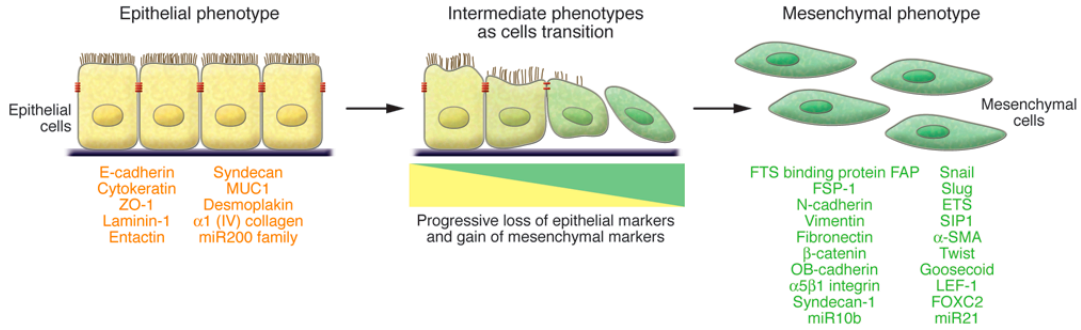

Figure 5. *Epithelial to Mesenchymal Transition.*

EMTs are encountered in three distinct biological settings that carry very different functional consequences (Figure 6) [49].

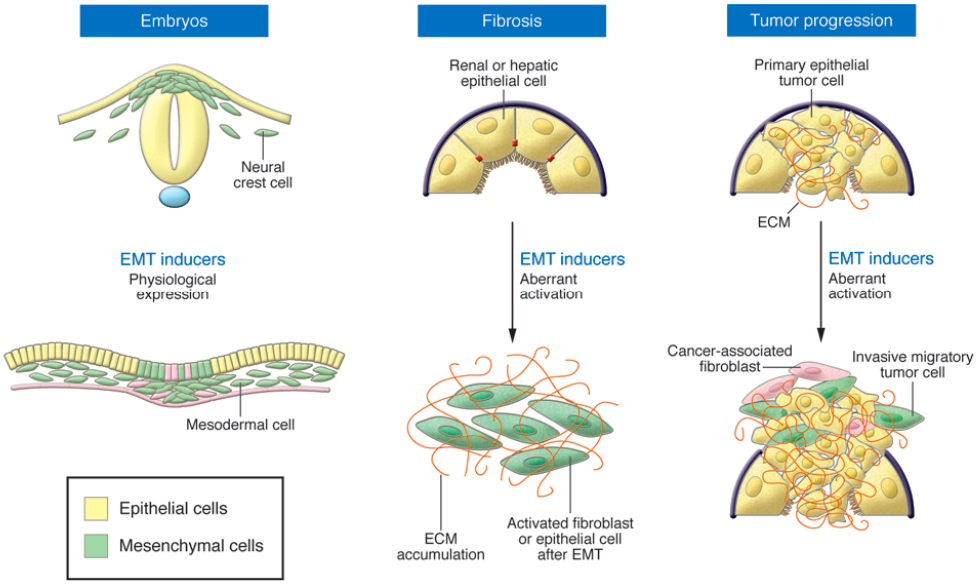

Figure 6. *EMT in development and diseases.*

The first class of EMTs, "type 1", is associated with implantation, embryo formation, and organ development. Primary EMT events (i.e., those that occur in tissues that have never undergone a previous EMT process) take place during implantation of the embryo into the uterus, during gastrulation, and during neural crest formation in amniotes. The EMTs associated with wound healing, tissue regeneration, and organ fibrosis are of a second type. In these "type 2" EMTs, the program begins to reconstruct tissues following trauma and inflammatory injury, and cease once inflammation is attenuated. In the setting of organ fibrosis, type 2 EMTs can continue to respond to ongoing inflammation, leading eventually to organ destruction. Tissue fibrosis is in essence an unabated form of wound healing due to persistent inflammation. Type 3 EMTs occur in neoplastic cells that have previously undergone genetic and epigenetic changes, notably affecting oncogenes and tumor suppressor genes that favor clonal outgrowth and the development of localized tumors. Carcinoma cells undergoing a type 3 EMT are typically seen at the invasive front of primary tumors and are considered to be the cells that eventually enter into subsequent steps of the invasion-metastasis cascade, i.e., intravasation, transport through the circulation, extravasation, formation of micrometastases, and ultimately colonization (the growth of small colonies into macroscopic metastases) (Figure 7) [50,51].

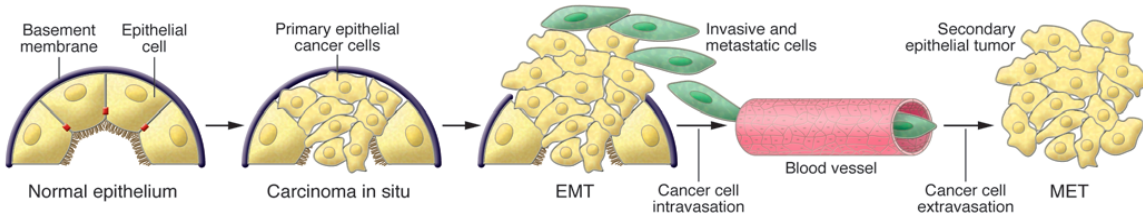

***Figure 7**. Contribution of EMT to cancer progression.*

EMT: Loss of Epithelial and Acquisition of Mesenchymal Characteristics

During EMT, expression of E-cadherin and cytokeratins, hallmarks of epithelial cell protein expression, is repressed, while the expression of vimentin, an intermediate filament component of the mesenchymal cell cytoskeleton, is induced. At the same time, expression of a typical fibroblastic marker, N-cadherin is often acquired in place of E-cadherin.

Of all these proteins, the transmembrane E-cadherin molecule plays the dominant role in influencing epithelial versus mesenchymal cell phenotypes. In normal epithelia, the ectodomains of E-cadherin molecules extend from the plasma membrane of one epithelial cell to form complexes with other E-cadherin molecules protruding from the surface of an adjacent epithelial cell. This enables homodimeric (and higher-order) bridges to be built between adjacent cells in an epithelial cell layer, resulting in the adherens junctions that are so important to the structural integrity of epithelial cell sheets (Figure 8). The cytoplasmic domains of individual E-cadherin molecules are tethered to the actin fibers of the cytoskeleton via a complex of α- and β-catenins and other ancillary proteins.

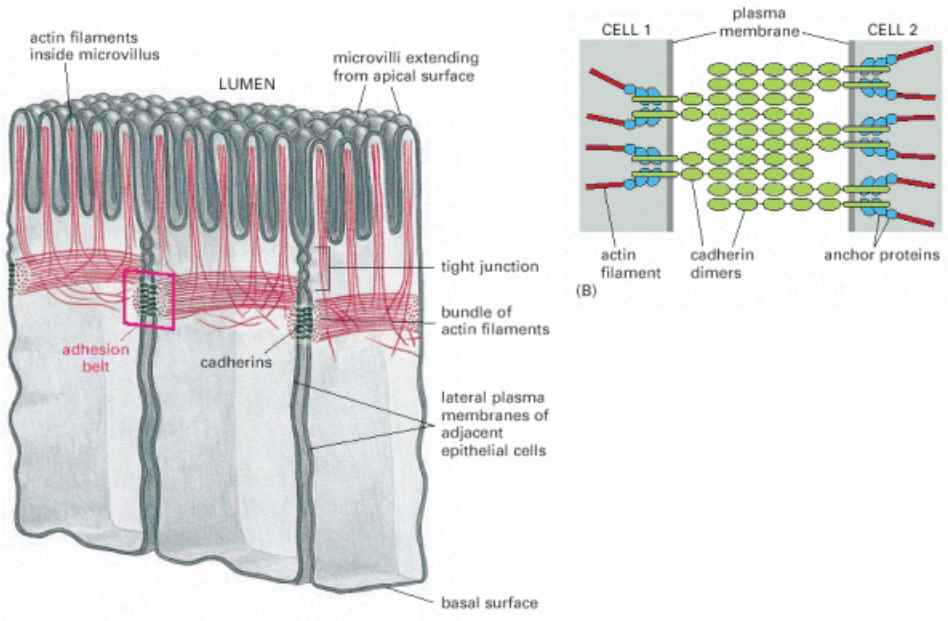

Figure 8. *Adherens junctions.*

The connection between loss of E-cadherin expression by cancer cells and passage through an EMT has been established by many studies. For example, induction of the *c-Fos* oncogene in normal mouse mammary epithelial cell lines induces an EMT and is associated with a decrease in E-cadherin expression [52]. Moreover, epithelial cell adhesion complexes reorganize and cell proliferation is suppressed when the full-length or the cytoplasmic portion of E-cadherin is ectopically expressed in cells that have passed through an EMT, causing such cells to lose their mesenchymal phenotype [52,53]. Cell lines that lack E-cadherin show increased tumorigenicity and metastasis when transferred into immunodeficient mice [54]. E-cadherin expression levels vary dramatically in different human tumors, and an inverse relationship between levels of E-cadherin and patient survival has been documented [55]. Once E-cadherin expression is suppressed, many of the other cell-physiologic changes associated with the EMT seem to follow.

Like E-cadherin, the N-cadherin that is produced in its stead binds to other molecules of the same type displayed by nearby cells, including the stromal cells that normally display N-cadherin. Importantly, the acquisition of N-cadherin expression does not result in the assembly of large sheets of cancer cells that might, in principle, be created by the formation of cell-cell N-cadherin bridges. It seems that the intermolecular bonds formed between pairs of N-cadherin molecules are far weaker than those formed by E-cadherin homodimers. This helps to explain why cell surface N-cadherin molecules actively favor cell motility, and thus behave very differently from E-cadherin, which function to immobilize cells within epithelial cell layers.

Mechanisms of EMT Activation

EMT is generally induced in epithelial cells by signals released by the mesenchymal cells that constitute the stroma of normal and neoplastic tissues. Although members of the TGF-β family of cytokines are the main and the best characterized inducers of EMTs, other EMT-inducing signals are becoming to emerge. Hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), Notch, integrins, Wnt, hypoxia and matrix metalloproteinases, all can induce EMT through multiple different signaling pathways, and the relative importance of each of these may depend on the particular cellular context. Each of these signaling pathways is responsible for the induction or functional activation of a series of EMT-inducing transcription factors. Once activated each of these transcription factors cooperate to choreograph the complex EMT program. Three families of transcription factors, the Snail, ZEB, and bHLH families have been involved in such process (Figure 9).

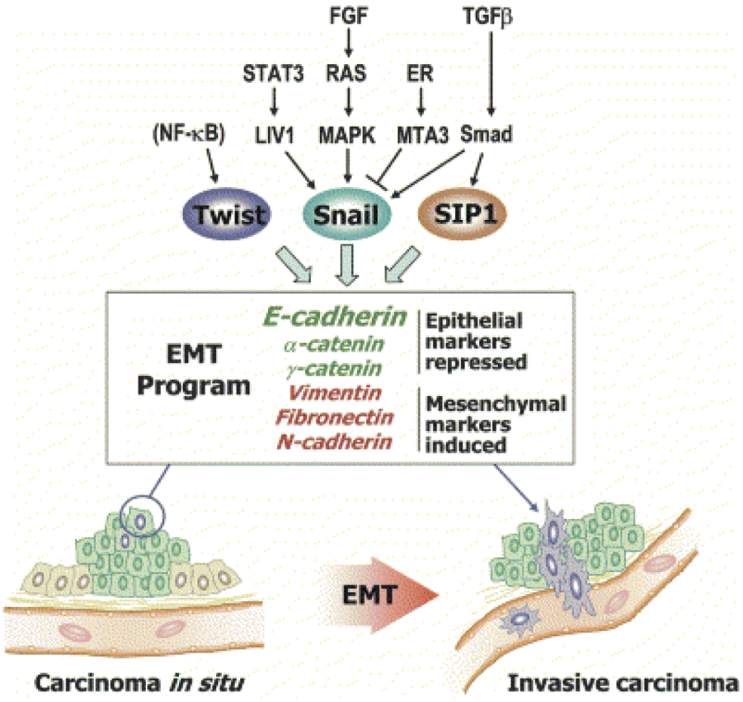

Figure 9. *Drivers and mediators of EMT.*

The Snail transcription factors function as repressors of transcription by recognizing and binding to E-box elements in their cognate target promoters. Three Snail family proteins have been identified in vertebrates: Snail1 (first described as Snail), Snail2 (also known as Slug) and a more recently characterized Snail3. Snail induction has been observed in several EMT processes. Snail expression is induced by TGF-β during skin and palate development, as well as during heart development, in mesothelial cells during pathological fibrosis, in cultured hepatocytes, and in multiple epithelial cell lines [56-61]. Induction of EMT by HGF, FGF, or EGF, also results in the induction of Snail expression [62,63]. Ectopic expression of Snail or Slug suppresses E-cadherin and plakoglobin expression and enhances vimentin and fibronectin expression, leading to a full EMT phenotype, whereas silencing of Snail expression reverses this process [64-68]. The expression of Snail and E-cadherin correlates inversely with the prognosis of patients with breast cancer or oral squamous cell carcinoma [69,70]. In addition to E-cadherin, Snail repress a spectrum of genes involved in maintaining epithelial structure and function, including genes encoding claudins and occludin, major transmembrane components of tight junctions, whereas alter the distribution, from peripheral localization to a diffused cytoplasmic pattern, of cytoplasmic components of tight junctions, such as ZO-1 and p120 [71]. Snail proteins regulate as well the expression of desmosome proteins and a subset of cytokeratins, thus affecting the epithelial cytoskeletal organization. While repressing epithelial gene expression, Snail proteins activate the expression of the mesenchymal proteins fibronectin, vitronectin and N-cadherin, the extracellular matrix proteins collagen type III and V, and proteins involved in migration and invasion, such as RhoB, plasminogen activator inhibitor-1 and matrix metalloproteinases. Snail also regulates the expression of multiple actin-modulating proteins to facilitate the rearrangement of actin

filaments from cortical distribution to stress fibers anchored to focal adhesions [72]. Consistent with the indirect induction of gene expression by Snails, Snail transcription factors induce the expression of other EMT-related transcription factors, such as Twist in MDCK cells, and ZEB1 and ZEB2 in squamous carcinoma cell lines [73-75].

Two ZEB family transcription factors are known in vertebrates: ZEB1, also known as δEF1 or AREB6, and ZEB2, also known as Smad-interacting protein 1 (SIP1). Similar to Snail and Slug, ZEB proteins can be induced by TGF-β and other growth factors that activate RAS-MAPK signaling and by Wnt/β-catenin signaling [76]. The expression of ZEB factors is also post-transcriptionally repressed by microRNAs, miR-200 family and miR-205 [77]. This group of microRNAs is dramatically downregulated in cells undergoing EMT, allowing expression of ZEB transcription factors.

The induction of ZEB proteins is necessary for the downregulation of E-cadherin expression and promotion of cell migration. ZEB2 directly represses the expression of the tight junction proteins claudin-4 and ZO-3 [78]. ZEB2 also suppresses the expression of the desmosome protein plakophilin-2 and induces the expression of the mesenchymal proteins vimentin, N-cadherin and matrix metalloproteinase-2 through as yet unknown mechanism(s) [75,78,79]. Both ZEB proteins promote cell migration and induce invasion and ZEB1 has also been implicated in the downregulation of epithelial polarity.

The HLH family is a large family of transcription factors controlling a wide array of developmental and pathological processes. HLH factors are divided into seven categories based on their tissue distribution, dimerization capability and DNA-binding specificity. Among these, the class I proteins E12 and E47, class II proteins Twists and class V proteins

Ids are involved in EMT. E12 and E47 directly repress E-cadherin expression through DNA binding at the E-box element in the proximal promoter. Ectopic expression of E12 or E47 represses E-cadherin and plakoglobin expression, induces vimentin and fibronectin expression, and promotes migration and invasion [67,80,81]. The HLH protein Twist1 is a major regulator of mesoderm formation in *Drosophila* and neural tube closure in mice, suggesting its involvement in developmental EMT [82]. Mutation of the gene are associated with an autosomal-dominant craniosynostosis (Saethre-Chotzen syndrome), which is characterized by cleft palate, and are possibly responsible for inhibition of EMT during palatal fusion [83]. Twist has been recently identified as an important regulator of EMT *in vitro* and *in vivo* in metastatic and invasive carcinomas. Ectopic expression of Twist in MDCK cells causes transcriptional repression of E-cadherin, α-, β- and γ-catenins, and induction of mesenchymal markers fibronectin, vimentin, and N-cadherin. *In vivo* studies suggest that elevated expression of Twist may be responsible for pulmonary metastases of mammary carcinoma [84].

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