

# TGF- $\beta$ and epithelial-to-mesenchymal transitions

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**Remarkable phenotype plasticity of epithelial cells underlies morphogenesis, epithelial repair and tumor invasiveness. Detailed understanding of the contextual cues and molecular mediators that control epithelial plasticity will be required in order to develop viable therapeutic approaches targeting epithelial-to-mesenchymal transition (EMT), an advanced manifestation of epithelial plasticity. Members of the transforming growth factor (TGF- $\beta$ ) family of growth factors can initiate and maintain EMT in a variety of biological systems and pathophysiological context by activating major signaling pathways and transcriptional regulators integrated in extensive signaling networks. Here we will review the distinct physiological contexts of EMT and the underlying molecular signaling networks controlled by TGF- $\beta$ .**

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On the basis of their distinct shape and organization during embryonic development, epithelial and mesenchymal cell types have already been described in the late 19th century. While interconversions between epithelial and mesenchymal states have been proposed almost a century ago, Greenburg and Hay were the first to describe that epithelial cells in culture may acquire mesenchymal features providing proof of principle for the process of epithelial-to-mesenchymal transition (EMT) (Greenburg and Hay, 1982). Early discoveries in EMT resulted largely from studies of embryonic development.

During development, EMT and the reverse process, mesenchymal-to-epithelial transition have been well-documented (reviewed in Hay, 1995). Developmental EMT is the process of disaggregating structured epithelial units to enable cell movement and morphogenesis.

The observation that conditioned fibroblast medium could induce ‘scattering’ of polarized epithelial cells into separate, single cells led to the isolation of hepatocyte growth factor (HGF) (Stoker and Perryman, 1985; Nakamura *et al.*, 1989) and demonstrated that EMT may be induced by extracellular stimuli through activation of kinase-dependent signaling cascades. Transforming growth factor- $\beta$  (TGF- $\beta$ ) was first described as

inducer of EMT in normal mammary epithelial cells by signaling through receptor serine/threonine kinase complexes (Miettinen *et al.*, 1994). During the 1990s, the process of EMT gained wide recognition as candidate mechanism in progression of malignant and chronic fibrotic disorders. In cancer progression, EMT was associated specifically with tumor invasiveness and intravasations and extravasations of metastatic cells (reviewed in Thiery, 2002). EMT may contribute to the degeneration of mature epithelial structures and to the generation of fibroblasts associated with accumulation of extracellular matrix (ECM) in chronic fibrotic disorders (reviewed in Kalluri and Neilson, 2003).

In parallel with the emergence of the EMT paradigm in carcinogenesis and fibrosis, a large body of work established new roles for TGF- $\beta$ s as important mediators of tumor progression, and in progression of chronic fibrotic disorders, in particular chronic kidney disease (reviewed in Border and Noble, 1994; Derynck *et al.*, 2001; Böttinger and Bitzer, 2002; Roberts and Wakefield, 2003). This review will examine the overlapping and distinct features of EMT in development, tumor progression, and nonmalignant degenerative disorders leading to tissue fibrosis with emphasis on overlapping and distinct mediators of TGF- $\beta$  signaling, depending on these three (patho)physiological contexts.

## Phenotypic elements (‘modules’) of EMT

EMT is a complex, extreme manifestation of epithelial plasticity (Thiery, 2002). In principle, polarized epithelial cells embedded in organized stratified or single cell layers convert into single fibroblastoid cells capable of locomotion. Prerequisite cellular changes include release of cells from epithelial polarity, remodeling of epithelial cell–cell and cell–matrix adhesion contacts and of their actin cytoskeleton. In addition, these motile fibroblastoid cells activate molecular programs capable of simultaneous degradation and *de novo* synthesis of ECM. These capabilities in matrix remodeling enable motile transitioning cells to invade through basement membranes and continue migration in ECM, a process that defines in particular tumor invasiveness. Here we term these distinct, inter-related cellular changes as phenotypic modules, which together define the complete EMT phenotype in both, nonmalignant and malignant cells subjected to morphogenic pressure (Table 1). Analysis of phenotypic modules of EMT stimulated by

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**Table 1** Phenotypic modules of EMT and metastasis

Category	Transitioning event	Phenotypic module
EMT	Epithelial release	Disintegration of cell–cell contacts Loss of cell polarity Repression of epithelial markers
	Migration	Cytoskeletal reorganization Locomotion
	Invasiveness	Basement membrane degradation Interstitial matrix degradation Interstitial matrix synthesis
Metastasis	Intravasation/ extravasation	Anoikis resistance
Apoptosis resistance	Metastatic growth	Autonomous growth potential
Endothelial cell adhesiveness		Angiogenesis factors

TGF- $\beta$  in various cultured epithelial cells suggests that they occur in a coordinated temporal sequence of disassembly of cell junctions, cytoskeletal reorganization, loss of epithelial polarity, and remodeling of cell–matrix adhesions (Zavadil *et al.*, 2001). Although it has not been determined whether these events are strictly interdependent *in vivo*, we will briefly introduce the key phenotypic elements of EMT as distinct modules in this order for the purpose of clarity. Malignant cells may acquire additional unique properties as an extension of common EMT that enable intravasations and extravasations and to sustain metastatic growth (Table 1). While ‘metastatic potential’ is often equated with EMT in tumors, it is clear that metastasis formation requires tumor cell properties that are not characteristic of EMT, including anoikis- and apoptosis-resistance, endothelial cell adhesiveness, autonomous growth, and angiogenic factors (Table 1). Thus, oncogenic EMT usually manifests in a genetic and cellular signaling background that is highly abnormal and distinct from nononcogenic EMT.

#### *Disintegration of cell–cell adhesions and loss of epithelial polarity*

A hallmark of EMT is the disintegration and disassembly of cell–cell junctions, including tight junctions, desmosomes, adherens junctions and gap junctions that maintain the integrity of epithelial units and support barrier function and juxtacrine cell–cell signaling.

*Tight junctions* are located in the most apical lateral regions and seal the space between adjacent cells to prevent transport of molecules through the intercellular space. At a molecular level, tight junctions are mediated by transmembrane claudins, occludins and scaffold proteins such as ZO1 (TJP1), which associates with the intracellular actin cytoskeleton and various signaling systems. During EMT, tight junctions dissolve and their essential protein components are downregulated. Recent studies yielded new detailed insights into the mechanisms of disassembly of tight junctions stimulated by TGF- $\beta$ . Par6 is a key component of epithelial polarity complexes regulating assembly of tight junctions (Hurd *et al.*, 2003). TGF- $\beta$  ligand binding enables type II

TGF- $\beta$  receptor kinase, that is associated with occludin at tight junctions, to phosphorylate Par6 (Ozdamar *et al.*, 2005). This protein–protein interaction is direct and independent of Smad proteins (Ozdamar *et al.*, 2005). Phosphorylation of Par6 allows it to recruit Smurf1 which in turn leads to ubiquitination and degradation of RhoA (Ozdamar *et al.*, 2005), a small GTPase family member responsible for stress fiber formation and for the maintenance of apico-basal polarity and junctional stability (Perez-Moreno *et al.*, 2003). The important new insight provided by these observations is that TGF- $\beta$  may alter cell surface protein complex structure and levels directly through its receptor complex independent of nuclear gene regulation.

*Adherens junctions* are mediated by homotypic interactions of the extracellular domains of E-cadherin. Intracellular domains link the protein with actin cytoskeleton via  $\alpha$ - and  $\beta$ -catenins. Extensive research has focused on the mechanisms of disintegration of adherens junctions associated with redistribution and repression of the E-cadherin gene product. Transcriptional repression of E-cadherin is mediated by members of the *Snail/Slug* family of transcriptional repressors, and their critical role in EMT has been confirmed by studies of ablation of snail in developing mouse (Carver *et al.*, 2001). Other repressors of E-cadherin have been identified, such as Slug, SIP1, Twist, LIV1, MTA3 or  $\delta$ EF, and we review their significance for EMT in additional detail in the following section on signaling. At the protein level, disassembly of E-cadherin-dependent adherens junctions is mediated by proteolytic processing by presenilin-1 (PS-1/ $\gamma$ -secretase) (Marambaud *et al.*, 2002). Disassembly of E-cadherin-mediated adherens junctions in TGF- $\beta$ -induced EMT in human keratinocytes is blocked by  $\gamma$ -secretase inhibitor (Zavadil *et al.*, 2004); however, whether this is caused by a specific action of PS1 on E-cadherin or by inhibition of Notch receptor activation needed for EMT remains to be established. Dysadherin, a membrane-associated glycoprotein, has been established as a novel regulator of E-cadherin protein levels and its elevated expression has been linked to metastatic properties of carcinomas (Ino *et al.*, 2002; Hirohashi and Kanai, 2003). Thus, modulation of E-cadherin expression levels is emerging as an important theme of epithelial plasticity in both, nononcogenic and oncogenic EMT.

*Desmosomes* support the integrity of the epithelial unit and cell–cell adhesions through cadherin molecules (desmoglein and desmocollin) and link the cell–cell adhesion molecules with cytoskeletal keratin fibers by desmosomal plaque proteins such as desmoplakin and plakoglobin (Getsios *et al.*, 2004). Conditional ablation of desmoplakin in the mouse epidermis reveals a role of the protein in formation and maintenance of epithelial sheets (Vasioukhin *et al.*, 2001). Disassembly of desmosomes at the onset of cytokine-induced EMT is regulated by Slug, a zinc-finger transcriptional repressor that inhibits the desmoplakin and desmoglein genes to facilitate progression of EMT (Savagner *et al.*, 1997).

As cell–cell junctions dissolve, epithelial units release individual cells with disappearing polarized organiza-

tion. Epithelial polarity markers such as apical actin-binding transmembrane protein mucin-1 (MUC1) are either redistributed, or downmodulated (Guaita *et al.*, 2002). In addition, integrin  $\alpha 6 \beta 4$ -mediated contacts between epithelial cells and basal lamina are replaced by mesenchymal integrin  $\alpha 5 \beta 1$  (Maschler *et al.*, 2005).

#### Cytoskeletal remodeling

Polarized epithelial cells are characterized by cortical filamentous actin bundles that are connected with intracellular juxtamembranous components of cell adherens junctions. Loss of apico-basolateral polarity and dissociation of intercellular junctions is accompanied by fundamental remodeling of the actin cytoskeleton from cortical actin to actin stress fibers, a hallmark of migratory, mesenchymal cells (Miettinen *et al.*, 1994; Piek *et al.*, 1999; Bakin *et al.*, 2000; Zavadil *et al.*, 2001). Active EMT signaling events induced by TGF- $\beta$  target guanine nucleotide exchange factors (GEFs) to activate GTPases of the Rho family, which in turn regulate the cytoskeletal remodeling associated with the gain of cell motility (Bhowmick *et al.*, 2001a; Shen *et al.*, 2001). Activation of Rho leads to *de novo* formation of stress fibers composed of polymerized F-actin filaments bundled with myosin II filaments responsible for contractility of these cytoskeletal structures (Ridley and Hall, 1992; Kimura *et al.*, 1996). Rho activation also leads to formation of focal adhesions harboring specific structural proteins and integrins that mediate the communication of fibroblastoid cells with ECM (Ridley and Hall, 1992). Activation of other members of the Rho GTPase family, Cdc42 and Rac, leads to actin polymerization associated with filopodia, or lamellipodia and membrane ruffles, respectively (Ridley and Hall, 1992; Hall, 1998). In addition to cytoskeletal remodeling, RhoA is involved in E-cadherin clustering during adherens junction formation while Rac1 and Cdc42 GTPases control interactions of adherens junctions and actin filaments (Fukata and Kaibuchi, 2001). Thus, Rho family GTPases and their regulatory molecules (GEFs and GAPs) exert important, pluripotent roles in epithelial plasticity and are essential effectors of EMT induced by TGF- $\beta$  and other stimuli.

#### Cell-matrix adhesion

In order to dissociate from their epithelial unit and to migrate through interstitial ECM, transitioning cells need to resolve integrin-mediated cell-matrix (basal lamina) contacts and digest/dissolve adjacent basal lamina. Basal lamina is a specialized ECM structure composed of collagen type IV, laminin and nidogen. Remodeling of the cell contact with basal lamina involves cooperation or direct activation of proteolytic enzymes, such as matrix metalloproteases MMP2 and MMP9 by cytokines such as TGF- $\beta$  or FGF2 (Strutz *et al.*, 2002; Li *et al.*, 2003). Increased synthesis and activation of MMP2 and MMP9 (gelatinase A and B, respectively) in response to TGF- $\beta$  leads to degradation of the collagen type IV component of basement membrane. Interestingly, inhibition of collagen IV

assembly is sufficient to induce EMT in murine renal epithelial cells (MCT) *in vitro* (Zeisberg *et al.*, 2001) and the activity of MMP2 is similarly required and sufficient to induce renal tubular EMT (Cheng and Lovett, 2003). The expression of specific integrins such as  $\alpha v \beta 6$  as a consequence of the EMT in colon cancer models enables invasive cells to interact with interstitial matrices and to sustain activation of TGF- $\beta$  (Bates and Mercurio, 2005). Importantly,  $\alpha v \beta 6$  integrin expression in human colon cancers is a marker of cells that have undergone EMT and it is prognostic for tumors that will progress more rapidly to terminal disease (Bates and Mercurio, 2005). TGF- $\beta$  induces the adapter protein Dab2 in epithelial cells concomitant with EMT. Dab2 binds integrin  $\beta 1$  and is required for activation of integrin  $\beta 1$  by TGF- $\beta$ . Block of Dab2 inhibits integrin activation, adherence, and results in apoptosis of transitioning cells and inhibition of EMT (Prunier and Howe, 2005).

#### Molecular marker proteins associated with EMT

EMT is characterized by loss of proteins associated with polarized epithelial phenotype and *de novo* synthesis of proteins associated with mesenchymal, migratory morphology of transitioning cells. Demonstration of absence or presence of select epithelial and mesenchymal proteins is widely used to demarcate states of epithelial plasticity of transitioning cells *in vitro* and *in vivo* in embryonic development, tissue homeostasis and epithelial stress/injury in mature organs, and in carcinoma progression. However, while loss of epithelial proteins such as MUC1, E-cadherin, ZO-1, desmoplakins, and cytokeratin 18, in cells of epithelial units clearly defines epithelial dedifferentiation, significant ambivalence and confusion complicates the use of gain of mesenchymal proteins to define transitioning states of cells in disintegrating epithelial units and in mesenchymal cells. An example is the mesenchymal intermediate filament protein vimentin. *De novo* expression of vimentin correlates with downmodulation of epithelial cytokeratins and has been proposed as canonical marker of the fibroblastoid state of transitioning cells (Franke *et al.*, 1982; Boyer *et al.*, 1989). Vimentin expression has been suggested as a critical marker to distinguish 'true, complete EMT' from 'cell scattering' or 'partial EMT' (Janda *et al.*, 2002a; Grunert *et al.*, 2003). However, vimentin expression is not specific for fibroblastoid cells, as it is also present in leukocytes, endothelial cells, and in some carcinomas without signs of dedifferentiation or EMT (Czernobilsky *et al.*, 1985; Auersperg *et al.*, 1994). These observations confound the use of vimentin as EMT marker *in vivo*, in particular in conditions that are associated with tissue inflammation. In addition, TGF- $\beta$  may induce *de novo* synthesis of vimentin in primary epithelial cells in the absence of EMT (J Zavadil, unpublished results).  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) is often used as marker for transitioning cells *in vivo*, however, its expression is limited to an 'activated' subpopulation of fibroblasts characterized by myofibroblast behavior (Serini and Gabbiani, 1999; Iwano *et al.*,

2002). Expression of Ca<sup>2+</sup>-binding protein fibroblast-specific protein 1 (FSP1), also known as S100A4 protein, has been described as fibroblast specific and is considered a marker for transitioning cells in EMT, in particular in EMT associated with renal epithelial stress or injury (Strutz *et al.*, 1995; Okada *et al.*, 1997; Iwano *et al.*, 2002). However, two recent reports indicate that FSP1 is expressed in cells expressing mononuclear markers in injured kidney, calling into question the specificity of FSP1 as marker for fibroblasts and EMT (Inoue *et al.*, 2005; Le Hir *et al.*, 2005). Therefore, widely reported interpretations of these markers as unequivocal molecular readouts for transitioning cells typical of EMT should be revisited as it is clear now that not all interstitial cells that carry these markers are EMT-generated transitioning cells of fibroblastoid lineage and, at a minimum, vimentin and  $\alpha$ -SMA can be induced in interstitial cells in contexts other than EMT.

**Physiological context defines distinct roles and features of EMT**

In general, EMT has been described in three major physiological and pathophysiological contexts, including embryonic development and morphogenesis, cancer progression and metastasis, and chronic degenerative, fibrotic disorders of mature organs. Based on its key morphologic and phenotypic features, it is widely assumed that EMT is a uniform process regardless of context. However, this assumption seems overly simplistic and may be misleading. A comparison of the basic scope and outcomes of EMT in embryonic development, cancer progression and chronic degenerative tissue injury highlights clearly distinct roles and features of EMT depending on physiological context (Table 2). In embryonic development, EMT affects tissues on a ‘global’ scale that is temporally and spatially highly coordinated. For example, EMT facilitates gastrulation and formation of a three-layered embryo. Whole epithelial sheets can undergo reversible or irreversible EMT in a highly synchronized process. EMT underlies organogenesis in particular of heart, musculoskeletal system, craniofacial structures such as palate, and peripheral nervous system (reviewed in Hay, 1995). *In vivo* evidence demonstrates an essential role for TGF- $\beta$ 3 in EMT of palate fusion in the mouse (Kaarinen *et al.*, 1995) and for TGF- $\beta$ 2 in EMT in the atrioventricular (AV) canal in the chick heart (Romano and Runyan, 2000).

In cancer progression, ‘oncogenic’ EMT refers to clusters of malignant cells that lose epithelial characteristics and acquire self-sustained migratory and highly matrix invasive cell phenotypes. Oncogenic EMT is

well-documented *in vivo* and typically considered as ‘complete’ and ‘irreversible’ EMT. However, transitioning carcinoma cell clusters are autonomous cellular entities that are uniquely characterized by self-sufficient autocrine loops of mitogenic signaling and mechanisms to evade apoptosis and anoikis (Derynck *et al.*, 2001; Gotzmann *et al.*, 2004). Thus, EMT in cancer cells describes merely one manifestation in the context of a spectrum of inter-related, fundamentally different and permanent reprogramming of cellular behaviors that is intrinsic to malignant cells. A growing number of *in vivo* studies demonstrate that inhibitors of TGF- $\beta$  or TGF- $\beta$  receptors may reduce the metastatic and/or invasive properties of a variety of experimental cancers presumably by preventing activation of EMT pathways (Dumont and Arteaga, 2003; Ge *et al.*, 2004; Subramanian *et al.*, 2004; Yingling *et al.*, 2004). Thus, there is broad experimental support for an important role of TGF- $\beta$  in oncogenic EMT associated with cancer progression, in particular in cooperation with oncogenic Ras (Derynck *et al.*, 2001; Dumont and Arteaga, 2003; Roberts and Wakefield, 2003; Gotzmann *et al.*, 2004).

Least well understood and least accepted is the process of EMT that is described in the context of epithelial stress and/or injury in kidney, liver and lung. With few exceptions, *in vivo* evidence for EMT in situations of epithelial stress is scant and difficult to document because of the isolated and possibly transient nature of the event. In contrast with oncogenic EMT, nonmalignant transitioning cells are difficult to track, making it difficult to determine their exact fate and scope. In fact, proof of concept has only recently been reported in an elegant study using genetic engineering in mice (Iwano *et al.*, 2002). In biopsies of diseased human kidney, single epithelial cells in tubular structures may show molecular evidence for EMT by coexpression of epithelial and mesenchymal markers (Rastaldi *et al.*, 2002; Vongwiwatana *et al.*, 2005). Although EMT has been widely adopted as an important mechanism that underlies epithelial degeneration and tissue fibrosis, the extent to which transitioning fibroblastoid cells contribute to accumulation of fibroblasts and ECM remains still unclear. The overwhelming majority of reports in support of EMT are based on the experimental model of unilateral ureteral obstruction (UUO) which is characterized by a strong inflammatory component and rapid progression to tubulointerstitial fibrosis of the obstructed kidney (Kalluri and Neilson, 2003). The UUO model is an acute renal injury model and mimics a very rare form of renal epithelial injury in humans. In contrast, the overwhelming majority of chronic renal disease is caused by primary and systemic glomerular

**Table 2** Physiological context of EMT

Context	Scope	Outcome	In vivo evidence	TGF- $\beta$ signaling
Embryonic development	Global (complete tissue)	Tissue conversion, morphogenesis	Widely accepted	In some morphogenic processes
Cancer progression	Focal (cell clusters)	Tumor invasiveness, metastasis	Widely accepted	In most common carcinoma
Epithelial stress/injury	Focal (single cells)	Epithelial degeneration, fibrosis	Scant, emerging	In most forms of tissue injury

disease, in particular diabetic nephropathy, glomerulonephritis and hypertension. Evidence for EMT in experimental models or human samples of these diseases is rare (Oldfield *et al.*, 2001; Rastaldi *et al.*, 2002). In addition, the ultimate fate of nonmalignant transitioning fibroblastoid cells remains unclear and it is not known whether the mesenchymal cell state is reversible or irreversible. In contrast, there is a large body of *in vitro* evidence to indicate that EMT manifestations may be triggered rather easily in nonmalignant epithelial cells of renal, pulmonary, or hepatic origins by hypoxic (Manotham *et al.*, 2004) or oxidative stress signals (Rhyu *et al.*, 2005), inflammatory stimuli (Fan *et al.*, 2001), wounding, and metabolic factors (Oldfield *et al.*, 2001) (also reviewed in Yang and Liu, 2001; Kalluri and Neilson, 2003).

In light of these considerations, EMT serves to describe phenotypic features (modules) that are common manifestations of distinct biological processes with fundamentally different scope and outcomes, depending on the (patho)physiologic context (developmental EMT, oncogenic EMT, nononcogenic EMT). Thus, the so-called 'universal' regulators and pathways controlling processes that manifest as EMT should to be viewed with caution. We assume that a variety of different and modifiable molecular signaling networks and mediators are capable to cooperate to induce manifestations consistent with EMT, depending on the physiological context and type of epithelia. At a minimum differentiation of oncogenic EMT and nononcogenic EMT provides an initial approach to organize context-dependent EMT signaling pathways and mediators, including TGF- $\beta$  (see Table 3 for overview).

### TGF- $\beta$ signaling in context-dependent networks associated with EMT

#### TGF- $\beta$ ligands

Depending on the physiological context, secreted inducers of EMT include TGF- $\beta$ , basic FGF (FGF2), EGF, IGF-II, and HGF (reviewed in Thiery, 2002). While TGF- $\beta$  signaling is typically associated with induction and maintenance of EMT, the effects of FGF2 and HGF are variable and highly context dependent. For example, FGF2 has been established as an inducer of partial EMT in kidney cells (Strutz *et al.*, 2002), but is not required for EMT associated with

injury of lens epithelium (Tanaka *et al.*, 2004). Interestingly, HGF signaling induces EMT in early developmental stages during somitogenesis and development of cardiac cushion through its receptor c-Met, while it may inhibit TGF- $\beta$ -induced EMT and fibrosis in the UOU injury of mature kidney (Mizuno *et al.*, 1998), possibly by inducing Smad transcriptional corepressor SnoN (Yang *et al.*, 2005). Importantly, BMP7, a member of the TGF- $\beta$  superfamily, inhibits TGF- $\beta$ -induced EMT *in vitro*, and prevents EMT manifestations associated with renal fibrosis *in vivo* in murine models (Zeisberg *et al.*, 2003a, b). In mature epithelia, EMT can be initiated by TGF- $\beta$  through autocrine or paracrine activation of intracellular signaling molecules that trigger transcription-independent regulatory events (Ozdamar *et al.*, 2005) and extensive transcriptional reprogramming of the epithelial cell (Zavadil *et al.*, 2001).

#### Role of Smads

In response to TGF- $\beta$ -ligand binding, TGF- $\beta$  receptors type I and II form tight complexes leading to phosphorylation of Smad2 and Smad3, members of the Smad protein family (Massague, 2000). Phosphorylated Smads partner with cytoplasmic Smad4 and translocate to the nucleus where Smad complexes control transcription of target genes through interaction with specific binding motifs in their gene regulatory regions (Derynck and Zhang, 2003). While the relative functional roles of Smad2 and Smad3 remain to be fully delineated, recent studies suggest that the majority of TGF- $\beta$  target genes are controlled through Smad3-dependent transcriptional regulation (Yang *et al.*, 2003). Experimental *in vivo* models of EMT and fibrosis, using Smad3 knockout mice, consistently demonstrate that Smad3 deficiency ameliorates epithelial degeneration and fibrosis, for example in a lens injury model (Saika *et al.*, 2004a), in proliferative vitreoretinopathy (Saika *et al.*, 2004b), and in UOU-induced tubulointerstitial fibrosis (Sato *et al.*, 2003) associated with EMT. Consistent with these observations, TGF- $\beta$  fails to induce EMT, and is unable to induce key transcriptional regulators of EMT, in primary tubular epithelial cells derived from kidneys of *Smad3* knockout mice (Zavadil *et al.*, 2004). In contrast, hepatocytes isolated from liver-specific *Smad2* knockout mice spontaneously transition to mesenchymal phenotype in the absence of TGF- $\beta$ , indicating that Smad2 may function to maintain an epithelial phenotype, which is contrary to the functional role of Smad3 (W Ju and EP Böttinger, unpublished results). Overexpression of inhibitory Smad7 consistently blocks Smad3-dependent EMT *in vitro*, including retinal pigment epithelium (Saika *et al.*, 2004b), mammary epithelial cells (Valcourt *et al.*, 2005), and in the renal UOU model *in vivo* (Lan *et al.*, 2003). The role of Smad3 in EMT and fibrosis has been extensively reviewed recently (Flanders, 2004).

Essential roles of Smad2 and/or Smad3 have also been demonstrated in EMT associated with tumor progression models. Smad2 and H-ras cooperate to

**Table 3** Modulators and effectors of TGF- $\beta$  signaling in EMT

Extracellular factors	Signaling	Nuclear regulators
HGF	Smad3	Snail/Slug
EGF	Smad7	Id2
FGF2	Rho GTPases	Twist
BMP7	Ras/Raf	Hey1, Hes1
Jagged	PI3K	$\delta$ EF1
Wnt	Notch	SIP1
	GSK3 $\beta$	Fos
	NF- $\kappa$ B	LEF1
	P38 MAPK	E2A

mediate EMT and metastasis formation (Oft *et al.*, 2002). Overexpression of Smad2 and Smad3 resulted in increased EMT in a mammary epithelial model (Piek *et al.*, 1999; Valcourt *et al.*, 2005). Reduction of Smad2 and Smad3 function, or overexpression of the type I TGF- $\beta$  receptor mutant that lacks the ability to bind Smad2/3 but is capable of activating non-Smad signaling, was associated with increased tumorigenicity of the primary tumors, but decreased metastatic potential of xenografted breast cancer cell lines (Tian *et al.*, 2003; Tian *et al.*, 2004). These studies support a central role for receptor-regulated Smads in TGF- $\beta$ -dependent EMT associated with tumor progression and metastasis. However, while Smads may be absolutely required for TGF- $\beta$ -induced EMT in carcinogenesis and epithelial stress/injury, context-dependent specification of EMT clearly involves the engagement of Smad-independent TGF- $\beta$  pathways and cooperation with TGF- $\beta$ -independent signaling systems.

#### TGF- $\beta$ and activated Ras

Numerous aspects of functional cooperation between Ras and TGF- $\beta$  suggest a rather complex relationship of these two major oncogenic pathways in EMT signaling (Oft *et al.*, 2002; Gotzmann *et al.*, 2004). Cooperative signaling between TGF- $\beta$  and Ras/Raf/MEK/MAPK is required for maintenance of complete EMT in various epithelial cell types. In mammary epithelial cells, mutant Ha-Ras activation promotes EMT through autocrine production of TGF- $\beta$  and continuous TGF- $\beta$  signaling through TGF- $\beta$ RI. Activated Raf can induce secretion of TGF- $\beta$  leading to autocrine TGF- $\beta$  stimulation and maintenance of irreversible invasive phenotypes *in vitro* (Lehmann *et al.*, 2000). Hyperactive Raf/MAPK activity is required for metastatic features of EMT *in vivo* (Oft *et al.*, 1998; Janda *et al.*, 2002a).

#### TGF- $\beta$ and ERK MAPK

Strong evidence exists for crosstalk and cooperation between TGF- $\beta$  and mitogen-activated protein kinase (MAPK) ERK. ERK (p44MAPK) is rapidly activated by TGF- $\beta$  in the context of growth arrest (Hartsough and Mulder, 1995). TGF- $\beta$  stimulates ERK activity in culture models of EMT (Ellenrieder *et al.*, 2001; Zavadil *et al.*, 2001; Xie *et al.*, 2004). ERK function is required for disassembly of adherens junctions as well as cell motility as part of the TGF- $\beta$ -induced EMT program (Zavadil *et al.*, 2001). In mammary gland epithelial cells undergoing EMT, TGF- $\beta$  transcriptionally induced components of MAPK signaling including Ras, Mek1/2 and Erk1/2 (Xie *et al.*, 2003) and activation of Erk1 (p42MAPK) (Xie *et al.*, 2004). Pharmacological inhibition of MEK upstream of ERK1/2 blocks key morphological features such as disassembly of E-cadherin-mediated adherens junctions in all these models (Ellenrieder *et al.*, 2001; Zavadil *et al.*, 2001; Xie *et al.*, 2004). A transcriptome screen of HaCaT keratinocytes stimulated to undergo EMT with TGF- $\beta$  in absence or presence of inhibitor of MEK/ERK MAPK

identified ~80 EMT-related targets of ERK MAPK (Zavadil *et al.*, 2001). This subset is enriched for genes with defined roles in cell–matrix interactions, cell motility, and endocytosis, suggesting that ERK controls cell motility and disruption of adherens junctions (Zavadil *et al.*, 2001). Interestingly, one of the targets of EMT-related function of ERK is tissue plasminogen activator (tPA, PLAT) (Zavadil *et al.*, 2001), a positive regulator of *MMP9*. *MMP9* deficiency in mice significantly reduces renal interstitial fibrosis and tubular EMT induced by UUO (Yang *et al.*, 2002).

#### TGF- $\beta$ and p38 MAP kinase

Induction of EMT by TGF- $\beta$  in cultured mouse mammary epithelial cells (NMuMG) involves rapid activation of the MKK3/6-p38MAPK-ATF2 pathway, dependent on both T $\beta$ RI and T $\beta$ RII receptor kinase activities (Bakin *et al.*, 2002). Interestingly, activation of p38 MAPK by TGF- $\beta$  in NMuMG cells depends on integrin-mediated cell adhesion (Bhowmick *et al.*, 2001b), and p38 is required for TGF- $\beta$ -induced EMT and apoptosis, but not growth arrest (Yu *et al.*, 2002). Synergy of TNF- $\alpha$  and TGF- $\beta$  signaling requires p38 MAPK activation to promote a rapid morphological conversion of a colonic carcinoma epithelium model to dispersed cells with a mesenchymal phenotype (Bates and Mercurio, 2003). Thus, current evidence supports a cell type-dependent role for p38 MAPK in TGF- $\beta$ -induced EMT in mammary gland epithelium and colon cancer.

#### TGF- $\beta$ and Jagged/Notch

Several recent reports suggest functional interactions between TGF- $\beta$ /Smad and Notch signaling in various tissues, based on hierarchical activation of one pathway by the other, or by coordinate regulation of common target genes. In keratinocytes induced to undergo EMT by TGF- $\beta$ , Notch pathway activation downstream of TGF- $\beta$  is suggested by early upregulation of the Notch ligand *Jagged1*, the Notch target genes *HES1*, and *TLE3* (Zavadil *et al.*, 2001). Follow-up studies demonstrate that a subset of the family of classical Notch target genes, basic-helix–loop–helix (bHLH) transcriptional repressors of the hairy/enhancer-of-split-related (H/Espl) family, including *HEY1*, *HEY2*, *HES1*, and *HES5*, and the Notch ligand *Jagged1*, are induced by TGF- $\beta$  at the onset of EMT in a panel of epithelial cells from mammary gland, kidney tubules, and epidermis (Zavadil *et al.*, 2004). TGF- $\beta$ -induced EMT is prevented by silencing of *HEY1* or *Jagged1*, and by chemical inactivation of Notch. These findings suggest functional integration of TGF- $\beta$ /Smad3 and *Jagged1*/Notch signaling in EMT (Zavadil *et al.*, 2004). Induction of *Jagged1* protein is upregulated in tubular epithelium subjected to UUO in mice (Morrissey *et al.*, 2002). Interestingly, an opposite hierarchy of the two pathways is observed during mouse embryonic heart development, where Notch and its downstream target and constitutive transcriptional repressor RBP-Jk (CBF1) are required for the expression of TGF- $\beta$ 2 and TGF- $\beta$  receptors and

for EMT of the endocardial cells mediated by positive regulation of Snail and repression of VE-cadherin (Timmerman *et al.*, 2004). It is possible that functional integration of TGF- $\beta$ /Smad and Jagged1/Notch is involved in skin cancer progression, as both, TGF- $\beta$  and Notch may exert overlapping oncogenic activities characterized by EMT (Cui *et al.*, 1996; Weijzen *et al.*, 2002).

#### TGF- $\beta$ and WNT/GSK3/ $\beta$ -catenin

$\beta$ -catenin exerts dual roles as a lateral membrane component of adherens junctions that colocalizes with E-cadherin and mediates its contact with actin cytoskeleton, and as a transducer and transcriptional coactivator of WNT signals. In renal proximal tubular cells, TGF- $\beta$  induced  $\beta$ -catenin is required for synthesis of  $\alpha$ -SMA as a marker of EMT (Masszi *et al.*, 2004). In a mammary gland model,  $\beta$ -catenin transcriptional activity is activated in FosER-induced EMT, concomitantly with loss of E-cadherin, and leads to activation of autocrine TGF- $\beta$  signals contributing to maintenance of the mesenchymal phenotype (Eger *et al.*, 2004). Glycerol synthase kinase (GSK3), a key member of the inhibitory complex targeting  $\beta$ -catenin for degradation in the absence of WNT signals, plays a role in EMT by regulating the nuclear localization and by inhibiting the activity of zinc-finger transcription factor Snail, an E-cadherin repressor. GSK3 thus maintains the integrity of adherens junctions and of epithelial phenotypes (Zhou *et al.*, 2004; Bachelder *et al.*, 2005). Interestingly, LEF1, the downstream target of WNT/ $\beta$ -catenin signaling is activated by TGF- $\beta$ 3 in a  $\beta$ -catenin-independent, Smad-dependent process, during EMT underlying the process of palatal fusion (Nawshad and Hay, 2003). Ectopic expression of LEF1 in the presence of stabilized nuclear  $\beta$ -catenin can also induce EMT directly (Kim *et al.*, 2002); however, the role of TGF- $\beta$  signaling in this process, if any, remains unclear.

#### TGF- $\beta$ and NF- $\kappa$ B

Recent studies identified NF- $\kappa$ B transcription factor as another key modulator of TGF- $\beta$ -induced EMT in mammary epithelial cells overexpressing Ras oncogene (Huber *et al.*, 2004). Inhibition of NF- $\kappa$ B blocked EMT in these cells, while its ectopic activation induced mesenchymal phenotypes independently of TGF- $\beta$  and its inhibition in mesenchymal cells restored the epithelial phenotype. Thus, a cooperation of TGF- $\beta$ , Ras and NF- $\kappa$ B is critical for epithelial plasticity manifested by EMT and the finding is consistent with the observation that NF- $\kappa$ B positively regulates expression of Snail and is itself inhibited by upstream GSK3, the main mediator of E-cadherin levels and of epithelial maintenance (Zhou *et al.*, 2004; Bachelder *et al.*, 2005).

#### TGF- $\beta$ and PI3K

During EMT in mammary epithelial cells TGF- $\beta$  activates phosphatidylinositol-3-OH kinase (PI3K) in a RhoA-dependent manner, and PI3K-Akt signaling is required for migration of breast cancer cells and for

ZO-1 downmodulation and disassembly of intercellular junctions (Bakin *et al.*, 2000). Interestingly, some of the features of EMT such as cell motility may overlap with PI3K-dependent cell scattering induced by HGF (Royal and Park, 1995; Day *et al.*, 1999). In contrast, in a model of multistep carcinogenesis in which TGF- $\beta$  cooperates with active oncogenic Ras to induce EMT, PI3K protects cells from TGF- $\beta$ -activated programmed cell death, but is not required for EMT (Janda *et al.*, 2002a). PI3K is not required for c-Raf1-activated EMT (Lan *et al.*, 2004) or for Ras-induced EMT (Janda *et al.*, 2002b). These studies suggest that the role of PI3K as effector of TGF- $\beta$ -induced EMT is probably limited.

#### Transcriptional regulators and control of EMT

A wide array of transcription factors are involved in regulating EMT, and, perhaps not surprisingly, TGF- $\beta$  is capable of controlling expression of a significant fraction.

#### *Snail/Slug*

The Snail/Slug zinc-finger proteins function as repressors of transcription by recognizing E-box elements in their cognate target promoters. Snail represses transcription of the E-cadherin gene in cultured cells (Battlle *et al.*, 2000; Cano *et al.*, 2000) and during mesoderm formation in early embryonic development (Carver *et al.*, 2001). Snail activity is regulated by various signaling pathways at multiple levels. For example, selective phosphorylation by GSK3 leads to its inhibition by ubiquitination and degradation, (Zhou *et al.*, 2004; Bachelder *et al.*, 2005; Yook *et al.*, 2005). WNT signals inhibit this function of GSK3, resulting in activation of Snail and repression of E-cadherin (Zhou *et al.*, 2004; Bachelder *et al.*, 2005; Yook *et al.*, 2005). TGF- $\beta$  activates both *Snail* and *Slug* directly through Smad3, however, comparison of multiple nonmalignant cell culture models of EMT indicates that the pattern of activation is mutually exclusive and cell type dependent (Zavadil *et al.*, 2004). Slug is also rapidly induced by FGF and HGF, and is responsible for downmodulation of desmoplakin and desmoglein and thus effectively regulates disassembly of desmosomes (Savagner *et al.*, 1997). In the developing chicken heart, Slug is required for the initiation of EMT as a downstream target of TGF- $\beta$ 2 (Romano and Runyan, 2000). While elevated expression of Slug frequently correlates with sole redistribution of E-cadherin from the adherens junction into cytoplasmic compartments rather than with its downmodulation (Savagner *et al.*, 1997; Zavadil *et al.*, 2001), ectopic expression of Slug results in repression of E-cadherin transcription via proximal E-box elements in the E-cadherin promoter and is sufficient to induce complete EMT in MDCK kidney cells (Hajra *et al.*, 2002; Bolos *et al.*, 2003). Interestingly, dissociation of E-cadherin-mediated junctions can lead to autoregulatory induction of Slug and subsequent repression of E-cadherin transcription (Conacci-Sorrell *et al.*, 2003). A role for Slug was also proposed in EMT associated with re-epithelization of cutaneous wounds, while Snail

was not involved (Savagner *et al.*, 2005), consistent with differential expression of these genes observed in TGF- $\beta$ -induced EMT in keratinocytes, renal tubular epithelial cells, and mammary epithelial cells (Zavadil *et al.*, 2004).

#### $\delta$ EF1, SIP1, Fos

Transcriptional downmodulation of E-cadherin in the context of carcinogenesis-related EMT is also exerted by two related zinc-finger transcriptional repressors of the  $\delta$ EF family,  $\delta$ EF1 (Eger *et al.*, 2005) and Smad-interacting protein-1 (SIP1, ZFX1B) (Comijn *et al.*, 2001). Similar to Snail and Slug, SIP1 can be induced by TGF- $\beta$  signals in a cell type-dependent manner (Zavadil *et al.*, 2004). SIP1 may associate with and regulate transcriptional activity of Smad proteins (Postigo *et al.*, 2003), and ectopic expression of SIP1 in MDCK cells is sufficient to induce dissociation of adherens junctions and increased motility (Comijn *et al.*, 2001). Similarly,  $\delta$ EF1 directly interacts with the E-cadherin promoter and its ectopic expression in mammary gland epithelial cells causes an invasive mesenchymal phenotype transition similar to that observed with ectopic expression of c-fos and oncogenic Ras (Eger *et al.*, 2005).

Inducible and sustained ectopic expression of Fos protein in mammary epithelial cells leads to EMT characterized by loss of cell polarity and increased invasiveness in collagen cultures (Reichmann *et al.*, 1992). Maintenance of EMT induced by sustained Fos activation depends on  $\beta$ -catenin/LEF1 signaling and autocrine production of TGF- $\beta$  (Eger *et al.*, 2000, 2004). In contrast, short-term activation of Fos in these cells results in reversible loss of cell polarity (Reichmann *et al.*, 1992). In human keratinocytes, TGF- $\beta$  stimulates ERK-dependent, rapid and transient induction of c-fos at the onset of sustained EMT (Zavadil *et al.*, 2001). Thus, the functional role of c-fos transcription factor appears to be associated with many aspects of epithelial plasticity and is cell type and context dependent.

#### Id, E2A, Twist

A microarray screen of epithelial cell response to TGF- $\beta$  or BMP7-identified HLH inhibitors of differentiation (inhibitors of DNA binding) *Id2* and *Id3* as early response targets (Kowanetz *et al.*, 2004). While TGF- $\beta$  represses *Id2*, BMP7 stimulates its synthesis (Siegel *et al.*, 2003; Kowanetz *et al.*, 2004). In mammary and lens epithelial cells, ectopic expression of *Id2* inhibits major features of TGF- $\beta$ -induced EMT such as adherens or tight junction dissolution, or synthesis of smooth muscle actin, a marker of myofibroblasts. In addition, siRNA silencing of *Id2* in lens epithelial cells enhanced the EMT-like response to TGF- $\beta$  and also permitted induction of EMT by BMP7 (Kowanetz *et al.*, 2004). Thus, the endogenous downregulation of *Id2* by TGF- $\beta$  signals is a part of the EMT transcriptional programs leading to mesenchymal phenotypes (Xie *et al.*, 2003; Kowanetz *et al.*, 2004) and the upregulation of *Id2* by BMP7, may prevent upregulation of smooth muscle actin (Valcourt *et al.*, 2005) and thereby inhibit the process of TGF- $\beta$ -induced EMT (Zeisberg *et al.*,

2003b). The molecular mechanism of Id-mediated regulation of EMT has been elucidated by the fact that Id proteins associate constitutively with the bHLH transcriptional regulator E2A, and maintain epithelial phenotypes by inhibiting E2A's function as repressor of E-cadherin (Perez-Moreno *et al.*, 2001; Kondo *et al.*, 2004). Downregulation of Id2 proteins by TGF- $\beta$  thus relieves this block, permitting conversion into mesenchymal phenotypes.

bHLH transcription factor Twist is a major regulator of embryonic morphogenesis and N-cadherin in *Drosophila* (Thisse *et al.*, 1987; Oda *et al.*, 1998). Mutations of the gene are associated with an autosomal-dominant craniosynostosis (Saethre–Chotzen syndrome) characterized by cleft palate, and are possibly responsible for inhibition of EMT during palatal fusion (el Ghouzzi *et al.*, 1997). Twist has been recently identified as a important regulator of EMT *in vitro* and *in vivo* in metastatic and invasive carcinomas (Yang *et al.*, 2004). Ectopic expression of Twist in MDCK cells causes transcriptional repression of E-cadherin,  $\alpha$ -,  $\beta$ - and  $\gamma$ - catenins, and induction of mesenchymal markers fibronectin, vimentin, smooth muscle actin and N-cadherin. *In vivo* studies suggest that elevated expression of Twist may be responsible for pulmonary metastases of mammary carcinoma (Yang *et al.*, 2004).

#### Hairy/Enhancer-of-split family

As described in a previous section, functional integration of TGF- $\beta$ /Smad and Jagged1/Notch signaling in TGF- $\beta$ -induced EMT of keratinocytes is associated with upregulation of a subset of H/Espl genes, including *HEY1*, *HEY2*, *HES1* and *HES5* (Zavadil *et al.*, 2004). The pattern of activation is biphasic, including an immediate early response that is mediated by Smad3 without involvement of Notch receptor, and a second wave that is mediated by classical Notch-dependent signaling induced through indirect and delayed induction of Jagged1 synthesis by TGF- $\beta$  (Zavadil *et al.*, 2004). Interestingly, the induction of H/Espl proteins by TGF- $\beta$  appears to be specific for EMT as it is observed in multiple cell types including human, murine and canine proximal tubular cells, mammary cells and immortalized keratinocytes (Zavadil *et al.*, 2004), while it is not detectable in cells that do not undergo EMT in response to TGF- $\beta$ , such as primary human keratinocytes (J Zavadil, unpublished results). Inhibition of *HEY1* activity by RNAi approaches or by pharmacological inhibition of Notch receptor signals prevents major features of TGF- $\beta$ -directed EMT such as disassembly of E-cadherin adherens junctions, disassembly of cortical actin and cell motility (Zavadil *et al.*, 2004). The significance of H/Espl as direct targets of TGF- $\beta$  signaling pathway in the context of EMT is further underscored by *Tgfb2* and *Hey2* gene ablation experiments in mice, revealing overlapping defects in cardiogenesis, suggesting that these proteins may be components of a functional pathway directing EMT in endothelial cushion remodeling during cardiac development (Camenisch *et al.*, 2002; Donovan *et al.*, 2002;

Sakata *et al.*, 2002). While TGF- $\beta$ -induced expression of H/Espl repressors is reminiscent of the rapid recruitment of other transcriptional regulators (Slug, Snail,  $\delta$ EF or Twist) in order to silence epithelial-specific genes such as E-cadherin and contribute to the overall pattern of TGF- $\beta$ -induced gene repression, the targets of H/Espl function in the specific context of EMT in development as well as disease pathogenesis remain to be identified.

## Conclusions

*In vitro* and *in vivo* evidence support important roles for TGF- $\beta$  as inducer of EMT in at least three distinct physiological contexts. First, TGF- $\beta$ 2 and TGF- $\beta$ 3 isoforms mediate specialized forms of EMT in cardiac and craniofacial development, respectively. The scope and outcome of these limited developmental processes are well defined.  $\beta$ -catenin and Slug have been implied as mediators of TGF- $\beta$ 2-induced EMT of the AV cushion structure of the developing heart (Camenisch *et al.*, 2002). LEF1 mediates the TGF- $\beta$ 3-induced EMT of medial edge fusion of the palate (Nawshad and Hay, 2003). While these observations imply (at least indirectly) interactions of Wnt and TGF- $\beta$  pathways in these specialized developmental EMT processes, details of specific signaling mechanisms downstream of TGF- $\beta$  isoforms that control their precise timing and location have not been elucidated.

Second, while it is now widely held that EMT underlies epithelial degeneration and fibrogenesis in chronic degenerative, fibrotic disorders, in particular of kidney, there is only limited definitive *in vivo* evidence to support this emerging paradigm (Iwano *et al.*, 2002). It is not known to what extent (if at all) and to what end (fibroblast and/or myofibroblast generation) EMT contributes to epithelial degeneration and fibrogenesis in response to epithelial stress/injury in mature kidney and

lens (or liver and lung). Other candidate mechanisms include proliferation and activation of resident interstitial fibroblasts, and extravasation of circulating bone marrow-derived fibroblasts and possibly circulating stem cells. In contrast, *in vitro* evidence clearly demonstrates that TGF- $\beta$  easily induces EMT in cultured epithelial cells of kidney, liver, and lung. In addition, TGF- $\beta$ 1 is typically expressed at sites of epithelial degeneration and adjacent fibrogenesis *in vivo*, and inhibition of TGF- $\beta$ 1 or TGF- $\beta$  signaling (Smad3 knockout) typically preserves tissues and prevents scarring. Therefore, the concept that TGF- $\beta$ -induced EMT underlies chronic degenerative, fibrotic disorders is attractive, but still requires broader definitive experimental support *in vivo* before it can be widely accepted.

Third, there is strong evidence, *in vitro* and *in vivo*, demonstrating that EMT endows dedifferentiated malignant epithelial cells with mesenchymal, migratory and proteolytic properties that are required for local tumor invasiveness, a prerequisite to metastasis formation. In this context, autocrine TGF- $\beta$  activity is essential for sustained TGF- $\beta$ -dependent signaling that cooperates with other oncogenic pathways, in particular activated Ras signaling, to maintain the mesenchymal phenotype of invasive/metastatic tumor cells. This mechanism may explain why several studies indicate a correlation between TGF- $\beta$  expression and invasiveness/metastatic potential of human cancers. New approaches to pharmacologic inhibition of TGF- $\beta$  and TGF- $\beta$  receptor function to eliminate these oncogenic activities and to prevent tumor progression and metastasis are being pursued vigorously.

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