

NEW INSIGHTS INTO THE REGULATION OF EPITHELIAL–MESENCHYMAL TRANSITION AND TISSUE FIBROSIS

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Abstract

Tissue fibrosis often presents as the final outcome of chronic disease and is a significant cause of morbidity and mortality worldwide. Fibrosis is driven by continuous expansion of fibroblasts and myofibroblasts. Epithelial–mesenchymal transition (EMT) is a form of cell plasticity in which epithelia acquire mesenchymal phenotypes and is increasingly recognized as an integral aspect of tissue fibrogenesis. In this review, we describe recent insight into the molecular and cellular factors that regulate EMT and its underlying signaling pathways. We also consider how mechanical cues from the microenvironment affect the regulation of EMT. Finally, we discuss the role of EMT in fibrotic diseases and propose approaches for detecting and treating fibrogenesis by targeting EMT.

Key Words: Epithelial–mesenchymal transition, Mechanotransduction, Myofibroblasts, Fibrosis, Epithelial plasticity, Pathogenesis. © 2012 Elsevier Inc.

ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
AV	atrioventricular
bHLH	basic helix–loop–helix
BMP	bone morphogenetic protein
CAF	cancer-associated fibroblast
DDR	discoidin domain receptor
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial–mesenchymal transition
EndoMT	endothelial–mesenchymal transition
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FOXC2	forkhead box C2
FSP	fibroblast-specific protein
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
ILK	integrin-linked kinase
IPF	idiopathic pulmonary fibrosis
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase
MET	mesenchymal–epithelial transition
miRNA	microRNA
MMP	matrix metalloproteinase

OT	outflow tract
PI3K	phosphoinositide-3-kinase
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SMA	smooth muscle actin
Sos	Son of sevenless
TGF	transforming growth factor
UTR	untranslated region
ZEB	zinc finger E-box binding
α SMA	alpha-smooth muscle actin

1. INTRODUCTION

The continuous polarized epithelial sheet is one of the most fundamental tissue forms of multicellular organisms. Epithelia establish a barrier that separates adjacent tissues from each other and maintains organ homeostasis and architecture during adult life. Epithelial sheets are remodeled during morphogenesis and wound repair through a combination of cell proliferation, shape changes, and local rearrangements, all of which are tightly regulated to maintain epithelial tissue integrity. Epithelial cells can also convert into mesenchymal cells through a process known as epithelial–mesenchymal transition (EMT). EMT and its reverse process, mesenchymal–epithelial transition (MET), regulate the early stages of development of most animals: EMT is required for gastrulation ([Thiery and Sleeman, 2006](#)) and MET occurs during somitogenesis, kidney development, and coelomic-cavity formation ([Christ and Ordahl, 1995](#); [Funayama et al., 1999](#); [Locascio and Nieto, 2001](#)). Reactivation of EMT in the adult is regarded as a physiological attempt to control inflammation and to heal damaged tissue. EMT is also co-opted by pathological processes such as fibrosis and cancer ([Kalluri and Weinberg, 2009](#); [López-Novoa and Nieto, 2009](#)). Developmental and pathological EMTs are typified by a common spectrum of changes in morphology, gene expression, and signaling pathways.

2. EPITHELIAL–MESENCHYMAL TRANSITION

EMT involves a series of changes through which epithelial cells lose their epithelial characteristics and acquire properties typical of mesenchymal cells. EMT facilitates cell movement and the generation of new tissue types during development and also contributes to the pathogenesis of disease.

2.1. Main features of epithelial and mesenchymal cells

Epithelial and mesenchymal cells are characterized by their unique phenotypes and the morphology of the multicellular structures that they create (Shook and Keller, 2003). Distinguishing features of epithelial and mesenchymal cells are summarized in Fig. 4.1. A typical epithelium is a sheet of cells, in which neighbors are adjoined by specific junctional complexes including tight junctions, adherens junctions, desmosomes, and gap junctions. These intercellular junctions allow an epithelial sheet to form a surface that encloses three-dimensional (3D) volumes and provide it with structural integrity. Epithelial sheets are polarized in a characteristic apical-basal pattern, which creates differences between the apical and basal surfaces; major determinants include (1) the specific localization and distribution of adhesion molecules (e.g., E-cadherin and integrins), (2) organization of specialized junctional structures, (3) polarization of the actin cytoskeleton, and (4) presence of a basement membrane. Epithelial cells normally associate tightly with their neighbors, which inhibit their potential for movement

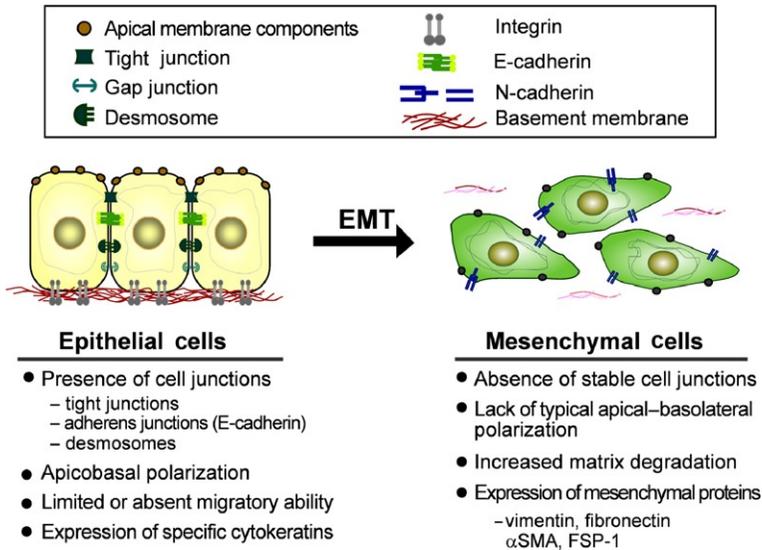


Figure 4.1 Major features of epithelial and mesenchymal cells. Epithelial cells contain specialized junctional proteins, exhibit apico-basal polarity, and have limited potential for dissociation and migration. In contrast, mesenchymal cells do not form specialized adhesion complexes and are irregular in shape with end-to-end polarity and focal adhesions resulting in increased migration capacity. During EMT, epithelial cells gain mesenchymal features which include changes in the expression of epithelial and mesenchymal markers (Table 4.1).

and dissociation from the epithelial layer. Epithelia contour the cavities and surfaces of organs throughout the body and also form many glands.

In contrast, mesenchymal cells do not form a regular layer of cells or specialized intercellular adhesion complexes. Mesenchymal cells are elongated in shape relative to epithelial cells and exhibit end-to-end polarity and focal adhesions, allowing for increased migratory capacity. Although mesenchymal cells may be polarized when migrating or interacting with neighboring cells, they lack the typical apical-basal polarity seen in epithelia. Moreover, mesenchymal cells migrate easily within tissues individually or collectively by forming a chain of migrating cells. Mesenchymal cells are essential for development as they can migrate large distances across the embryo to give rise to a particular organ. In the adult, the main function of fibroblasts, prototypical mesenchymal cells that exist in many tissues, is to maintain structural integrity by secreting extracellular matrix (ECM).

2.2. Types of EMT

Mature tissues arise from a series of conversions using EMT and its reverse process, MET. These processes endow cells with defined functions through the expression of specific genes, and thereby permit functional diversity. EMT is an example of cell plasticity that generates new mesenchymal cell types from epithelial cells (Kalluri and Weinberg, 2009). The process of EMT results in (1) loss of epithelial polarity due to the loss of organized intercellular junctions, (2) cytoskeletal reorganization, and (3) acquisition of mesenchymal features. It was long thought that a state of terminal differentiation is necessary for epithelia to carry out their specialized functions (Yeaman et al., 1999). This concept has been challenged by the observation of postnatal dedifferentiation of epithelial cells during tissue morphogenesis (e.g., mammary gland development), repair (wound healing), and pathogenesis (cancer and organ fibrosis), suggesting that epithelial cells may also be plastic in adult tissues. EMT is now considered as a mechanism to generate morphologically and functionally distinct cell types.

EMT may be classified into three subtypes based on context (Fig. 4.2) (Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009). Type 1 EMT involves the transition of primordial epithelial cells into motile mesenchymal cells and is associated with the generation of diverse cell types during embryonic development and organogenesis. These type 1 EMTs neither cause fibrosis nor induce invasion, and in many cases, the mesenchymal cells that are generated later undergo MET to give rise to secondary epithelia. Type 2 EMT involves transition of secondary epithelial cells to resident tissue fibroblasts and is associated with wound healing, tissue regeneration, and organ fibrosis. In contrast to type 1, type 2 EMT is induced in response to inflammation, but stops once inflammation is attenuated, especially during wound healing and tissue regeneration (López-Novoa and Nieto, 2009;

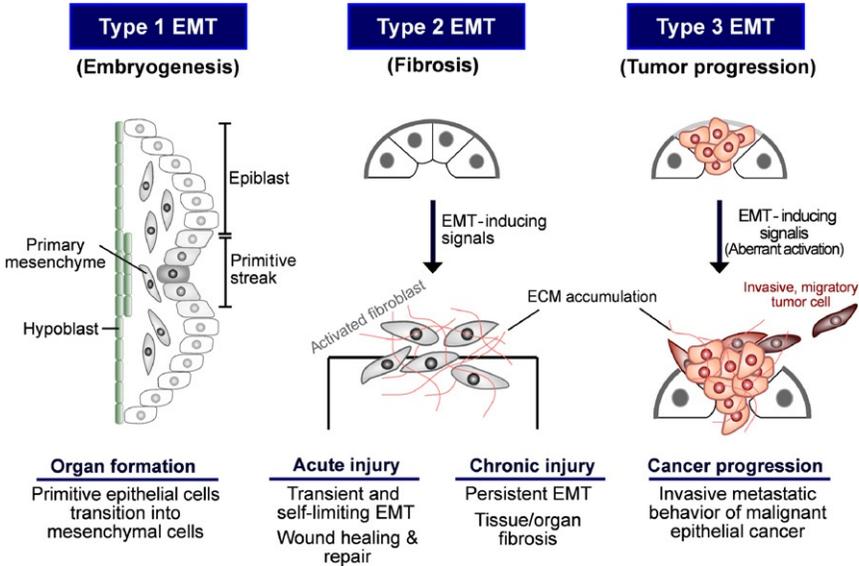


Figure 4.2 Different types of EMT. Type 1 EMT is associated with gastrulation and generation of mesoderm, endoderm, and neural crest. The primitive epithelium gives rise to primary mesenchyme through an EMT. Type 2 EMT begins as part of tissue repair to generate fibroblasts. Type 2 EMT can contribute to organ destruction if it is persistent if inflammation insult is not attenuated. Type 3 EMT occurs in epithelial cancer cells and affects oncogenes and tumor suppressor genes which conspire with the EMT proteome to result in increased invasiveness and migration.

Wynn, 2008). During organ fibrosis, type 2 EMT continues to respond to persistent inflammation, resulting in tissue destruction (López-Novoa and Nieto, 2009). Type 3 EMT occurs in carcinoma cells that have formed solid tumors and is associated with their transition to metastatic tumor cells that have the potential to migrate through the bloodstream and, in some cases, form secondary tumors at other sites through MET (Miyazawa et al., 2000; Thiery, 2002). During type 3 EMT, some cells retain epithelial traits while acquiring mesenchymal features and other cells shed most epithelial features and become fully mesenchymal (Thiery, 2002; Zeisberg and Neilson, 2009).

Although these three classes of EMT represent distinct biological outcomes, the specific signals that delineate these subtypes are unclear. However, these different EMT programs may be induced and regulated by a common set of stimuli, signal transduction pathways, transcription factors, and posttranslational regulations (Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009).

2.3. Endothelial–mesenchymal transition

Vascular endothelial cells share several common traits with epithelial cells and can generate fibroblasts by undergoing a phenotypic transition similar to EMT, referred to as endothelial–mesenchymal transition (EndoMT). EndoMT is characterized by the loss of endothelial markers including CD31 and vascular endothelial cadherin (VE-cadherin) and the expression of mesenchymal proteins including α -smooth muscle actin (SMA) (Nakajima et al., 2000; Zeisberg et al., 2007a). During embryonic development of the heart, a subset of endothelial cells located in the atrioventricular (AV) and conoventricular regions loses the expression of VE-cadherin, detaches from the endothelial sheet, and invades the cardiac jelly to form the cardiac cushions, which later form the cardiac valves and septae (Eisenberg and Markwald, 1995). Lineage tracing of endothelial cells in mice revealed that endocardial cushion mesenchyme is derived from endothelial progenitors (Kisanuki et al., 2001). Moreover, the presence of cells expressing both CD31 and α SMA in the cardiac valve suggests that endothelial cells have the potential to form mesenchyme through EndoMT (Armstrong and Bischoff, 2004). Additionally, EndoMT contributes to cardiac fibrogenesis which results in progressive stiffening of the ventricular walls, loss of contractility, and abnormalities in cardiac conductance (Goumans et al., 2008; Zeisberg et al., 2007a). EndoMT is also involved in pulmonary fibrosis (Hashimoto et al., 2010), idiopathic hypertension (Kitao et al., 2009), and corneal fibrosis (Nakano et al., 2008). Many growth factors and signaling pathways that govern EMT also regulate EndoMT in the embryonic heart and during cardiac fibrosis (Armstrong and Bischoff, 2004; Goumans et al., 2008). However, as compared to EMT, relatively little is known about EndoMT.

3. MAJOR CRITERIA AND RELEVANT MARKERS TO DETECT EMT

The conversion of epithelium into mesenchyme requires alterations in cellular morphology, adhesion, and migratory capacity. A variety of biomarkers have been suggested to define all three subtypes of EMT (Table 4.1). The spectrum of changes that occurs during EMT is not always identical and may be determined by integration of the extracellular signals.

3.1. Morphological changes

The initial step of classical EMT involves a disruption of intercellular junctions in the epithelium. The most direct approach to appreciate EMT is to follow time-dependent changes in cell morphology: in culture, epithelial cells dissociate from their neighbors and acquire a fibroblast-like,

Table 4.1 Major criteria to detect EMT include established markers, phenotypes

Phenotypic markers of EMT
<ul style="list-style-type: none"> – Spindle shape, fibroblast-like phenotype – Increased motility and migratory capacity – Increased resistance to anoikis and apoptosis – Maintain phenotype after removal of triggering stimuli
EMT proteome
<i>Proteins decreased during EMT</i>
<ul style="list-style-type: none"> – E-cadherin, ZO-1, mucin1, cytokeratin, occludin, desmoplakin, collagen IV, laminin 1, MiR-200 family
<i>Proteins increased during EMT</i>
<ul style="list-style-type: none"> – <i>Transcription factors:</i> Snail (Snai1/Snai1), Slug (Snai2/Snai2), ZEB1 (TCF8/δEF1), ZEB2 (SIP1), E47 (TCF3), E2-2 (TCF4), Twist1, FOXC2 – <i>Matrix metalloproteinases:</i> MMP2, MMP3, MMP9 – <i>Cell-surface proteins:</i> N-cadherin, OB-cadherin, α5β1 integrin, αVβ6 integrin, DDR2 – <i>Cytoskeletal markers:</i> vimentin, fibronectin, αSMA, FSP1 – <i>Transcription factors that translocate into nuclei:</i> β-catenin, NF-κB, Smad 2/3 – <i>miRNA:</i> miR10b, miR-21x – HSP-47
Minor changes
<ul style="list-style-type: none"> – Abundant intermediate filaments and microfilaments – Loss of chromatin condensation associated with gain of multiple nucleoli – Gain of rough ER, abundant lysosomal granules
EMT-triggering signals
<ul style="list-style-type: none"> – Growth factors and cytokines: TGFβ, EGF, HGF, FGF – ECM components through integrins – Wnt proteins, Notch – Hypoxia – ROS – Mechanical stress

spindle-shaped morphology, and often scatter from their original monolayers (Fig. 4.3). EMT is also characterized by increased cell motility and has therefore emerged as a key event in cancer metastasis (Ishigaki et al., 2011).

3.2. The EMT proteome

The alterations in cell morphology characteristic of EMT are associated with changes in the expression of several molecules, as indicated in Table 4.1 (Kalluri and Nelson, 2003; Zeisberg and Nelson, 2009). These molecules are often used as biomarkers to detect EMT.

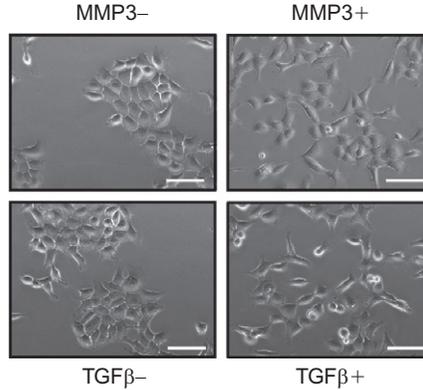


Figure 4.3 Change in cell morphology through EMT. Treatment of mammary epithelial cells with MMP3 (top) or TGF β (bottom) results in dissolution of intercellular adhesions, cell scattering, and spindle-shaped morphology. Scale bar, 50 μ m.

3.2.1. Cell-surface markers

E-cadherin maintains cell–cell contacts and epithelial tissue architecture. Decreased expression of E-cadherin has been found in all three types of EMT and is thought to be the prototypical marker of EMT (Kalluri and Neilson, 2003). Loss of E-cadherin contributes to EMT both by modulating cell–cell adhesion and by altering signaling through the sequestration of associated cytoplasmic proteins, including β -catenin. The expression of E-cadherin is highly controlled during normal development, both at the transcriptional and posttranscriptional levels (Daniel and Reynolds, 1997; Peinado et al., 2004b). Cadherin switching, a change in the expression of different cadherins, has emerged as a marker for EMT. In particular, EMT is often associated with a switch from E-cadherin to N-cadherin, which is expressed in mesenchymal cells, cancer cells, and neural tissue (Cavallaro and Christofori, 2004; Nakagawa and Takeichi, 1998). Dynamic and reciprocal changes in E- and N-cadherin expression occur when mouse embryos undergo EMT at the primitive streak (Nakagawa and Takeichi, 1995). Similarly, L-CAM, the avian homologue of E-cadherin, is substituted by N-cadherin during neural plate invagination in the chick embryo (Nakagawa and Takeichi, 1995). Overexpression of N-cadherin has been observed in breast, prostate, and intestinal gastric carcinomas and often correlates with decreased levels of other cadherins, such as E- and P-cadherin (Peinado et al., 2004b; Rosivatz et al., 2002). In addition, the switch from E-cadherin to OB-cadherin, which is expressed in myofibroblasts, is of interest for type 2 EMT associated with fibrogenesis (Rastaldi et al., 2002). Although the mechanisms underlying cadherin switching in development and disease remain unclear, the heterogeneous pattern of switching between

cadherins suggests that environmental cues provoke a shift to a more dynamic adhesion state through the expression of new cadherins.

During EMT, cells relocate from a microenvironment rich in basement membrane to one rich in fibrillar ECM. An integrin switch often reflects these changes in cell–ECM interactions. Although cell–ECM signaling facilitates EMT (Li et al., 2003), integrins, in general, have limited utility as biomarkers because many are expressed ubiquitously by both epithelial and mesenchymal cells. However, there are specific examples in which integrins are used as biomarkers. During gastrulation, EMT is associated with *de novo* expression of $\alpha 5 \beta 1$ integrin, which binds to fibronectin and controls the orientation of cellular protrusions (Davidson et al., 2006). Increased $\alpha 5$ integrin also promotes EMT during kidney fibrosis (White et al., 2007), and the expression of $\beta 6$ and $\alpha 5$ integrins correlates with EMT in colon carcinoma cells and melanoma cells, respectively (Bates et al., 2005; Qian et al., 2005).

Expression of discoidin domain receptor (DDR), the collagen-specific receptor tyrosine kinase (RTK), also reflects adaptation to the altered ECM microenvironment associated with EMT (Vogel et al., 1997). DDR2 expression increases during EMT and, upon binding to collagen, mediates upregulation of matrix metalloproteinase (MMP)-1 and cell motility (Goldsmith et al., 2010; Vogel et al., 1997). *De novo* expression of DDR2 is associated with type 2 EMT (Zhang et al., 2010) and also correlates with increased invasiveness, suggesting its possible application to demonstrate type 3 EMT (Vogel et al., 1997). Similarly, DDR1 expression correlates with type I collagen-induced EMT (Shintani et al., 2008).

3.2.2. Cytoskeletal markers

There are several cytoplasmic proteins that are used as markers for EMT. Vimentin is an intermediate filament protein present in most mesenchymal cells. Vimentin is responsible in part for the strength and integrity of these cells and is necessary for tissue movements that require traction forces (Eckes et al., 2000). Vimentin is commonly used as a marker for EMT during embryogenesis. In mice, vimentin is first expressed in the cells of the parietal endoderm and also in those cells that delaminate through the primitive streak to become mesoderm (Colucci-Guyon et al., 1994; Eckes et al., 2000). However, in adult tissues vimentin is not only expressed in fibroblasts, endothelial cells, and hematopoietic lineages but is also upregulated in epithelial cells in response to various stimuli, so it is considered questionable as a marker of type 2 EMT in the setting of fibrosis. In contrast, vimentin expression correlates with increasing tumor grade, invasiveness, and metastasis of carcinomas and has been used to identify EMT during cancer progression (Heatley et al., 1993; Yang et al., 2004).

α SMA is an actin isoform expressed by vascular smooth muscle cells and myoepithelial cells. EndoMT that gives rise to the cardiac cushions is

characterized by *de novo* expression of α SMA (Nakajima et al., 1997a). α SMA is especially well defined as a marker for myofibroblasts, cells that represent an advanced stage of EMT and that are associated with fibrosis (Masszi et al., 2003). In type 3 EMT, α SMA expression has been detected in basal-type breast cancer (Sarrió et al., 2008).

β -Catenin is an adhesion plaque protein that plays a dual role during EMT. In quiescent epithelium, β -catenin is located in the cytoplasm and either bound to cadherin or targeted for degradation (Gavert and Ben-Ze'ev, 2007). During EMT, β -catenin translocates into the nucleus and functions as a transcriptional activator together with T cell factor (TCF/LEF) complex to regulate the expression of genes associated with EMT including Snail (Yook et al., 2006). Nuclear accumulation of β -catenin has been detected in cells undergoing EMT in embryonic development, fibrosis, and cancer and has been used as a biomarker for all three types of EMT (Kalluri and Neilson, 2003; Nawshad et al., 2005).

Fibroblast-specific protein-1 (FSP1) belongs to the S100 superfamily of calcium-binding proteins and is a widely appreciated marker for EMT in fibrogenesis and cancer (Iwano et al., 2002; Xue et al., 2003). Mice expressing an FSP-driven reporter revealed that FSP1-positive fibroblasts arise in large numbers through local EMT during kidney and renal fibrosis (Iwano et al., 2002; Zeisberg et al., 2007a). FSP1-positive cells coexpress heat-shock protein (HSP)-47, a chaperone molecule indicative of collagen synthesis, suggesting that these cells are directly involved in fibrogenesis (Iwano et al., 2002). In cancer, FSP1 is often expressed in metastatic cells and plays a role in determining the latency of tumor dispersion (Xue et al., 2003). FSP1 is expressed after E8.5 and is associated with cells of mesenchymal origin or fibroblast phenotype. However, this molecule has limited utility for the detection of type 1 EMT because it is restricted to epithelial cells that are transitioning to fibroblasts rather than to primitive mesenchymal cells.

3.2.3. Extracellular proteins

The basement membrane components type IV collagen, laminin, nidogen, and sulfated proteoglycans are all downregulated during EMT. Laminins are heterotrimeric glycoproteins of which 15 different heterodimers have been identified (Colognato and Yurchenco, 2000). Loss of laminin-111 (α 1 β 1 γ 1) is associated with EMT during gastrulation, palatal fusion (Zagris et al., 2005), and renal fibrosis (Zeisberg et al., 2002). In contrast, increased laminin-332 (α 3 β 3 γ 2) is associated with idiopathic pulmonary fibrosis (IPF) (Chilosi et al., 2006) and invasive cancers (Carpenter et al., 2008).

Fibronectin is a glycoprotein that serves as a scaffold for fibrillar ECM and has been used as a marker of EMT during gastrulation, palatal fusion, and neurulation (Zeisberg and Neilson, 2009). Fibronectin is limited as a biomarker for types 2 and 3 EMT because it is expressed by various cell types including fibroblasts, mononuclear cells, and epithelial cells (Zeisberg

et al., 2001). However, increased levels of fibronectin have been reported during fibrogenesis and cancer progression (Yang et al., 2007; Zeisberg et al., 2001).

3.2.4. Transcription factors

Despite the distinct environmental stimuli that can induce EMT, the response is relatively uniform. This raises interest in key regulators that commonly function downstream of various signaling pathways to control EMT. As noted above, one of the key molecular changes is repression of E-cadherin (Kalluri and Neilson, 2003). Several transcriptional repressors of E-cadherin have been identified, and these include members of the Snail and basic helix–loop–helix (bHLH) families and double zinc finger E-box-binding (ZEB) transcription factors (Peinado et al., 2007). These proteins function downstream of the EMT-inducing signaling pathways activated by transforming growth factor (TGF)- β , fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and others.

The Snail family of transcription factors, which in mammals includes Snail, Slug, and the less characterized SMUC, is prominent downstream of EMT-inducing stimuli. These family members share a highly conserved carboxy-terminus containing C₂H₂-type zinc fingers that bind to a subset of E-box regions (Peinado et al., 2007) and an amino-terminal SNAG domain, which is essential for their nuclear localization and for transcriptional repression (Grimes et al., 1996). The Snail family of transcription factors is most widely appreciated for its repression of E-cadherin and has been implicated in both normal and pathological development. In vertebrates, Snail and Slug play an essential role in the migration of neural crest cells (del Barrio and Nieto, 2002) and in formation of the mesoderm (Barrallo-Gimeno and Nieto, 2005). Increased Snail expression is also associated with fibrosis. Snail activation in mice results in pathological type 2 EMT, with prolonged activation resulting in death, presumably due to renal failure (Boutet et al., 2006). Moreover, high levels of Snail were detected in fibrotic human kidney tissue, accompanied by deposition of collagen I and expression of vimentin (Boutet et al., 2006). Snail expression also increases during liver fibrosis, and its levels correlate with disease progression, reaching highest values in patients with advanced liver fibrosis (Scarpa et al., 2011). In addition to E-cadherin repression, Snail transcription factors contribute to other aspects of EMT. Snail and Slug induce the expression of mesenchymal markers and decrease that of epithelial markers (Kalluri and Neilson, 2003; Lee et al., 2011). Snail transcription factors also regulate cell cycle progression and survival during EMT (Liu et al., 2010; Vega et al., 2004).

ZEB1 and SIP1 also contain a C₂H₂-type zinc finger domain that interacts with E-box elements and negatively regulates E-cadherin (Vandewalle et al., 2009). Increased expression of ZEB proteins results in a rapid EMT encompassing a loss of epithelial polarity and adherens junctions and

desmosomes with concomitant upregulation of mesenchymal markers (Comijn et al., 2001; Eger et al., 2005). In addition, expression of ZEB proteins induces cell scattering, migration, and invasiveness in Matrigel (Comijn et al., 2001; Vandewalle et al., 2005). In contrast to the Snail family, ZEB1 and SIP1 are capable of interacting with the transcriptional co-activators p300 and pCAF, suggesting that they may use a different mechanism to activate the expression of mesenchymal markers (Peinado et al., 2007). Similar to the Snail family, ZEB transcription factors also regulate key cellular behaviors, including proliferation, susceptibility to apoptosis, and senescence (Vandewalle et al., 2009).

Twist is a member of the bHLH-family of transcription factors and is upregulated during mesoderm development (Yu et al., 2008), neural tube formation (Chen and Behringer, 1995), tissue fibrosis (Kida et al., 2007), and tumor metastasis (Yang et al., 2004). Aberrant expression of Twist potently induces EMT in kidney and mammary epithelial cells (Kida et al., 2007; Yu et al., 2008). Twist1 also promotes the formation of invadopodia and invasion (Eckert et al., 2011). In addition, Twist1 increases the expression of the proto-oncogene AKT2 that in turn induces survival, invasiveness, and migration of breast cancer cells (Cheng et al., 2007) and activates microRNA (miRNA) associated with prometastatic signals, including miR-10b (Ma et al., 2007). Therefore, Twist appears to be a *bona fide* metastatic gene by promoting migration of cells through EMT.

E47 is a transcription factor that is produced by alternative splicing of the exon encoding the DNA-binding domain of the E2A gene and has been shown to promote EMT during mammary epithelial branching morphogenesis (Lee et al., 2011) and renal fibrosis (Slattery et al., 2006). E2A is a member of the E-protein family that encodes bHLH transcription factors that bind E-box elements. Therefore, the expression of E2A represses E-cadherin and is associated with increased invasiveness and migration. The mRNA of E2A is absent in mature and embryonic epithelia but is present in E-cadherin-negative invasive carcinoma cells (Pérez-Moreno et al., 2001).

Forkhead box C2 (FOXC2) is another transcription factor known to induce EMT. FOXC2 is required for angiogenesis, musculogenesis, and organogenesis of the kidney, heart, and urinary tract (Kume et al., 2000). Expression of FOXC2 correlates with the highly aggressive basal-like human breast cancer and is associated with metastatic progression (Mani et al., 2007). Moreover, overexpression of TGF β , Snail, or Twist increases the expression of FOXC2, suggesting an importance for this transcription factor in type 3 EMT (Mani et al., 2007). A role for FOXC2 in type 1 and type 2 EMT is yet to be established.

These EMT-inducing transcription factors are often activated simultaneously. The expression of Snail, Slug, Twist, and SIP1 is increased during EMT in neural crest cells (Casas et al., 2011; Nieto, 2002), and Twist1 and Slug are frequently coexpressed in human breast tumors (Casas et al., 2011).

We also demonstrated that Snail, Slug, and E47 are concurrently expressed in nascent branches of mammary ducts and activate the EMT program during branching morphogenesis (Lee et al., 2011). The expression of Snail, Slug, and E47 changed dynamically during the branching process and depleting any of these inhibited branching (Lee et al., 2011), suggesting that EMT-inducing transcription factors may function coordinately to activate EMT. Although the various mechanisms involved in the repression of E-cadherin make it difficult to define a simple model, recent studies provide insight into how these transcription factors coordinate the EMT program. A comprehensive binding analysis has revealed the possibility of a hierarchy: Snail is dominant over E47 or Slug in silencing E-cadherin (Bolós et al., 2003b). However, EMT-inducing transcription factors all function as E-cadherin repressors and can contribute to maintenance of the mesenchymal phenotype. It is tempting to speculate that EMT-inducing transcription factors have specific roles at different stages of development and pathogenesis: the initial stage of EMT probably requires a rapid and more effective repression of E-cadherin, such as through Snail; in contrast, subsequent maintenance of dedifferentiated features during migration may be attained by weaker, but more widely expressed, repressors such as Slug, E47, and SIP1. This hypothesis is supported by the expression pattern of these transcription factors during mouse embryogenesis, as Snail is expressed specifically at the areas where EMT occurs whereas Slug and E12/E47 are expressed in the cells that are already migratory (Pérez-Moreno et al., 2001; Sefton et al., 1998). Snail is also expressed at earlier stages of mammary epithelial branching morphogenesis than E47 and Slug and may be involved in initiation of branching (Lee et al., 2011). Moreover, during tumorigenesis, Snail and ZEB1 promote EMT to initiate invasion, whereas Slug, E47, and SIP1 favor the maintenance of the motile phenotype in invading tumor cells, and Twist1 plays a role in distant metastasis (Comijn et al., 2001; Peinado et al., 2004b; Pérez-Moreno et al., 2001). Therefore, the different EMT-transcription factors not only regulate E-cadherin expression in specific cellular contexts but also coordinately control the EMT program.

Coexpression of these transcription factors during EMT also suggests that regulatory feedback may be involved. Twist1 regulates the expression of Snail which is required for axis control and mesoderm formation in *Drosophila* (Leptin, 1991), and loss of Twist1 decreases the expression levels of Snail and Slug in the early *Xenopus* embryo (Zhang and Klymkowsky, 2009). Twist1 directly binds to the Slug promoter and activates its transcription (Casas et al., 2011). This Twist1-Slug network is essential for the role of Twist1 in promoting invasion and metastasis of breast cancer (Casas et al., 2011). We also found an interaction between Snail and Slug during mammary epithelial branching morphogenesis (Lee et al., 2011), and this finding, together with previous reports demonstrating the cross-activation of Snail and Slug (Aybar et al., 2003; Sakai et al., 2006), suggests that Snail

and Slug interact during EMT to trigger and maintain mesenchymal phenotypes. Thus, these transcription factors might be regulated by the presence of, and interaction with, other EMT-inducing factors. These mechanisms add an additional level of complexity to our understanding of the EMT-inducing transcription factors. Therefore, a more detailed analysis of the different EMT-transcription factors is needed to obtain a comprehensive view of the transcriptional network during EMT.

3.2.5. MicroRNAs

MiRNAs regulate gene expression posttranscriptionally and are involved in many biological processes including embryogenesis, organogenesis (Wienholds et al., 2003; Yi et al., 2006), and disease progression (Gregory et al., 2008). Some miRNAs are expressed ubiquitously, whereas others are expressed in a specific cell, tissue, or developmental stage. Genome-wide analysis for miRNAs has revealed that the miR200 family and miR205 are highly associated with EMT (Gregory et al., 2008; Park et al., 2008). This change is reflected in a strong correlation between the expression of the miR200 family and E-cadherin across numerous cell lines and epithelial tissues (Burk et al., 2008; Gregory et al., 2008; Park et al., 2008). The miR200 family binds to the 3' untranslated regions (UTRs) of RNA and suppresses the expression of ZEB1 and SIP1, which repress E-cadherin. The miR200 family is thereby capable of enforcing epithelial phenotypes. Additional EMT-related downstream targets of the miR200 family have been identified: miR141 inhibits TGF β 2 (Burk et al., 2008) and miR200a suppresses β -catenin (CTNNB1) (Xia et al., 2010).

MiRNAs are also associated with the TGF β signaling pathway. The expression of miR155 increases during TGF β -induced EMT in mammary epithelial cells through Smad4-mediated transcriptional upregulation and facilitates loss of cell polarity and tight junctions (Kong et al., 2008). Moreover, epithelial cells expressing miR155 responded more rapidly to TGF β . A key downstream target of miR155 is RhoA, which plays a role in the formation and stabilization of cell junctions. RhoA contains three conserved regions that may serve as binding sites for miR155 (Kong et al., 2008). These data suggest that miR155 may provide further inhibitory effects on RhoA during EMT, in addition to TGF β -mediated ubiquitination and degradation (Wang et al., 2003). The expression levels of miR29a and miR21 also increase upon TGF β -induced EMT in mammary epithelial cells (Gebeshuber et al., 2009; Kong et al., 2008), although their role in EMT has not been completely elucidated. Overexpression of miR29a suppresses the expression of tristetraprolin and leads to EMT in cooperation with the Ras signaling pathway (Gebeshuber et al., 2009). The levels of pre-miR21 and mature miR21 are increased by TGF β treatment in breast cancer cells through increased processing of the miR21 primary transcript and in a Smad4-independent manner (Davis et al., 2008).

It was recently shown that miR9 directly targets the mRNA encoding E-cadherin (Ma et al., 2010). Ectopic expression of miR9 led to EMT in human mammary epithelial cells (Ma et al., 2010). Moreover, a significant number of breast carcinoma cells located at the edge of miR9-expressing tumors expressed mesenchymal markers including vimentin, whereas few cells located in intratumoral regions were vimentin-positive, suggesting that miR9 may sensitize cells to EMT-inducing signals from the tumor microenvironment (Ma et al., 2010).

The EMT-inducing transcription factors have recently emerged as transcriptional regulators of miRNAs. MiR21 is highly expressed in various tumors and known to induce metastasis through EMT. The promoter regions of miR21 include consensus E-box sequences that serve as binding sites for ZEB1 (Du et al., 2009). Binding of ZEB1 induces transcription of miR21 and also blocks bone morphogenetic protein (BMP)-6-mediated inhibition of EMT in breast cancer cells (Du et al., 2009). MiR10b is also highly associated with cell migration, invasion, and metastasis of breast cancer cells. A recent study revealed that Twist binds to the E-box element proximal to the predicted promoter of miR10b and activates its transcription, which in turn contributes to Twist-mediated EMT (Ma et al., 2007). Nonetheless, the regulation of miR10b is unclear. ZEB1 increases the expression of miR10b in colorectal cancer cells but decreases expression in breast cancer cells (Burk et al., 2008). Further, Snail reduces the expression of miR10b in human mammary epithelial cells (Ma et al., 2007). These data suggest that the regulation of miR10b expression may be cell-type specific and context dependent.

4. INDUCTION AND REGULATION OF EMT

Initiation of EMT requires external stimuli, including growth factors, cytokines, and hormones that activate intracellular signal transduction pathways and alter the expression of downstream target genes. Studies over the past few decades have revealed the molecular and biochemical mechanisms involved in the initiation and regulation of EMT.

4.1. Growth factor receptors and signaling pathways

The primary inducers of EMT are specific growth factors that bind to their cognate cell-surface receptors. RTKs are cell-surface transmembrane proteins that transduce extracellular signals into the cytoplasm, or as non-RTKs, that relay intracellular signals. The HGF receptor, Met, was among the first identified RTKs to promote scattering of epithelial cells (Birchmeier et al., 2003). HGF-induced activation of Met enhances cell migration and

leads to epithelial scattering (Birchmeier et al., 2003). Met receptor-mediated signaling has been linked to the regulation of Snail expression (Grotegut et al., 2006) and has also been shown to affect the localization of adherens and tight junction proteins (Brembeck et al., 2004; Hollande et al., 2001). Similarly, FGF signaling through its receptor, FGFR1, promotes EMT (Savagner et al., 1997). Desmosomal proteins, including desmoplakin and desmoglein, were found to be recruited away from the cell surface shortly after FGF treatment and cells underwent active migration after longer treatment with FGF (Boyer et al., 1989). FGF signaling regulates migration and patterning of mesoderm at gastrulation: in mice lacking FGFR1, epiblast cells in the primitive streak fail to undergo EMT due to the absence of Snail expression and subsequent failure to repress E-cadherin (Ciruna and Rossant, 2001). FGF signaling is also associated with tissue regeneration and wound healing through the transdifferentiation of epithelial cells to myofibroblasts and can cause organ fibrosis, which greatly enhances the risk of cancer (Kalluri and Weinberg, 2009; Ortega et al., 1998). Activation of the EGF receptor family (EGFR, ErbB, or HER) also stimulates EMT and has been implicated in gastrulation, heart development, and mammary gland morphogenesis (Hardy et al., 2010; Thiery et al., 2009). EGF signaling represses E-cadherin by promoting its endocytosis (Lu et al., 2003) and also by inducing the expression of Snail and Twist (Lee et al., 2011; Lo et al., 2007). Additional growth factors that bind to RTKs, including PDGF/PDGFR, IGF/IGFR, and neuregulin/ErbB2 and ERbB3, also induce cell scattering. The overlapping effects of different growth factors and their receptors suggest that activation of RTKs initiate signaling cascades needed for cell scattering.

Upon binding of growth factors, RTKs are activated through autophosphorylation on tyrosine residues which, in turn, act as docking sites for SH2-domain-containing proteins such as Grb2, phosphoinositide-3-kinase (PI3K), and Src. Ras is activated following Grb2-mediated recruitment of the guanosine nucleotide exchange factor (GEF), son of sevenless (Sos), and induces the Ras-Raf-MEK1 signaling cascade. This ultimately results in the nuclear localization of mitogen-activated protein kinase (MAPK) and regulation of gene expression by phosphorylating transcription factors including Slug (Conacci-Sorrell et al., 2003). MAPK also activates the transcription factors AP-1 and Ets which are putative mediators of EMT (Davies et al., 2005; Hsu et al., 2004). Moreover, MAPK phosphorylates GSK3 β and suppresses its activity, thus potently inducing expression of Snail (Ding et al., 2005). Similarly, PI3K/Akt phosphorylates and inactivates GSK3 β to prevent proteosomal degradation of Snail and β -catenin. Stabilized Snail and β -catenin then induce EMT (Zhou et al., 2004).

Growth factors also affect the activity of the Rho family of small GTPases, including Cdc42, Rho, and Rac by Ras and PI3K/AKT mediators as well as other EMT-inducing signaling pathways (Bakin et al., 2000;

Edme et al., 2002). The Rho GTPases play a crucial role in the actin cytoskeleton rearrangements, cell motility, and cell–cell dissociation that accompany EMT (Edme et al., 2002; Keely et al., 1997). RhoA is required for differentiation of coronary smooth muscle cells, and inhibition of p160 rho-kinase (ROCK) leads to a failure of epicardial-derived mesenchymal cells to migrate into the myocardium (Lu et al., 2001). Activation of Rho family members also increases migration and invasiveness of various cell lines in culture and is associated with EMT during metastasis (Edme et al., 2002; Keely, 2001). Rho GTPases regulate integrin signaling that mediates cellular attachment to and migration across connective tissue and are also involved in the activation of proteases that remodel the ECM, such as MMPs (Zhuge and Xu, 2001). Many downstream mediators of RTKs, such as MAPK, PI3K, and Rho GTPase, cooperate with TGF β signaling to affect EMT (Bakin et al., 2000; Bhowmick et al., 2001).

TGF β is a prominent regulator of EMT during developmental morphogenesis and migration of normal and cancer cells (Nawshad et al., 2005; Nelson et al., 2006). TGF β has also been implicated as a master switch of fibrosis in many tissues (López-Novoa and Nieto, 2009; Zeisberg et al., 2007a). TGF β signals through type I and type II receptor serine/threonine kinases; upon ligand binding, the type II receptor phosphorylates the type I receptor, which then phosphorylates cytoplasmic Smad2/3. Activated Smad2/3 forms complexes with Smad4 and regulates the expression of genes involved in cell proliferation, differentiation, migration, and ECM production (Nawshad et al., 2005). TGF β represses the expression of Id which inhibits EMT; Id repression is required for subsequent downregulation of E-cadherin and zonula occludens (ZO)-1 (Kondo et al., 2004). TGF β also regulates E-cadherin by inducing the expression of Snail and Slug through either Smad signaling or activation of PI3K and ERK pathways (Peinado et al., 2003; Thuault et al., 2006). TGF β increases the expression of ZEB1 and SIP1; pSmads form repression complexes with SIP1 that promote repression of E-cadherin (Comijn et al., 2001; Shirakihara et al., 2007). In addition, Smad complexes induce the expression of N-cadherin, fibronectin, and α SMA (Nawshad et al., 2005; Xu et al., 2009). Furthermore, TGF β induces cell migration and EMT in a Smad-independent manner by activating MAPK, PI3K, integrin-linked kinase (ILK), and Rho small GTPases (Cordenonsi et al., 2007; Moustakas and Heldin, 2005). TGF β is considered to be the prototypical cytokine for induction of EMT because different isoforms mediate various aspects of EMT in many diverse cellular contexts, whereas the effects of other EMT inducers are often context dependent and variable (Sanford et al., 1997; Xu et al., 2009).

EndoMT is also mediated by TGF β (Nakajima et al., 2000; Zeisberg et al., 2007a). TGF β 2 is expressed in the AV and OT myocardium at the onset of and during endocardial cushion formation (Dickson et al., 1993). In addition, TGF β 2-null mice showed abnormal AV endocardial cushion

morphogenesis and defects in OT development (Sanford et al., 1997). Further, in an AV explant culture, treatment with neutralizing TGF β antibodies inhibited formation of the mesenchyme (Nakajima et al., 1997b). Additionally, when premigratory chicken AV endothelium was cultured in the presence of TGF β , cells displayed phenotypic changes characteristic of EndoMT, including scattering and hypertrophy (Nakajima et al., 1998).

The canonical Wnt/ β -catenin pathway is another major signaling pathway involved in EMT. Upon binding of Wnt proteins to Frizzled family receptors, the APC/Axin/CK1/GSK3 β destruction complex is inhibited, leading to stabilization of β -catenin (Nelson and Nusse, 2004). As mentioned above, stabilized β -catenin induces the expression of genes associated with EMT (Conacci-Sorrell et al., 2003; Nelson and Nusse, 2004). Alternatively, β -catenin signaling can be activated by mechanisms that cause it to accumulate in the cytoplasm or by pathways that promote phosphorylation of GSK3 β , including PI3K/AKT, MAPK/Ras, and ILK (Nelson and Nusse, 2004; Yang et al., 2006; Zhou et al., 2004).

Notch is a key regulator in the induction of EMT (Timmerman et al., 2004; Wang et al., 2010). Upon binding of ligands such as Jagged-1, the intracellular domain of Notch is cleaved and released (Miele, 2006). Indeed, Notch is expressed in the embryo where EMT occurs, and overexpression of Notch1 in endothelial cells induces the expression of Snail and a mesenchymal morphology (Noseda et al., 2004; Timmerman et al., 2004). In addition, Notch directly regulates the activity of the Slug promoter through its nuclear partner *CBF1/Suppressor of Hairless/Lag-1* (CSL), resulting in upregulation of Slug (Niessen et al., 2008). Further, Notch cross talks with TGF β and synergistically regulates EMT markers including Snail; TGF β induces the expression of Notch ligands and Jagged-1 contributes to the activation of TGF β (Niessen et al., 2008; Niimi et al., 2007).

Other signals that trigger EMT include matrix-degrading proteases, ECM components, and integrins (Jo et al., 2009; Schedin and Keely, 2011). MMPs, cysteine proteases, and urokinase promote EMT not only by altering the extracellular milieu favoring cell migration via ECM degradation but also by promoting the release of growth factors and cytokines stored in the ECM (Jo et al., 2009; Lochter et al., 1997). ECM proteins have also been shown to induce cell scattering and migration. Increased collagen deposition and structural changes in collagens are well-recognized characteristics of fibrotic diseases (Wynn, 2008) and tumor progression (Levental et al., 2009). These changes in the ECM affect the mechanical environment of the cells and presumably lead to mechanical disruption of intercellular contacts (Schedin and Keely, 2011). Cells undergo EMT and become motile and invasive when cultured on fibrillar collagens such as type I and III (Menke et al., 2001). The finding that mechanical signals from the ECM regulate cell behaviors suggests a role for integrins in transmitting

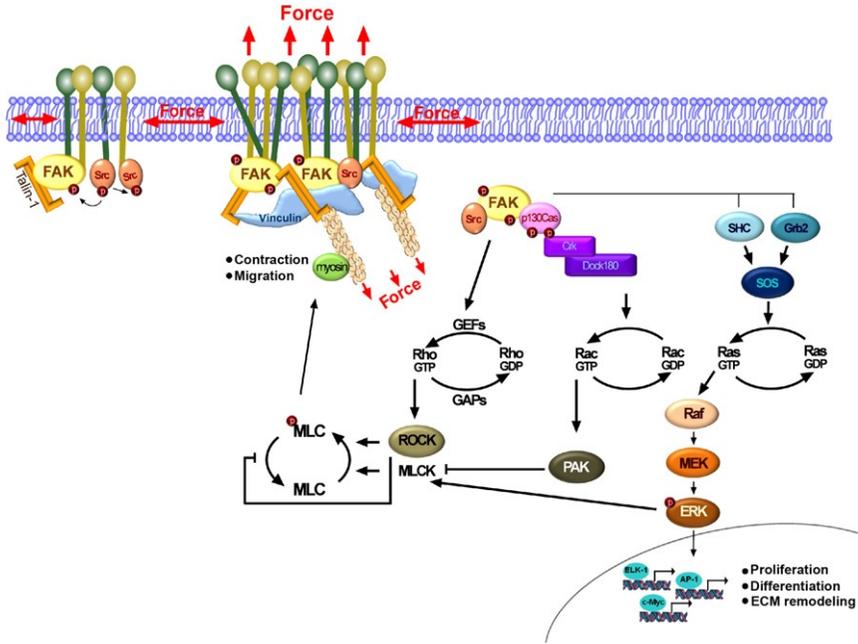


Figure 4.4 Mechanotransduction. Integrin-mediated focal adhesion complexes are assembled in response to local mechanical stress and transmit physical signals from the ECM to intracellular biochemical signals. When cells are exposed to mechanical stress, integrins become activated, which leads to integrin clustering, conformational changes in talin-1 and p130Cas, association of talin-1 and vinculin, and phosphorylation of FAK by Src to stimulate Rho GTPase-mediated actomyosin contractility and actin remodeling. Mechanically induced unfolding of p130Cas followed by phosphorylation through Src also stimulates the activation of Dock180 (dedicator of cytokinesis, also known as DOCK1) to promote Rac activity. In addition, activation of FAK leads to the phosphorylation of the adaptor protein SHC which promotes recruitment of the Grb2-Sos complex to the membrane, resulting in activation of Ras. This activates the Ras/Raf/MEK/ERK signaling cascade to regulate the expression of genes associated with proliferation, differentiation, and ECM remodeling.

signals (Fig. 4.4). Binding to ECM leads to integrin clustering at adhesion sites and the subsequent recruitment and activation of signaling proteins, including focal adhesion kinase (FAK), Src, Ras, PI3K, RhoA, and ILK (Chiquet et al., 2009; Levental et al., 2009). In particular, FAK is recruited to nascent focal adhesions, either directly or through the cytoskeletal proteins talin and paxillin (Giancotti and Ruoslahti, 1999). Upon activation, FAK undergoes autophosphorylation, leading to binding and activation of Src that, in turn, phosphorylates FAK. This results in activation of PI3K, inducing the PI3K-PIP3-AKT pathway and creating binding sites for Grb2/Sos, thereby activating Ras-MAPK signaling (Giancotti and Ruoslahti,

1999). FAK-dependent activation of Src also leads to phosphorylation of a number of focal adhesion components including paxillin (Vallés et al., 2004). Phosphorylated paxillin associates with the adaptor protein Crk and induces paxillin-Crk-DOCK1 signaling complex to activate Rac1 (Vallés et al., 2004).

4.2. Reactive oxygen species

Reactive oxygen species (ROS), such as superoxide, hydroxyl radical, and hydrogen peroxide, have been implicated in a large number of pathological conditions (Clerkin et al., 2008). ROS are conventionally thought to be cytotoxic and genotoxic and at high levels lead to irreversible cell damage. Recently, a number of studies have indicated that ROS also function as second messengers in signal transduction pathways for a variety of cellular processes, including proliferation, differentiation, and migration (Clerkin et al., 2008; Poli et al., 2004; Radisky et al., 2005). ROS have well-defined roles in fibrogenesis and cancer and have also been implicated in EMT (Novo and Parola, 2008). Treatment of mammary epithelial cells with repeated low doses of hydrogen peroxide, a protocol mimicking the chronic inflammation that is common to many human diseases, leads to a fibroblast-like phenotype (Mori et al., 2004). This morphological change is associated with dissolution of cell-cell contacts, redistribution of E-cadherin, upregulation of MMPs and integrins, and activation of Rac1 (Mori et al., 2004). ROS cross talk with TGF β and HGF signaling: the generation of ROS is increased intracellularly by TGF β and HGF (Ferraro et al., 2006; Rhyu et al., 2005); antioxidants and ROS scavengers block TGF β -induced EMT (Rhyu et al., 2005); ROS regulate signaling downstream of HGF (Ferraro et al., 2006). ROS also mediate MMP3-induced EMT: MMP3 increases the generation of ROS through the expression of Rac1b, a constitutively activated splice variant of Rac1 that was initially found in breast and colorectal tumors (Radisky et al., 2005); treatment of mammary epithelial cells with MMP3 promotes loss of E-cadherin, activation of β -catenin signaling, and increased expression of Snail (Lochter et al., 1997; Radisky et al., 2005). In addition, ROS induce the expression of Snail by enhancing its mRNA stability (Dong et al., 2007) and promote hypermethylation of E-cadherin through Snail-mediated recruitment of histone deacetylase (HDAC)-1 and DNA methyltransferase (Lim et al., 2008). Moreover, ROS trigger the actin cytoskeletal rearrangements and tight junction impairment that are essential for cell migration (Werner and Werb, 2002). Several studies demonstrated that ROS generated by the integrin-Rac pathway promote formation of stress fibers through glutathionylation of actin (Fiaschi et al., 2006; Nimnual et al., 2003). Therefore, the role of ROS in EMT might be associated not only with its critical impact on signaling pathways but also its oxidative modifications of structural proteins.

4.3. Oxygen tension

Capillary rarefaction is a hallmark of fibrotic disease and reduces blood perfusion and oxygen delivery (Higgins et al., 2008). Alterations in intracellular pO_2 have profound effects on cellular metabolism, proliferation, differentiation, and tissue-specific function (Lee et al., 2007; Semenza, 2003). Hypoxia is a state of decreased oxygen availability and is associated with normal development as well as pathological conditions (Lee et al., 2008; Saini et al., 2008). Hypoxia-inducible factor (HIF) is the master transcription factor that regulates cellular adaptation to changes in oxygen tension (Semenza, 2003). HIF is a heterodimer of HIF α and HIF β subunits. In contrast to constitutively expressed HIF1 β , HIF1 α is oxygen sensitive. HIF1 α is constantly synthesized and in well-oxygenated cells, is hydroxylated on proline residues which leads to ubiquitination and proteosomal degradation. Under hypoxic conditions, hydroxylation decreases, HIF1 α accumulates and dimerizes with HIF1 β to form a functional transcription factor that binds to DNA at hypoxia response elements (HREs) and activates transcription of target genes. It has become apparent that hypoxia and HIF affect EMT by either regulating EMT-triggering signaling pathways or by directly regulating EMT inducers.

The close proximity of DNA-binding sequences for HIF and Smads suggests that hypoxia and TGF β may cooperate in the transcriptional regulation of target genes, as has been shown for vascular endothelial growth factor (VEGF) (Sánchez-Elsner et al., 2001). Hypoxia increases the expression of Smad3 and promotes the release of latent TGF β 2 thus activating TGF β signaling (Zhang et al., 2003), and blocking HIF1 α transcription decreases the TGF β -stimulated expression of type I collagen (Basu et al., 2011). Moreover, HIF and TGF β co-regulate connective tissue growth factor (CTGF), which promotes EMT and fibrosis (Higgins et al., 2004; Shi-Wen et al., 2008). Hypoxia also influences the activity of HGF, Wnt, and Notch (Kaidi et al., 2007; Pennacchietti et al., 2003; Sahlgren et al., 2008). Hypoxia enhances HGF signaling through an HIF-dependent increase in the expression of c-Met (Pennacchietti et al., 2003). With regard to Wnt, β -catenin binds to HIF1 α and enhances HIF-mediated transcriptional activity (Kaidi et al., 2007). HIF1 α also interacts with Notch intracellular domain and enhances Notch signaling causing EMT as a result of increased Snail expression (Sahlgren et al., 2008).

There is increasing evidence indicating that HIF directly regulates the expression of EMT-related transcription factors. Hypoxia attenuates the expression of E-cadherin through HIF-induced expression of Snail (Imai et al., 2003). Renal carcinoma cells that constantly express HIF1 α in an oxygen-independent manner exhibited increased expression of E12/E47, ZEB1, and SIP1 (Krishnamachary et al., 2006). The upregulation of these transcription factors by HIF is associated with decreased E-cadherin, loss of

cell–cell adhesion, and increased migration (Krishnamachary et al., 2006). HIF also induces the expression of Twist by binding directly to the HRE within its proximal promoter and thus mediates hypoxia-induced EMT in breast cancer, nasopharyngeal cancer, and lung tumor cells (Gort et al., 2007; Yang et al., 2008). Moreover, HIF induces the expression of lysyl oxidase (LOX) and lysyl oxidase like (LOXL) that induce the stabilization of Snail and thereby promotes renal fibrosis (Higgins et al., 2008; Peinado et al., 2005).

Hypoxia affects the composition and integrity of the ECM that is essential for epithelial homeostasis (Cowden Dahl et al., 2005; Higgins et al., 2008). In the kidney, HIF promotes transdifferentiation of tubular epithelial cells into myofibroblasts which increases cell migration and ECM turnover and causes renal fibrosis (Haase, 2009). These events are likely regulated by the HIF-mediated expression of $\alpha_v\beta_3$ integrin, chemokine receptor CXCR4, and its receptor SDF-1 (Cowden Dahl et al., 2005; Haase, 2009; Lee et al., 2009). Hypoxia also promotes ECM turnover: HIF regulates the expression of collagen I, MMP1 and 2, tissue-inhibitor of metalloproteinases (TIMP)-1, plasminogen activator inhibitor (PAI)-1, and CTGF (Haase, 2009; Higgins et al., 2008).

4.4. Epigenetic regulation

A large body of evidence suggests that epigenetic modifications, such as DNA methylation, chromatin remodeling, and posttranscriptional and posttranslational modifications, function as key mechanisms responsible for regulating the EMT proteome (Dumont et al., 2008; Herranz et al., 2008; Peinado et al., 2004a). Hypermethylation of E-cadherin is frequently seen in type 3 EMT and is associated with breast cancer progression (Lombaerts et al., 2006). When E-cadherin is silenced by hypermethylation, mammary epithelial cells exhibit a mesenchymal morphology through upregulation of the EMT proteome (Lombaerts et al., 2006). In contrast, cells maintain an epithelial phenotype with minimal change in the expression of genes involved in EMT when E-cadherin is inactivated by mutation (Lombaerts et al., 2006). This suggests that molecular changes leading to type 3 EMT may be primarily modulated epigenetically. Recent work has revealed that premalignant cells can acquire *de novo* DNA methylation at sites including E-cadherin early in tumor progression (Dumont et al., 2008). This DNA methylation is heritable and subsequently generates cell progeny that exhibits an invasive phenotype associated with sustained activation of EMT (Dumont et al., 2008). Snail expression is associated with hypermethylation of the E-cadherin promoter in several types of carcinoma (Cheng et al., 2001; Lim et al., 2008), suggesting a link between Snail and epigenetic modification. Snail binds to the E-cadherin promoter through local modification of chromatin structure by recruiting a repressor complex

formed by the Sin3A/HDAC1 and HDAC2 (Peinado et al., 2004b). The recruitment of this complex is mediated by the SNAG domain of Snail, and the presence of this complex results in a decrease in acetylated histones H3/H4 and an increase in methylated histone H3 in the E-cadherin promoter. Snail also induces DNA methylation through recruiting DNA methyltransferase-1 (Lim et al., 2008) and by forming a ternary complex with the scaffold protein AJUBA and the arginine methyltransferase-5 (Hou et al., 2008). These, in turn, lead to a condensed repressive chromatin structure and prevent transcriptional initiation of E-cadherin.

The transcriptional activity of Snail is also tightly regulated by posttranslational modifications that control its stability and nuclear localization. Phosphorylation of Snail by GSK3 β facilitates its nucleocytoplasmic transport by exposing a nuclear export sequence (Dominguez et al., 2003; Zhou et al., 2004). Snail is very unstable in the cytoplasm with a half life of \sim 30 min. Once in the cytoplasm, Snail is further phosphorylated on other residues which promote Snail ubiquitination and degradation (Zhou et al., 2004). This phosphorylation is counteracted by small C-terminal domain phosphatase that interacts and colocalizes with Snail in the nucleus (Wu et al., 2009). Conversely, phosphorylation of Snail by protein kinase-A and casein kinase-2 increases its stability and enhances its interaction with Sin3A corepressor, thereby stimulating repression of E-cadherin (MacPherson et al., 2010). Moreover, p21-activated kinase (PAK)-1 phosphorylates Snail which results in its retention in the nucleus (Yang et al., 2005). Snail is also posttranslationally regulated by oxidation. LOXL2/3 catalyses oxidative deamination of Snail that leads to a conformational change which masks GSK3 β phosphorylation sites and prevents further degradation (Peinado et al., 2005).

Compared to Snail, the biochemical characteristics of Slug are less established. Slug lacks most of the residues that are phosphorylated in Snail (Kataoka et al., 2000). Although Slug does not undergo phosphorylation and subsequent ubiquitination and degradation, its stability is also tightly regulated. In *Xenopus*, Partner of paired, an F-box-containing component of a modular E3 ubiquitin ligase, binds to Slug and promotes its degradation (Vernon and LaBonne, 2006). Slug is also a target of the Mdm2 ubiquitin ligase (Wang et al., 2009). Slug shares structural similarity with Snail in its carboxy-terminal DNA-binding domain and amino-terminal regulatory domain, and the SNAG domains are almost identical (Kataoka et al., 2000). Nonetheless, proteins that interact with the SNAG sequence in Snail, for example, Sin3A, have not been reported to bind to Slug. Conversely, very few proteins that bind to Slug cannot interact with Snail; so far, only the anti-apoptotic protein Puma seems to meet this condition (Wu et al., 2005). The lack of interaction of Slug with other proteins may explain its lower binding affinity to target genes and less robust induction of EMT as compared to Snail (Bolós et al., 2003a).

5. EMERGING MECHANICAL CUES INVOLVED IN THE TRIGGERING OF EMT

While it has long been appreciated that biochemical cues regulate many cellular processes including EMT, there is growing recognition that mechanical aspects, such as applied forces or the rigidity of the ECM, crucially influence cellular behavior and function (Butcher et al., 2009; Hoffman et al., 2011; Schedin and Keely, 2011). Cells within tissues constantly experience physical forces. Cells in heart, lung, and bone are exposed to hydrostatic pressure, shear stress, and compressive and tensile stress (Butcher et al., 2009). Cells in mechanically static tissues, such as the breast and the brain, are also exposed to isometric physical stress that is transmitted through cell–cell and cell–ECM interactions (Butcher et al., 2009; Gjorevski et al., 2011). These mechanical cues have profound effects on cell survival (Chen et al., 1997), proliferation (Nelson et al., 2005), and EMT (Gomez et al., 2010). Mechanical cues also regulate stem cell fate (Engler et al., 2006; Pajerowski et al., 2007), embryonic development (Czirók et al., 2004; Krieg et al., 2008), and tissue-specific organization and function (Alcaraz et al., 2008; Paszek et al., 2005). Disrupting mechanical homeostasis is associated with pathological conditions including cancer (Butcher et al., 2009; Levental et al., 2009).

5.1. Mechanosensing and mechanotransduction

To cope with the constant mechanical stress, cells have evolved specialized mechanosensing mechanisms. Several proteins undergo conformational changes in response to applied force, including mechanically gated ion channels (Brakemeier et al., 2002), the cytoskeletal network (Helmke et al., 2003), and ligand–receptor binding (Vogel and Sheetz, 2006). These force-induced conformational changes stimulate downstream signaling. Unfolding cryptic binding sites promotes the self-assembly of fibronectin into fibrils in the ECM (Smith et al., 2007). Mechanical tension promotes unfolding of talin, which associates with vinculin to connect integrins within focal adhesions to filamentous actin, thereby transmitting forces between ECM and the actin cytoskeleton (del Rio et al., 2009). Direct application of force can stimulate the mechanical extension of p130Cas, which enhances its susceptibility for phosphorylation by Src (Sawada et al., 2006). Phosphorylated p130Cas then binds to GEFs to activate small GTPases and propagate integrin signaling (Tamada et al., 2004).

Once mechanical stress has been detected, cells convert these physical cues into biochemically relevant information and translate the signal into transient or sustained responses. Integrins interact with both ECM and focal

adhesion proteins and function as ubiquitous mechanotransducers (Butcher et al., 2009; Schedin and Keely, 2011) (Fig. 4.4). Mechanical force, either exogenous or endogenous, activates integrins by facilitating their nucleation and clustering into focal adhesions (Hoffman et al., 2011; Paszek et al., 2005). Integrin clustering leads to the phosphorylation of FAK to stabilize focal adhesions (Shi and Boettiger, 2003). The assembly of focal adhesions initiates cytoskeletal remodeling through the nucleation of assorted adhesion plaque proteins including talin and vinculin and induces downstream signaling through kinases and Ras, Rac, and Rho (Schedin and Keely, 2011). Ras links force-induced integrin signaling to MAPKs such as ERK (Chess et al., 2000; Plotkin et al., 2005). Mechanical stress is associated with sustained alterations in cellular behavior: compression changes microtubule assembly, thereby altering cell shape (Dennerll et al., 1988); shear determines cell shape and fate during condensation of mesenchymal stem cells (McBride et al., 2008). Further, in response to mechanical stimuli, fibroblasts synthesize and secrete fibronectin and collagen and remodel the ECM by activating MMPs and matrix cross-linking enzymes, which results in sustained changes in the cellular environment that may further alter cell shape, growth, migration, and differentiation (Levental et al., 2009; Paszek et al., 2005).

5.2. Mechanical regulation of EMT

Alterations in cell morphology, induced by changes in cytoskeletal organization, are also associated with EMT. Treatment of mammary epithelial cells with MMP3 induces cell spreading and this morphological change is required for the activation of downstream signaling and induction of EMT (Nelson et al., 2008). As described above, cytoskeletal architecture is sensitive to mechanical aspects of the microenvironment. Cyclic mechanical stretch significantly increases actin polymerization and promotes EMT in type II alveolar epithelial cells (Heise et al., 2011). We also found a link between mechanical stress and EMT within sheets of mammary epithelial cells (Fig. 4.5). Cells within tissues experience spatial variations in mechanical stress that play a critical role in development, differentiation, and wound healing (Gjorevski and Nelson, 2010; Gomez et al., 2010; Nelson et al., 2005; Ruiz and Chen, 2008). We showed that EMT preferentially occurred in response to TGF β at locations within the tissue where mechanical stress was concentrated (Gomez et al., 2010) (Fig. 4.5). Increased cytoskeletal tension induced nuclear localization of myocardin-related transcription factor-A and thereby increased the expression of EMT markers (Gomez et al., 2010).

Similarly, mechanical stress is distributed nonuniformly in 3D tissues (Fig. 4.6), and this patterned mechanical force plays a critical role in determining branch sites of mammary epithelium (Gjorevski and Nelson,

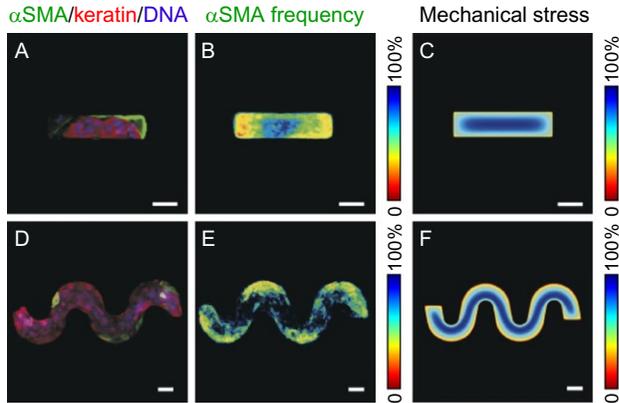


Figure 4.5 Spatial patterning of EMT correlates with gradients of cytoskeletal tension. In various shapes of epithelial sheets, TGF β -induced EMT, as determined by the expression of α SMA using immunostaining (A, D) and frequency map (B, E), was restricted to regions of high mechanical stress that were predicted computationally (C, F). Scale bar, 25 μ m. Adapted from Gomez et al. (2010).

2010). Regions of high mechanical stress correlate with patterned expression of EMT markers during branching morphogenesis (Lee et al., 2011). Moreover, disrupting actomyosin contractility significantly reduces the expression and nuclear localization of Snail (Fig. 4.6). Conversely, increasing contractility induces Snail expression (Fig. 4.6). We also found patterned activation of FAK, suggesting that FAK may mediate the transmission of mechanical stress into biochemical signals related to EMT. Consistently, knocking-down of FAK inhibits EMT in renal tubular epithelial cells (Deng et al., 2010). However, the pathways downstream of FAK that promotes EMT are currently unknown.

6. EMT IN FIBROSIS AND DISEASE

During injury and repair, the boundaries of the tissue disintegrate and the protective architecture of the ECM is disturbed, thereby exposing cells to drastic changes in the mechanical environment. Under this mechanical imbalance, cells are exposed to an overwhelming cocktail of cytokines, initially derived from damaged cells, inflammatory cells, and myofibroblasts which drive tissue repair by secreting collagen and reorganizing the ECM (Gurtner et al., 2008; Hinz, 2010). Fibrosis is characterized by the massive deposition of ECM as a reactive process initiated to protect the tissue from injury. Nevertheless, fibrosis causes serious damage when it becomes

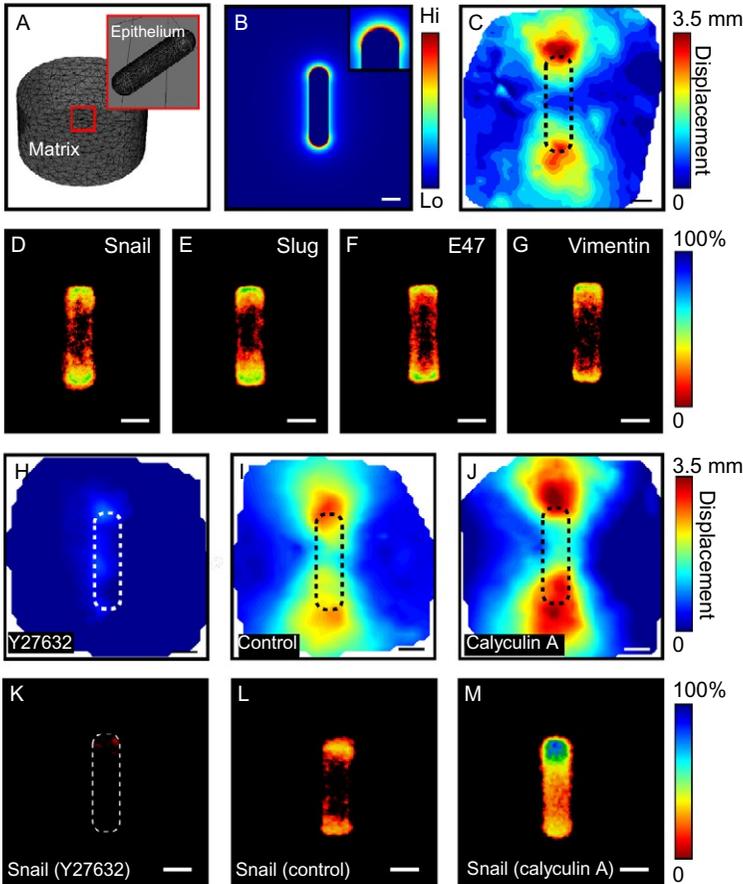


Figure 4.6 The correlation between mechanical stress and the expression of EMT markers in mammary epithelial tubules. Mesh shows the epithelium and ECM (A) and the stress profile of a mammary epithelial tubule (B, C). The expression of Snail (D), Slug (E), E47 (F), and vimentin (G) were concentrated at regions of high mechanical stress in the epithelium. Decreasing mechanical stress (H) led to reduced Snail expression (K) and enhancing mechanical stress (J) increased the expression of Snail (M), compared to control (I, L). Scale bar, 25 μm . Panels (A–C) adapted from [Gjorevski and Nelson \(2010\)](#); (D–F) adapted from [Lee et al. \(2011\)](#).

uncoupled from its initial stimulus ([Wynn, 2008](#)). Fibrosis is associated with the overgrowth, hardening, and scarring of tissues and is frequently observed in chronic diseases of the lung, liver, kidney, and heart ([Guarino et al., 2009](#); [Wynn, 2008](#)). Advanced stages of fibrosis result in organ dysfunction and eventually organ failure ([Guarino et al., 2009](#)). Further, in cancer, desmoplasia causes dense fibrosis around the tumor and is usually associated with malignancy ([Acloque et al., 2009](#); [Arendt et al., 2010](#)).

6.1. Fibroblasts and myofibroblasts

Fibroblasts are responsible for producing interstitial ECM. Fibroblasts are spindle-shaped cells found in the stroma of most tissues and characterized by the expression of vimentin. When activated, fibroblasts synthesize and secrete ECM and proteases capable of degrading ECM. Under nonpathological conditions, fibroblasts maintain homeostasis of the tissue (Powell et al., 1999; Turner and Grose, 2010).

When engaged in fibrogenesis, fibroblasts display the highly activated phenotype characteristic of myofibroblasts. Myofibroblasts are widely distributed throughout the embryo and are co-opted during tissue remodeling (Powell et al., 2011). In adult tissues, myofibroblasts are activated by inflammation and are involved in restoring tissue homeostasis and wound healing (Eckes et al., 2000; Wynn, 2008). Myofibroblasts express α SMA and differ from fibroblasts by the presence of cytoplasmic bundles of contractile microfilaments or stress fibers, which are similar but not identical to those in smooth muscle cells (Desmouliere et al., 2003). These cytoskeletal features not only enable the myofibroblast to remodel and contract the ECM but also to adapt to changes in the mechanical microenvironment. Myofibroblasts are also characterized by an increased proliferation, migratory ability, production of cytokines, and greater capacity to produce interstitial matrix (Desmouliere et al., 2003; Guarino et al., 2009). Myofibroblasts are present in large numbers in sites with ongoing inflammation and repair, and effectively close wounds through the contraction of connective tissue (Guarino et al., 2009; Hinz, 2010). However, due to the inability of myofibroblasts to regenerate tissue, they often create a collagenous and stiff scar. This scar tissue frequently disrupts the function of intact residual tissues and alters the biochemical and biophysical microenvironment, turning healthy neighboring cells into fibrotic and dysfunctional cells (Hinz, 2009). Therefore, deregulated activity of myofibroblasts results in impaired tissue function and even organ failure (Hinz, 2009; McAnulty, 2007).

6.2. Origin of myofibroblasts

Myofibroblasts were originally believed to be generated by proliferation and activation of local fibroblasts (Barnes and Gorin, 2011; Grillo, 1963). This was supported by the presence of fibroblasts positive for proliferation markers at the periphery of the wound (Grillo, 1963) that acquire smooth muscle features during wound healing and progressive organ fibrosis (Barnes and Gorin, 2011). However, an exclusive role for resident stromal cells in development of myofibroblasts has been reconsidered, and it is now thought that myofibroblasts can be derived from multiple sources (Abe et al., 2001; Zeisberg et al., 2007a) (Fig. 4.7). During pulmonary fibrosis, circulating fibroblast-like cells derived from bone marrow influx to the site of tissue

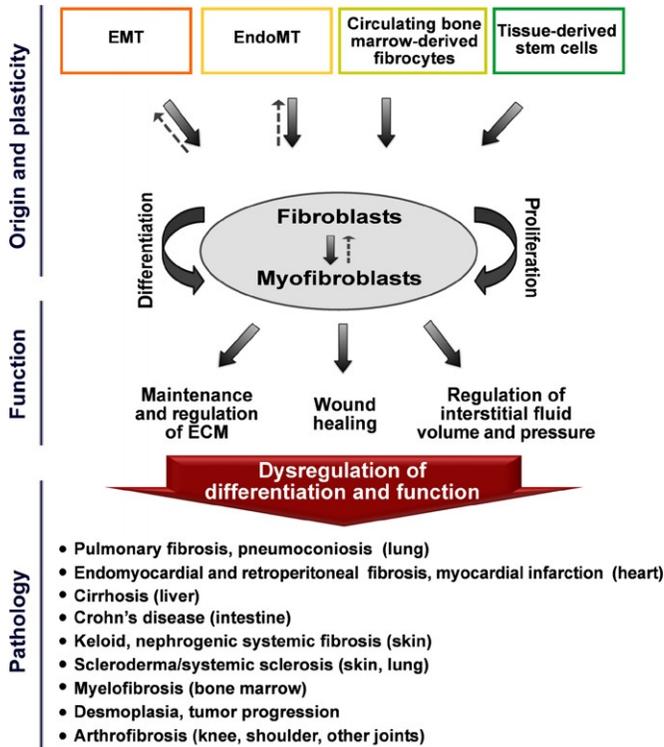


Figure 4.7 Myofibroblast: origin, function, and role in disease. In addition to proliferation and activation of local fibroblasts, myofibroblasts are generated from epithelial and endothelial cells through EMT and EndoMT and bone marrow- and tissue-derived stem cells. Myofibroblasts are responsible for ECM homeostasis and tissue remodeling and repair after injury. Nevertheless, myofibroblasts lead to serious organ damage when they become independent from the initial stimulus, which results in various human diseases.

injury (Abe et al., 2001). These blood-borne mesenchymal stem cell progenitors, termed fibrocytes, have myofibroblast-like features (Abe et al., 2001; Phillips et al., 2004). Fibrocytes represent a systemic source of contractile myofibroblasts in various fibrotic lesions such as lung, keloids, scleroderma, and kidney (Abe et al., 2001; Gressner et al., 2007). Similarly, bone marrow-derived hepatic stellate cells (HSCs) appear to be a source of myofibroblasts in liver fibrosis (Baba et al., 2004).

Myofibroblasts may also be generated by the transdifferentiation of epithelial cells through EMT. This possibility was initially suggested by the neo-expression of FSP1 in tubular epithelium at sites of inflammation and in epithelial cells undergoing a transition to fibroblasts in collagen gels

(Strutz et al., 1995). This finding was supported by histological evidence *in vivo* that epithelial cells at fibrotic regions acquired the phenotype of FSP1⁺/HSP47⁺ collagen-producing fibroblasts (Okada et al., 2000). Later, *in vivo* lineage tracing studies using transgenic mice provided direct evidence that myofibroblasts arise in large numbers through EMT during renal fibrosis (Iwano et al., 2002). A similar process occurs with endothelial cells undergoing EndoMT: lineage tracing studies showed that EndoMT contributes to the accumulation of cardiac fibroblasts and recapitulates pathways associated with cardiac development (Zeisberg et al., 2007a).

6.3. Contribution of EMT to organ fibrosis

EMT promotes the progression of fibrotic disease both by generating new mesenchymal cells that may expand the pool of interstitial fibroblasts/myofibroblasts, and by causing a loss of epithelial cells that probably leads to the destruction of parenchyma seen in advanced fibrosis.

Since it was first described that the renal interstitium in end-stage renal disease contains a population of cells with epithelial characteristics (Nadasdy et al., 1994), the role for EMT has been intensively investigated in this context. The expression of FSP1 in tubular epithelial cells during kidney fibrosis and fate-labeling tubular epithelium provided direct evidence that epithelium can contribute to fibrosis through EMT (Iwano et al., 2002; Strutz et al., 1995). The clinical relevance of EMT has also been demonstrated in a study characterizing kidney biopsies: a significant correlation was found between epithelial cells containing EMT features, extent of interstitial fibrosis, and renal functional impairment (Rastaldi et al., 2002). Moreover, expression of EMT markers including Snail has been observed in areas with significant collagen deposition in nephrectomy specimens from patients with urinary obstruction (Boutet et al., 2006). In these clinical settings, the expression of EMT markers is often seen before histological signs and is correlated with the risk of progression to chronic fibrosis, suggesting that EMT may be used to predict progression toward interstitial fibrosis (Hertig et al., 2008).

Pulmonary fibrosis is recognized as the end stage of tissue responses to injury including toxic, autoimmune, and infectious insults (Chapman, 2011). Histopathologically, IPF displays fibroblast foci, aggregates of proliferating fibroblasts and myofibroblasts, which are considered the site of active disease progression (Chapman, 2011). Fibroblast foci are frequently associated with metaplastic alteration of overlying epithelia and may be derived from abnormal proliferation of epithelial cells (Willis et al., 2005). Lung epithelial cells from patients with IPF coexpress epithelial and mesenchymal markers, suggesting EMT (Kim et al., 2006; Willis et al., 2005). This finding was supported by genetically modified mice in which the fate of alveolar epithelial cells can be tracked; vimentin-positive cells in injured

lung were mostly of alveolar epithelial origin, indicating epithelial cells as the main source of mesenchymal expansion during pulmonary fibrosis (Kim et al., 2006). In addition, significant nuclear β -catenin and secretion of TGF β were detected in bronchiolar and alveolar epithelial cells in biopsies from patients with IPF, suggesting aberrant activation of Wnt/ β -catenin and TGF β signaling (Chilosi et al., 2003; Willis et al., 2005).

Hepatic fibrosis is a scarring response to liver damage from various stimuli including viral hepatitis, alcohol abuse, drugs, congenital abnormality, and metabolic and autoimmune disease. Hepatic fibrosis is characterized by an increased pool of interstitial myofibroblasts derived from proliferation and activation of HSCs as described above. Epithelial cells including hepatocytes and cholangiocytes have been suggested as an additional source of myofibroblasts in liver fibrosis; treatment of primary rat hepatocytes with TGF β leads to downregulation of epithelial genes, upregulation of mesenchymal α SMA, collagen, FSP1, and increased migration (Kaimori et al., 2007); lineage tracing analysis has revealed that a substantial population of FSP1+ fibroblasts is derived from hepatocytes via EMT (Zeisberg et al., 2007b); cholangiocytes undergo EMT in response to conditioned medium from myofibroblastic HSC (Omenetti et al., 2008). Further, colocalization of epithelial and mesenchymal markers was detected in liver tissue from patients with biliary atresia as well as other liver diseases (Díaz et al., 2008).

TGF β is also believed to promote fibrotic disease in the eye. Such ocular fibrotic diseases include scarring in cornea and conjunctiva, fibrosis in the corneal endothelium, and fibrosis of the lens capsule following cataract surgery (Saika et al., 2008b). Unlike fibrotic lesions in other organs, myofibroblasts in the lens are derived only from EMT of lens or retinal pigment epithelium (Johar et al., 2007; Saika et al., 2008a). Anterior subcapsular cataract consists of irregular plaques of fibrous tissue that are formed by transition of lens epithelial cells to collagen-producing myofibroblasts, culminating with significant deposition of ECM (Guarino et al., 2009; Johar et al., 2007). EMT in retinal pigment epithelium is involved in the development of ocular fibrotic disease, proliferative vitreoretinopathy, and fibrosis in the retina (Saika et al., 2008a). TGF β /Smad signaling is responsible for these reactions; blocking Smad pharmacologically or through anti-Smad gene therapy suppresses the fibrotic reaction (Saika et al., 2008a,b).

Since it was first noticed that tumors are located near scar tissue, fibrosis has been investigated for its role in tumor formation and progression (Radisky et al., 2007). Myofibroblasts are abundant in the reactive tumor stroma where they are referred to as carcinoma-associated fibroblasts (CAFs) (Egeblad et al., 2005). CAFs have been shown to promote epithelial carcinogenesis; nontumorigenic epithelial cells form tumors when co-inoculated with CAFs (Olumi et al., 1999). CAFs are largely responsible for the desmoplastic response (Elenbaas and Weinberg, 2001) and in many cancers, including breast cancer, these cells play a role in upregulation of fibrillar

ECM. In fact, some of these changes occur before the carcinoma develops: high mammographic density which is reflective of excess collagen deposition is a strong predisposing factor for the development of breast cancer (Maskarinec et al., 2010). Although some studies suggested that CAFs may arise independently from carcinomas (Moinfar et al., 2000), CAFs may in fact be derived from epithelial cells that have undergone EMT (Petersen et al., 2003; Radisky et al., 2007). Indeed, immortal fibroblast-like cells that had the same X-inactivation pattern as the carcinoma cells in the tumor have been isolated from human breast cancer, and these cells behave like CAFs, nontumorigenic by themselves but causing transformation of mammary epithelial cells in culture and tumor growth *in vivo* (Petersen et al., 2003). Therefore, EMT may also affect the tumor microenvironment.

7. THERAPEUTICS THAT TARGET EMT AND FIBROSIS

Because of its potent role in pathogenesis of fibrotic diseases, detection of EMT in biopsy specimens could be useful diagnostically, and anti-EMT therapy has emerged as a target for drug development (Díaz et al., 2008; Galichon and Hertig, 2011). The EMT-inducing transcription factors that repress E-cadherin may be obvious targets. However, transcription factors are difficult to target with classical approaches such as small molecule inhibitors (Redell and Tweardy, 2006). Moreover, most genes including E-cadherin are regulated by two or more transcription factors that often act cooperatively (Comijn et al., 2001; Franco et al., 2011; Peinado et al., 2004b). MiRNA and siRNA would be potent alternatives in terms of specificity; however, further work is needed to increase stability and to improve efficacy in cell targeting and intracellular delivery. Nevertheless, recent studies showed that systemic administration of miRNA inhibits metastatic progression in mouse models (Kota et al., 2009; Ma et al., 2010). Other alternatives include use of negative regulators of EMT. Unfortunately, little is currently known about such regulators.

It is now clear that TGF β is an EMT inducer and profibrotic molecule, and many strategies to block TGF β have been used in animal studies (de Gouville and Huet, 2006; Huang et al., 2006; Liu et al., 2006). TGF β antibodies (Yu et al., 2004), antisense oligonucleotides (Isaka et al., 2000), inhibitors (Border et al., 1992), the negative regulatory signaling molecule Smad7 (Lan et al., 2003), and gene therapy using TGF β receptor chimera (Isaka et al., 1999) have all shown therapeutic efficacy. Among these, neutralizing antibodies against TGF β are the best developed. Studies *in vivo* have demonstrated their antifibrotic effects in renal fibrosis (Sharma et al., 1996; Ziyadeh et al., 2000), pulmonary fibrosis (Giri et al., 1993), arterial restenosis (Wolf et al., 1994), and skin scarring and thickening

(Yamamoto et al., 1999). Moreover, chronic inhibition of TGF β effectively prevents glomerulosclerosis and renal insufficiency resulting from type 2 diabetes without deleterious side effects (Ziyadeh et al., 2000). A potential clinical therapy can also be developed by using the antifibrotic effect of BMP7 that counteracts TGF β -induced EMT; application of BMP7 inhibits fibrosis in rat (Kinoshita et al., 2007) and stimulates regeneration of tissue and MET (Sugimoto et al., 2007; Zeisberg et al., 2005); administration of recombinant BMP reduces cardiac fibrosis by reversing EndoMT (Zeisberg et al., 2007a). Determination of the ratio of TGF β and BMP7, in serum or plasma, has also been suggested as a potential noninvasive diagnostic, since this ratio might reflect the progress of EMT (Damião et al., 2007; Gressner et al., 2007). However, the cytokine ratio in the circulation may not be an accurate reflection of that in the tissue.

Another strategy currently under investigation is to target the RTKs that activate EMT. Small molecule inhibitors targeting EGFR, Met, PDGFR, and VEGFR were initially developed as inhibitors of cell proliferation or angiogenesis and have been evaluated in preclinical and clinical trials against cancer (Mejias et al., 2009; Piechocki et al., 2008; Tugues et al., 2007). It was recently demonstrated that these inhibitors also prevent EMT and fibrogenesis. Gefitinib and erlotinib, competitive inhibitors of EGFR currently used for the treatment of advanced carcinomas, protect against pulmonary fibrosis and hepatic fibrosis/cirrhosis (Ishii et al., 2006; Kim et al., 2009). In addition, antiangiogenic drugs, sorafenib and sunitinib that inhibit VEGFR and PDGFR, have shown antifibrotic effects in liver (Mejias et al., 2009; Tugues et al., 2007).

8. CONCLUDING REMARKS AND PERSPECTIVES

Current health statistics suggest that nearly 45% of all deaths in the western world can be attributed to some type of chronic fibroproliferative disease (Wynn, 2007). Fibrosis can affect most organs and is a major cause of morbidity and mortality (Chilosi et al., 2006; Díaz et al., 2008; Johar et al., 2007; Rastaldi et al., 2002). Fibrotic tissue remodeling also influences cancer progression (Petersen et al., 2003; Radisky et al., 2007). EMT has become a key topic in the study of organ fibrosis, since stressed and injured epithelium can give rise to myofibroblasts and thereby contribute to fibrogenesis.

A growing number of the extracellular factors and intracellular mediators that control EMT have been identified and could be exploited in developing therapeutics for fibrosis. However, given the diversity of known EMT regulatory factors and complexity of the underlying signaling pathways, there is likely profound cross talk and feedback. Moreover, EMT

in vivo is often integrated with other processes that may occur during development and pathogenesis. This complexity and apparent redundancy impede the identification of novel targets and effective treatment for diseases associated with EMT. One of the current challenges is to elucidate a comprehensive view of the molecular mechanisms controlling EMT, and in so doing to identify a “master switch” that integrates various inputs and controls the EMT proteome. High-throughput mapping of signaling networks and time-resolved analysis may be required to uncover connectivity in these dynamic signaling pathways (Barrios-Rodiles et al., 2005; Vetter et al., 2009). Better culture models are also required to study EMT. Although conventional 2D culture systems allow the identification of pathways that are involved in the morphological conversion of epithelial cells, they have limitations; for example, most cells are not fully polarized in 2D models. *In vivo* studies are invaluable; however, they are much more demanding than those in culture, especially in mice. 3D cultures, where epithelial cells polarize and generate functional structures, hold particular promise (Lee et al., 2011; Leroy and Mostov, 2007; Nelson et al., 2006).

EMT might alter the mechanical aspects of a tissue through both accumulation of matrix-producing myofibroblasts and destruction of the epithelial parenchyma (Guarino et al., 2009; Hinz, 2010). The physical properties of tissues are crucial determinants of normal development and changes in the topology, and material properties of the microenvironment constitute a positive feedback loop that promotes disease progression (Levental et al., 2009; Schedin and Keely, 2011). EMT is also controlled through mechanical feedback from the ECM. A proto-myofibroblast phenotype is only produced on stiff substratum, whereas the development of stress fibers by fibroblasts is suppressed on softer substrata or in collagen gels (Tamariz and Grinnell, 2002; Yeung, 2005). Consistently, fibrotic tissues and contracting wound granulation tissues have been shown to be quite stiff, and this tissue is mainly populated by proto-myofibroblasts (Hinz, 2009). Stiff scar tissue further modulates the character of the healthy resident cells by driving the differentiation of a variety of precursor cells into myofibroblasts (Hinz, 2009). This mechanical cue for the differentiation of myofibroblasts may establish a vicious cycle because the excessive ECM-secreting and remodeling activities of myofibroblasts cause further connective tissue contraction (Lopez et al., 2011). Therefore, defining the unique local and global matrix properties within specific differentiated tissues is needed to understand how cells coordinate and adapt to their environment and how physical signals might modulate biochemical signaling pathways.

Finally, perhaps the most difficult challenge ahead is a coherent plan to translate experimental innovations into clinically effective regimes. Early pathologic detection of EMT markers might be relevant for patient prognosis, clinical decision making, or therapeutic options. Obstacles include the design of effective clinical trials with well-defined end points. Because

fibrosis typically progresses slowly in most diseases, clinical trials could be long and expensive. Therefore, there is a desperate need to develop noninvasive methods to differentiate between different fibrosis stages and reflect treatment outcome.

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