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ORIGINAL PAPER

Regulation of Epithelial-Mesenchymal Transition by Transmission of Mechanical Stress through Epithelial Tissues

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Abstract Epithelial-mesenchymal transition (EMT) is a phenotypic shift wherein epithelial cells lose or loosen attachments to their neighbors and assume a mesenchymal-like morphology. EMT drives a variety of developmental processes, but may also be adopted by tumor cells during neoplastic progression. EMT is regulated by both biochemical and physical signals from the microenvironment, including mechanical stress, which is increasingly recognized to play a major role in development and disease progression. Biological systems generate, transmit and concentrate mechanical stress into spatial patterns; these gradients in mechanical stress may serve to spatially pattern developmental and pathologic EMTs. Here we review how epithelial tissues generate and respond to mechanical stress gradients, and highlight the mechanisms by which mechanical stress regulates and patterns EMT.

Keywords Force · Contractility · MRTF · Morphogenesis

Abbreviations

ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
MET	Mesenchymal-epithelial transition
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MRTF	Myocardin-related transcription factor
SRF	Serum response factor
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
TGFβ	Transforming growth factor-beta
2D	Two-dimensional
3D	Three-dimensional

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Introduction

Epithelial-mesenchymal transition (EMT) is critical for embryonic development [1]. During gastrulation, the embryonic epithelium undergoes EMT to give rise to the mesoderm. During delamination of the neural crest, EMT is used to form a population of highly motile cells that ultimately incorporate into many different tissues [2, 3]. Induction of EMT alters cytoskeletal structure and leads to the breakdown of interactions between cells, their neighbors, and the underlying substratum. These phenotypic changes are driven by alterations in the expression of many genes including cytoskeletal components, transcription factors, and enzymes. Although the role of EMT in tumorigenesis and metastasis is currently a topic of active debate [4, 5], processes related to developmental EMTs are involved in key steps of tumor development [6, 7]. The biochemical and mechanical signals that regulate EMT are thus of critical interest.

The role of the mechanical microenvironment in the regulation of morphogenesis and pathogenesis is becoming ever more recognized. Mechanical cues from the environment direct basic cellular processes such as cell survival [8], proliferation [9], stem cell lineage commitment [10, 11] and EMT [12, 13]. At the tissue-level, mechanical signals have emerged as indispensable regulators of embryogenesis. Development of *Xenopus laevis* embryos requires that

mesoderm and notochord be sufficiently mechanically stiff so as to resist buckling [14, 15]. Actomyosin contractility is necessary for dorsal closure of the *Drosophila* embryo [16, 17], and forces due to apoptosis significantly contribute to the process [18]. External forces applied to the *Drosophila* embryo enhance expression of Twist, a key morphogenetic protein and promoter of EMT [19, 20]. Mechanical influences have also been implicated in the branching development of the mammalian lung [21], kidney [22] and mammary gland [23], and in breast involution following engorgement [24]. Importantly, dysregulation of mechanical signals has been shown to contribute to malignant transformation and progression. Increased activity of the small GTPase Rho—responsible for regulating cellular contractility—has been observed in human breast tumors [25] and increase in Rho-generated contractility promotes malignant progression by inducing tumor dissemination and angiogenesis [26]. Enzymatic crosslinking of the extracellular matrix (ECM) and its subsequent stiffening induce invasive behavior by otherwise non-metastatic breast cancer cells and drive tumor progression and malignancy [27–29]. Here we discuss the origins of mechanical stress within epithelial tissues, and describe how endogenous stresses may cooperate with biochemical signals in the microenvironment to regulate and spatially pattern EMT.

EMT as a Phenotypic Switch

Epithelial cells form functional polarized tissues by maintaining close contacts with their neighbors. These interactions are disrupted during EMT, which is characterized by the loss of cell-cell adhesion, loss of apical-basal polarity and the acquisition of migratory properties resulting in loosely organized mesenchymal cells [30]. Changes in gene expression associated with EMT include down-regulation of epithelial markers including E-cadherin and up-regulation of mesenchymal markers including vimentin, α-smooth muscle actin, N-cadherin, and fibronectin [31]. The reverse of this process, mesenchymal-epithelial transition (MET), occurs during the formation of epithelial organs and secondary tumors [32].

EMT plays an important role in normal developmental processes including embryogenesis, organogenesis, wound healing, and tissue regeneration as well as in diseases such as fibrosis and cancer [30]. During gastrulation, EMT governs the cell migration and rearrangement required for the formation of the three distinct germ layers [3, 30, 33]. EMT is also responsible for development of the neural crest and heart valves [30]. In cancer, EMT has been noted at the invasive front of the tumor mass, and may be instrumental in the acquisition of motility required for invasion and metastasis. Once tumor cells have circulated, MET may allow migratory cancer cells to establish a secondary tumor [30, 32].

It has been proposed that EMT occurs in three distinct biological settings (type 1, type 2, and type 3), each of which result in fundamentally different functional consequences. Type 1 EMT is associated with the developmental processes of implantation, embryogenesis and organ development. Diverse cell types are generated, including the primary mesenchyme which can later undergo MET to generate secondary epithelia. Importantly, type 1 EMT does not cause fibrosis and cannot induce an invasive phenotype. Inflammation induces type 2 EMT, which is involved in wound healing and tissue regeneration. Chronic inflammation results in persistent type 2 EMT which leads to fibrosis. Genetic and epigenetic abnormalities of neoplastic cells conspire with the EMT regulatory circuitry to generate type 3 EMT, a program by which epithelial carcinoma cells acquire the ability to invade and metastasize. Remarkably, a common set of genetic and biochemical elements is thought to underlie and enable these three types of EMT with fundamentally different functional consequences and outwardly diverse phenotypic programs [33, 34].

Biochemical Induction of EMT

Just as there are several different EMT programs with different functional consequences, there are also several different biochemical signals that can induce EMT, including cytokines, growth factors, and matrix metalloproteinases (MMPs) [30]. Perhaps the most well-studied EMT stimulus is transforming growth factor (TGF)-β, which initiates and maintains EMT in a variety of biological systems [35]. In response to activation by binding to TGFβ, type I and type II TGFβ receptors dimerize and induce signaling that results in phosphorylation of receptor-activated Smad2 and Smad3. These phosphorylated Smads partner with cytoplasmic Smad4 and translocate to the nucleus where Smad complexes control the transcription of target genes that regulate EMT [35–38]. The expression of the Snail family of transcription factors is induced directly in response to TGFβ [38, 39]. Snail transcription factors are structurally similar, containing a characteristic zinc finger-rich C-terminal domain that mediates sequence-specific binding to E-box elements within the regulatory regions of different genes [39, 40]. The activation of Snail transcription factors represses the expression of epithelial markers (claudins, occludin, E-cadherin, cytokeratins, etc.) and upregulates that of mesenchymal markers (fibronectin, N-cadherin, Twist, etc.) [38]. For example, both Snail1 and Snail2 repress the expression of *CDH1* (the gene that encodes E-cadherin) by binding to E-box elements in the promoter and recruiting a combination of co-repressors [41–45].

TGFβ also activates Rho-family GTPases by targeting guanine nucleotide exchange factors, thereby affecting actin cytoskeletal dynamics, stress fiber formation, and the acquisition of mesenchymal characteristics [46]. Activation of the Rho pathway is fundamental to the formation of stress fibers and cytoskeletal contractility. Furthermore E-cadherin clustering, adherens junction maturation, and linkage to the actin cytoskeleton are controlled by RhoA, Rac1 and Cdc42 [42], respectively [47]. TGFβ-induced EMT can also be modulated by signaling through phosphatidylinositol 3-kinase, mitogen-activated protein kinase, Jagged/Notch (reviewed in [35]), and myocardin-related transcription factor (MRTF)-A (discussed below).

Similarly, MMP3-induced EMT is modulated by members of the Rho GTPase family. In the presence of MMP3 in culture or in vivo, mammary epithelial cells scatter, downregulate epithelial markers, upregulate mesenchymal markers [48], form large lamellipodia, and spread out substantially [13]. Radisky and colleagues have shown that MMP3-induced EMT is not dependent on RhoA and Cdc42; however, the expression of Rac1b, an alternatively spliced variant of Rac1, causes an increase in cellular reactive oxygen species (ROS), which ultimately stimulates the expression of Snail and EMT [48]. MMP3-induced spreading is a consequence of Rac1b expression and is required for downstream EMT. Blocking cell spreading by plating cells at high density effectively inhibits induction of Rac1b and EMT-related gene expression. Therefore, cell shape regulates MMP3-induced EMT [13], suggesting that cytoskeletal contraction and mechanical stress play an indirect role in this process.

Endogenous Mechanical Stress

Generation of Mechanical Stress

Before we discuss the role of mechanical stress in EMT, we begin with a review of how cells create stresses. In physics, force is defined as an influence that accelerates a given mass. In simple terms, force is a mechanical quantity associated with a "push" or a "pull". A closely related (but not universally interchangeable) quantity is mechanical stress, defined as force per unit area. Mechanical stress arises within deformable bodies from the application of internal or external forces. Stresses acting perpendicular to a surface are called normal stresses, whereas those acting parallel to a surface are called shear stresses. For example, blood pressure is a normal stress that prevents veins from collapsing, whereas blood flow induces a shear stress across the surface of endothelial cells lining the vessels.

The ability of biological systems—cells and tissues—to generate and transmit mechanical forces over a distance has long been recognized. Harris and co-workers visualized cell-generated forces nearly three decades ago by demonstrating that fibroblasts plated on silicone membranes pull on their substratum, creating wrinkles [49]. Such forces have since been extensively analyzed and measured [50–52]. Endogenous forces arise from the tendency of cells to contract. In response to various stimuli, non-muscle myosin II motors undergo ATP-dependent activation and "walk" along actin filaments, thus creating contractile, force-generating actomyosin bundles [53, 54]. The best-described regulators of myosin II and the overall contractile machinery of the cell include myosin light chain kinase (MLCK) and the Rho effector Rho-associated kinase (ROCK) [55–57]. MLCK directly phosphorylates the regulatory myosin light chain (MLC), whereas ROCK has a dual role: it promotes myosin activation both by phosphorylating MLC and by inactivating MLC phosphatase. These regulatory proteins thus form the machinery that enables cells to contract and pull.

Cellular contraction alone is not sufficient for the generation of stress. Mechanical stress necessitates cellular attachment and contraction against a substratum capable of resisting deformation [58, 59]. The ability of a substratum to resist deformation, thus balancing cytoskeletal forces and giving rise to stress, is quantified by its elastic modulus (stiffness), a physical parameter implicated in the regulation of normal and pathologic processes [29, 60]. How matrix stiffness affects the generation of mechanical stress has been tested by plating cells on a substratum comprised of ECM-coated beads of submicrometer (i.e. subcellular) size. The beads were not physically linked, allowing the cells to displace them without encountering resistance. These experiments demonstrated that cells plated on such substrata fail to produce stress, which suggests that substratum stiffness is necessary for generation of stress [59] (Fig. 1a). In fact, matrix stiffness not only maintains cell-generated mechanical stress, but also modulates it: stiffer two-dimensional (2D) substrata and three-dimensional (3D) matrices lead to activation of the Rho pathway, stronger cell-matrix adhesion and ultimately enhanced generation of force [29, 60] (Fig. 1b). Cell-generated stresses thus require both cytoskeletal contraction and attachment to ECM or neighboring cells.

Transmission and Concentration of Mechanical Stress

Epithelial cells rarely function individually in vivo. Instead, they are connected to their neighbors via cell-cell junctions and to the ECM via cell-matrix adhesions, thus forming functional epithelial tissues. Importantly, the junctional proteins are directly or indirectly linked to the force-generating cytoskeletal machinery, thus creating a supra-structure capable of long-range transmission of mechanical stresses produced at the cellular level.

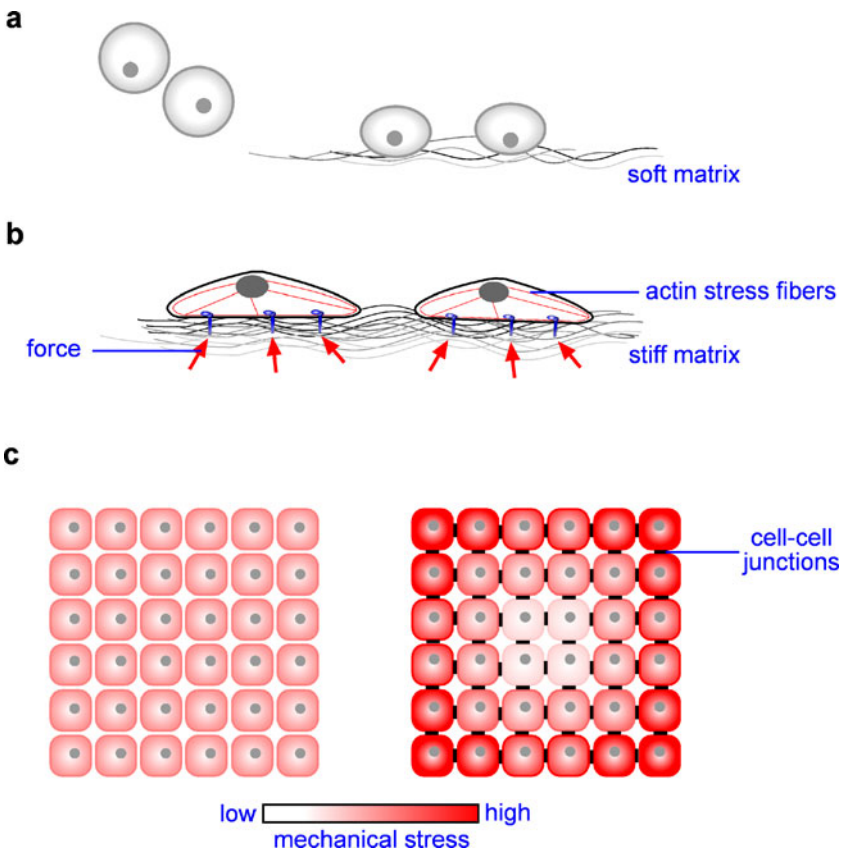

Fig. 1 Intercellular transmission of endogenous contraction. **a** Cells in suspension or attached to soft matrices incapable of resisting deformation fail to generate mechanical stress. **b** Cells attached to a stiff substratum contract isometrically, giving rise to mechanical stress locally. **c** When cells are connected into cohorts, mechanical stresses generated at the single cell level are transmitted over a distance via intercellular junctions. Transmission of stress across tissues of anisotropic geometry results in concentration of stress and formation of gradients; for example, maximum stress occurs at corners of square monolayers

It is by now widely accepted that the actin cytoskeleton is physically linked to the ECM at focal adhesions, comprised of transmembrane integrin receptors and numerous scaffolding proteins [61, 62]. This link not only tethers the cell to the ECM, which resists deformation leading to mechanical stress, but also serves as a conduit for inside-out channeling of mechanical force. Techniques used to quantify cell-generated forces, such as 2D or 3D traction force microscopy, rely upon the transmission of force from cells to 2D substratum or 3D matrix [23, 50]. Here, matrix deformations induced by cells are visualized, measured and, when possible, converted to mechanical stresses. Cells may transmit stress over long distances through compliant matrices in order to communicate mechanically with adjacent cells or tissues. Hammer and co-workers have demonstrated that endothelial cells in culture can detect and respond to substratum deformation due to stresses originating from neighboring cells [63]. The extent of matrix deformation depends upon its stiffness, suggesting that ECM stiffness determines the maximum distance over which cells can communicate mechanical signals.

Cells can also transmit stresses directly to coherent neighbors. Adherens junctions are a type of intercellular junction maintained by calcium-dependent homophilic interactions between cadherins. The engagement between the extracellular cadherin domains of adjacent cells triggers the recruitment of structural and signaling proteins on the cytoplasmic face, which anchor the junction to actin creating physical continuity between the cytoskeletons of adjacent cells [64]. Actin cables which circumscribe wounds in epithelial sheets appear to be continuous from cell to cell and connected by clusters of E-cadherin at cell-cell contacts [65, 66]. The collective contraction of the interlinked actin cables generates force which is transmitted at ranges that are considerably longer than the length of a single cell and span the entire perimeter of the wound, driving wound closure [65, 66].

Collective cellular contraction and transmission of the resulting stress within tissues of anisotropic (i.e. non-spherical) geometries leads to concentration of stress and formation of spatial stress gradients. The existence of mechanical gradients in cellular monolayers and engineered 3D epithelial tissues has been predicted computationally and confirmed experimentally [9, 12, 23]. Here, lithography-based microfabrication methods were used to control the 2D or 3D geometry of the tissues, and maximum stress was observed at sharp corners and regions of high convex curvature (Fig. 1c). As expected, preventing transmission of stress by disrupting the physical link between the cadherins and the actin cytoskeleton abrogated the gradients, rendering the mechanical stress spatially uniform [23]. Tissue-level heterogeneities in the distribution of mechanical stress have also been demonstrated in amphibian embryos and correlated with morphological patterns and mechanochemical processes in vivo [67]. Cellular contraction may thus be used as a microenvir-

onmental cue to signal over large distances during development.

Mechanosensing and Mechanotransduction

Endogenous and exogenous mechanical forces influence a variety of biological behaviors. High local mechanical stress has been correlated and causatively linked with basic cellular processes such as proliferation [9], stem cell differentiation [68], EMT [12], and morphogenetic processes such as branch formation [23]. However, we still have a poor grasp of cellular mechanosensing and mechanotransduction, the mechanisms whereby cells and tissues sense and interpret physical signals and convert them into a functional response.

A number of cellular structures are emerging as mechanosensors, including the focal adhesion machinery. Numerous proteins are recruited at focal adhesions and phosphorylated in a stress-dependent manner [58]. Focal adhesion kinase (FAK) and Src, in particular, have been implicated as mechanosensors. FAK undergoes enhanced phosphorylation in response to mechanical stress [23, 69, 70] and is required for sensing of substratum stiffness during fibroblast migration [71]. Similarly, fluorescence-resonance energy transfer analysis has shown that Src is activated at adhesion sites in response to mechanical stress [72]. Active FAK and Src direct a plethora of cellular processes including proliferation, differentiation, adhesion, motility and invasion [71, 73, 74]. It must be emphasized, however, that mechanosensitive pathways often feed back to regulate the generation of force, thus serving as more than passive sensors. This feedback complicates studies aimed at defining specific roles within the mechanobiological machinery of the cell [75, 76].

We discussed the cellular structures likely responsible for sensing mechanical stress, but the question remains: how does mechanotransduction occur? That is, what are the molecular-level effects of force responsible for causing biochemical and functional response? One relatively well-documented mechanism is force-induced changes in protein conformation. Studies in molecular mechanics report stress-triggered alteration in a number of protein structural motifs (reviewed in [77, 78]). For instance, cellular contractile activity is sufficient to partially unfold fibronectin, exposing otherwise hidden (cryptic) regions [79]. Physical forces also open ion channels tethered to the ECM or cytoskeletal filaments, and thus influence the flux of ions into or out of the cells [77, 78]. The nucleus itself has been implicated as another potential site for mechanotransduction. Accumulating evidence suggests the existence of physical continuity between the ECM, the cytoskeleton, the nuclear lamina, and chromatin [80–82]. One could speculate that force transmitted to the nucleus may directly unravel chromatin, allowing access to transcription factors and thereby regulating gene expression. Identification of mechanosensing and mechanotransduction machinery is an active area of investigation.

Mechanical Regulation of EMT

Mechanosensing and mechanotransduction have recently been implicated as playing crucial roles in the regulation of EMT events. Biochemical cues such as TGFβ induce EMT, but these signals cooperate with mechanical stress to yield spatial patterns within tissues [12]. Indeed, spatial variations and patterns in EMT are widely observed and may be necessary for development [3] and disease progression [30, 32]. The relationship between mechanical stress and EMT is demonstrated by signaling through MRTF-A, a cofactor of serum response factor (SRF) [83, 84]. Rho activation triggers the nuclear translocation of MRTF-A, which can thereby activate two parallel pathways during EMT in cooperation with SRF. First, the expression of EMT-regulating genes is enhanced, leading to the dissolution of cell-cell contacts. Second, the expression of cytoskeletal genes is up-regulated, thereby affecting remodeling of the cytoskeleton [84].

The activation of MRTF-A thus connects mechanical stress and actin dynamics to the regulation of transcription [85, 86]. Under conditions of low mechanical stress, the actin cytoskeleton is in a relaxed state, with a relatively high pool of actin monomers. Monomeric actin associates with the three Arg-Pro-X-X-X-Glu-Leu (RPEL) motifs (RPEL1, RPEL2, and RPEL3) located in the amino-terminus of myocardin-family proteins [85–88]. Point mutations to RPEL2 and RPEL3 abolish the association between actin and MRTF-A; therefore, these regions of the amino-terminal sequence are essential for MRTF-A to bind to monomeric actin and be sequestered in the cytoplasm [87, 89]. Activation of the Rho-actin signaling pathway due to increased mechanical stress is necessary and sufficient to promote the nuclear accumulation of MRTF-A [87]. Under conditions of high mechanical stress, the activation of Rho small GTPases and subsequent cytoskeletal polymerization reduces the cytoplasmic pool of G-actin, thereby favoring the dissociation of MRTF-A from G-actin and ultimately resulting in nuclear translocation (Fig. 2) [84, 87, 90–92].

Cell deformation or perceived tension thus regulates the nuclear accumulation of MRTF-A [90], which can thereby determine which cells within a tissue will undergo EMT. As described above, the endogenous contractility and cohesion of epithelial sheets causes mechanical stresses to be transmitted between cells. If the epithelium has any degree of geometric asymmetry—that is, if the epithelium is not spherical—then mechanical stresses will concentrate within

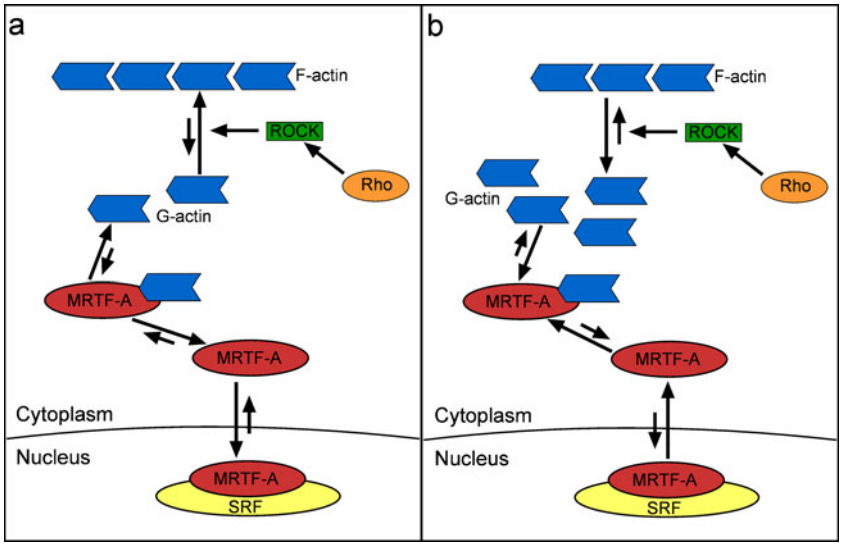

Fig. 2 Regulation of MRTF-A by mechanical stress. **a** Increased mechanical stress causes increased actin polymerization, thereby decreasing the cytoplasmic pool of G-actin and increasing the nuclear localization of MRTF-A by triggering its dissociation from G-actin. **b** Under conditions of low mechanical stress a larger cytoplasmic pool of G-actin sequesters MRTF-A in the cytoplasm

subpopulations of cells. This spatial patterning of endogenous mechanical stress would be expected to template patterns of Rho activation and cytoskeletal assembly within the epithelium, which could result in patterning of the activation of MRTF-A and initiation of transcription. We recently found that this is indeed the case, at least for mammary epithelial sheets in culture. When treated with TGFβ, microfabricated monolayers of mammary epithelial cells undergo EMT at regions of high mechanical stress; cells located within these regions show increased nuclear localization of MRTF-A [12] (Fig. 3). Disrupting the connections between cells removes the patterning of mechanical stress and leads to uniform activation of EMT across the entire epithelium. Conversely, forcing MRTF-A to translocate to the nucleus of cells located within low stress regions of the monolayer induces aberrant EMT. Intercellular transmission of mechanical stress between cells within epithelial tissues may thus serve to pattern the response of constituent cells to biochemical inducers of EMT, even those as potent as TGFβ. The relationships between these signals is complex, and complicated by recent findings that Smad3 may also act to inhibit transcription downstream of TGFβ [93, 94].

Implications for Development and Disease

Tissue morphogenesis is by nature a highly patterned, intensely physical process. Tissues are sculpted and pulled into the final architectures that comprise mature organs; these mechanical stresses are likely involved in the realization of developmental EMTs. Gene expression changes consistent with EMT have been proposed to play a role in the branching morphogenesis process that builds the arborous structures of the epithelial ducts in the kidney, lung, and mammary gland [95–97]. We recently found that branching regions of mammary epithelial tissues are templated by patterns of endogenous mechanical stress such that regions of high stress induced the local emergence of nascent multicellular branches [23]. The branch sites also express genes within the EMT proteome [98, 99], consistent with the possibility that patterns of endogenous mechanical stress play a role in EMT gene expression during branching morphogenesis. This possibility awaits further exploration.

In pathological conditions, EMT has been observed to be spatially segregated to specific subpopulations of cells as well. During re-epithelialization of the skin, EMT events

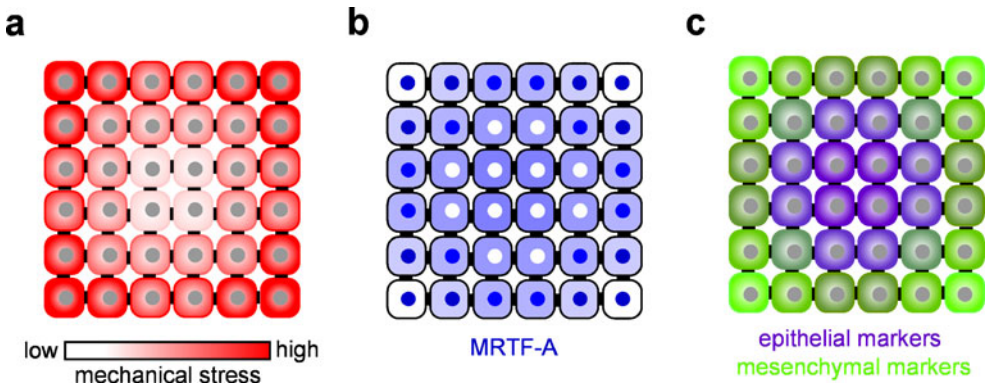

Fig. 3 Endogenous mechanical stress patterns EMT. **a** In monolayers of epithelial cells, stress is concentrated at the free edges and corners of the tissues. **b** Under these conditions, MRTF-A accumulates in the nuclei of cells located at the free edges and corners of the tissue, but is sequestered in the cytoplasm of cells located in the center of the tissue. **c** When treated with TGFβ, cells located in high stress regions undergo EMT

were found to be localized to the edges of the healing wounds [100]. As discussed above, the free edges of epithelial tissues would be expected to experience the highest amounts of mechanical stress simply from endogenous cellular contraction. Indeed, strain-gauge devices have shown significant forces generated by skin wounds as they heal [101]. It remains unclear whether mechanical stress plays a role in the EMT events occurring at the free edges of healing wounds, but mechanical stress, MRTF-A, and CArG boxes are all involved in the generation and maintenance of myofibroblasts present within granulation tissue [102, 103]. Mechanical signaling may augment the EMT pathway in the epithelial regions of skin wounds.

Patterns of mechanical stress may also play a role in the induction of EMT during tumor progression. Tumors are invariably stiffer than the surrounding normal tissue [28], and this increase in stiffness plays a fundamental role in the generation of the tumorigenic phenotype [28, 29]. As discussed above, the stiffness of the surrounding substratum affects cellular contractility. Increased stiffness enhances cellular contractility, which would be expected to increase the magnitude of endogenous mechanical stresses within the tumor tissue. Mechanical stress helps to induce EMT downstream of at least two different stimuli common to the tumor microenvironment (TGFβ and MMPs), so it is plausible that the physical properties of the tumor enhance EMT pathways thought to be necessary for tumor invasion and metastasis. During tumor invasion itself, EMT has been localized to the leading edges of metastatic cohorts of colorectal carcinomas [104, 105]. This patterned localization of EMT may be due to transmission and concentration of intercellular tension.

Conclusions

Mechanical stress arises from the contractile nature of the actin cytoskeleton and is transmitted through and concentrated within epithelial tissues by virtue of the cohesion between neighboring cells. Mechanical stress acts as an independent signal that can integrate with other (soluble) signals within the microenvironment to direct the phenotypes of constituent cells. By altering cytoskeletal dynamics, mechanical stress directly impacts the regulation of transcription through modulation of the subcellular localization of proteins including the transcription factor MRTF-A. High mechanical stress—from exogenous application, from increased ECM stiffness, or from endogenous contractility—can thus influence the subpopulations of cells within a tissue (normal, healing, or tumorigenic) that will undergo EMT. Because the EMT process generates cells of mesenchymal, migratory, contractile phenotype, these signals may cause both feed-back and feed-forward loops that dynamically impact the patterning of the tissues. Future studies investigating the signaling and mechanical regulatory networks within tissues undergoing EMT may reveal points and targets to augment the process (in the case of wound healing) or cut it short (in the case of metastasis).

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Conflict of interest The authors declare they have no conflict of interest.

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