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Mechano-reciprocity is maintained between physiological boundaries by tuning signal flux through the Rho-associated protein kinase

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Mechano-reciprocity is maintained between physiological boundaries by tuning signal flux through the Rho-associated protein kinase

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Abstract

The mechanical properties of the ECM strongly influence the behavior of all cell types within a given tissue. Increased matrix tension promotes epithelial cell proliferation by engaging mitogenic mechanotransduction signaling including the Salvador/Warts/Hippo, PI 3-kinase, Rho, Wnt and MAP kinase pathways. The Rho signaling pathways in particular are capable of increasing intra-cellular tension by elevating the production and contractility of the actomyosin cytoskeleton, which counteracts tension changes within the matrix in a process termed mechano-reciprocity. We have discovered that Rho-ROCK signaling increases the production of ECM through paracrine signaling between the epithelium and fibroblasts and also the remodeling of the ECM by regulating focal adhesion dynamics in fibroblasts. These two phenomena together cause increased ECM tension. Enhanced mechano-reciprocity results in ever-increasing intra- and extra-cellular tension in a vicious cycle that promotes cell proliferation and tumor progression. These insights reveal that inhibiting mechano-reciprocity, reducing ECM tension and targeting cancer-associated fibroblasts in a coordinated fashion has potential as cancer therapy.

Introduction

Mechanical forces in biology

The capacity to exert mechanical force upon an object is a fundamental requirement for physical interaction with the environment. Flowing from this is the need to detect and measure external forces exerted upon the body. Everyday activities like sitting, standing and handling objects require not only the exertion of mechanical force, but the ability to detect mechanical signals and coordinate a timely response. This is facilitated by the sense of proprioception, which is mediated by mechanoreceptors, nerve endings specialized for the detection of forces. However, it has been clear for some time that mechanical force can be detected not only by specialized nerve receptors, but also by all other cell types. Indeed, the generation and detection of mechanical force is a key aspect of cell and developmental biology. Cells interact with their environment by exerting and sensing mechanical forces between themselves and the extra-cellular matrix (ECM). These mechanical forces greatly influence cell behavior, by guiding decisions about cell migration, growth, division and differentiation.

Mechanical signaling and cancer

Whereas the biochemical aspects of unrestrained cell growth and proliferation in cancer have been extensively studied over many decades, an understanding of the biophysical mechanisms underlying how force and mechanical stress influence tumor development is only just emerging. However, as early as 1972, reports appeared that mechanical force has the capacity to influence tumor growth and metastasis[1]. Throughout the late 70s and early 80s, reports emerged that the application of mechanical stress upon cells is capable of eliciting distinct phenotypic

responses[2-4]. The first clues into how mechanical force may be converted to a biochemical signal for propagation within the cell were provided by Carvalho et al.[5], who demonstrated that integrin expression and subcellular distribution is markedly altered upon the application of external force on cells. As integrins were well known to link cells to the extra-cellular matrix[6] and were dysregulated in cancers, they then became the prime candidates for the mechanosensing **(Figure 1)** receptors transducing mechanical signals to the intra-cellular biochemical machinery. This pioneering work caused a flurry of papers providing evidence for a link between extra-cellular mechanical stresses and changes in actin cytoskeletal structure, adhesion complex composition and number, cell migration and spreading, and proliferation via integrin ligation (reviewed in [7]), thereby linking extra-cellular biophysical signals to intra-cellular biochemical changes. This process became known as mechanotransduction (**Figure 1**) and several signaling pathways including those mediated by Salvador/Warts/Hippo[8], PI 3-kinase[9], Rho small GTPases[10], Wnts[11,12] and MAP kinases[10] have been shown to be activated downstream of changes in the mechanical properties of the ECM.

It therefore follows that changes in the mechanical properties of the ECM have implications for tumor progression, as mitogenic pathways are directly linked to changes in ECM stiffness via mechanotransduction. However, the observation that ECM was increased in many different cancers and directly influenced tumor growth and spread[13,14] suggested that tumors themselves were capable of strongly promoting changes in the ECM that facilitated their growth and spread. Several growth factors of tumor origin, including TGFβ, CTGF, IL6 and LIF are known to directly act upon tumor-associated fibroblasts and other stromal cells to increase the production and remodeling of ECM molecules to increase ECM stiffness[15-18]. While

some of the mechanisms underlying mechano-sensation, mechanotransduction and conversely the direct involvement of tumors in regulating ECM stiffness are beginning to be uncovered, a precise understanding of how these processes are integrated and coordinated still remains elusive.

The Rho family of small GTPases and the regulation of intracellular tension

Rho (Ras homology) GTPases are monomeric GTP-binding proteins and are a subset of the Ras superfamily, comprising 22 members of which the best characterized are Rho, Rac and Cdc42. Rho family members act as molecular regulators of signal transduction by switching between GDP-bound inactive and GTP-bound active states, and have numerous molecular targets including kinases, transcription factors and scaffold proteins that mediate diverse cellular processes such as migration, proliferation, adhesion and apoptosis in cell type and temporal context-dependent ways (reviewed in [19]). The regulation of actomyosin cytoskeletal dynamics by these proteins is the best studied aspect of their biology.

Dynamic remodeling of the actin cytoskeleton to generate intra-cellular force is key to cell migration. The extension of actin filament networks by actin polymerization generates intracellular forces, as filaments in the leading edge are compressed between transient associations with the cell membrane and the bulk of the actin cytoskeletal network behind them. As protrusions grow and retract, individual actin filaments undergo tension from transient bonds with the membrane, and are bent or compressed depending on their orientation[20]. Myosin-mediated contraction of the actin cytoskeleton results in a reduction in plasma membrane surface area and causes clustering of cell surface integrins, which are responsible for attachment to the ECM. Integrin-mediated ECM adhesion activates guanine nucleotide exchange

factors (GEFs), which enhance RhoA activity[21]. During ameboid cell movement, Rac interacts with lamellipodin at the leading edge to extend actin filaments and generate lamellipodia, sheet-like cell protrusions supported by short actin filaments in a branched network, which adhere to substrates and determine the direction of cell movement[22]. Cdc42 controls the formation of filopodia, thin protrusions from the cell membrane that contain parallel actin bundles for mechanosensing the environment in order to determine cell polarity – another mechanism by which the direction of movement is determined[23]. In order to direct migration, actin polymerization must be restricted to a portion of the plasma membrane. Cdc42 directs this polarity, by acting through the Par polarity complex, localizing Rac activity and stabilizing microtubules within the cytoskeleton. At the trailing edge of cells exhibiting mesenchymal motility, and to a lesser extent those exhibiting ameboid motility, RhoA controls retraction through activation of effector kinases such as Rho-associated kinase (ROCK)[24,25]. Furthermore, Rho-mediated activation of ROCK is required for the formation of membrane ruffles and lamellae and in particular membrane blebs. Membrane blebs are a feature of ameboid motility (also termed blebbing motility), a mode of cell migration that is independent of lamellipodia and frequently observed in cancer cells[26]. Rho signaling is also responsible for the generation of intra-cellular tension, which is required for the morphological changes associated with other cellular processes such as apoptosis[27,28] and cytokinesis[29].

The effector proteins of Rho signaling, Rho-associated kinases 1 and 2 (ROCK1 and ROCK2), are serine-threonine kinases containing a Rho-binding domain and are activated upon interaction with Rho-GTP[30]. Activation of ROCK by Rho induces stress fiber assembly in a number of ways. ROCK directly phosphorylates and activates the regulatory myosin light chain (MLC)[31], it indirectly increases MLC

phosphorylation by phosphorylating and inhibiting the myosin targeting subunit of MLC phosphatase (MYPT)[32] and directly phosphorylating and activating the LIM kinases (LIMK1 and LIMK2), which subsequently phosphorylate and inhibit cofilin and stabilizes actin structures[33]. In cultured cells, parallel bundles of actin filaments form stress fibers that link sites of focal adhesions, permitting tension to be transmitted between focal adhesions and thereby to the ECM. As well as being the product of intracellular tension, stress fibers themselves are able to exert force upon focal adhesions, permitting forces exerted by the external environment to be counteracted[21].

Rho-ROCK signaling and the regulation of extracellular tension

Not surprisingly, cells are not merely passive reactors to changes in the mechanical properties of the ECM, but are active players in the remodeling of their environments. Protein components of the ECM can be degraded by a variety of proteinases such as the families of matrix metalloproteinases (MMPs)[34] and a disintegrin and metalloproteinases (ADAMs)[35] that are produced by cancer cells to remodel the ECM. While most proteinases acting on the ECM are secreted, a subset of MMPs are membrane tethered, permitting cells to exert a greater degree of spatial control over their deployment[36]. The ECM may also be remodeled by changes to its composition and level of cross-linking. Lysyl oxidases and lysyl hydroxylases are produced by cells to crosslink collagen chains, thereby increasing ECM stiffness[37]. The mechanical properties of the ECM are also significantly altered in diseased states, such as chronic wounds, cancer and fibrosis, by changes to ECM composition[38,39].

Recently, we demonstrated a mechanism by which epithelial cells and tumor cells of epithelial origin are capable of increasing the mechanical stiffness of the ECM by elevating the production of ECM components[14,39,40]. Activation of ROCK in the context of tumor cells or the hyper-proliferation of epidermal cells in the context of wound healing results in paracrine signals arising from the proliferating epithelia that act on stromal fibroblasts, increasing their production of collagen, fibronectin, periostin and tenascin C, components of the ECM, and thereby increasing matrix stiffness[14,39]. While the nature of the specific paracrine signals caused by activation of ROCK remains to be uncovered, cytokines and other secreted molecules of tumor origin have been previously implicated in the recruitment and activation of cancer-associated fibroblasts, the key regulators of ECM composition and stiffness in the tumor context[15-17]. Nevertheless, ROCK activation in tumor cells results in increased ECM stiffness of the magnitude frequently observed in epithelial cancers including cutaneous squamous cell carcinoma[14], breast cancer[13] and pancreatic cancer[41] and promotes tumor progression. We therefore propose that ROCK integrates inputs from growth factor and mechanotransduction signaling to produce the appropriate cellular response, be it migration, proliferation, ECM production or remodeling.

Biochemical changes arising from increased extra-cellular tension

External forces drive clustering of integrin molecules at the cell membrane, stabilizing focal adhesions and linking the intracellular actin cytoskeleton to the ECM. The stiffer the ECM or substrate to which a cell is attached, the greater the size and strength of those focal adhesions, and the greater the cellular response, as intra-cellular forces are generated to balance the extra-cellular forces. Upon integrin activation, talin and vinculin are recruited to the complex, and focal adhesion kinase (FAK) is auto-phosphorylated at Y397 and/or Y925 and activated. FAK

phosphorylation results in the recruitment of Src-homology-2 (SH2) domain-containing proteins Src and Shc that link FAK to the Ras pathway and Rho-ROCK pathway and, via PI 3-kinase and Akt activation, to the stabilization of β-catenin and the transcriptional activity of TCF/LEF transcription factors. Extracellular signal-regulated kinase (ERK) is also regulated by the FAK-Src complex, and is responsible for mechanical signal transduction from the microenvironment, to regulate intracellular processes[10,14,42].

Responses to cell-matrix interactions are governed by a complex interplay between signaling pathways involving FAK, ERK, β-catenin, Rho and others, and cells are therefore able to respond to extracellular forces in myriad ways. For example, in the mammary gland, where development and homeostasis occur within the context of a pliable ECM, Rho activity in fibroblasts was increased upon heightened matrix rigidity. This led to an increase in focal adhesion assembly and growth factor-dependent ERK activation, suggesting that a mechano-regulatory "circuit" amalgamates stimulatory extracellular tension with focal adhesion formation through ERK and Rho-dependent cytoskeletal changes, to maintain homeostasis[13].

Mechano-reciprocity

Mechano-reciprocity (**Figure 1**)is the ability of cells to enhance or moderate intra-cellular tension in order to adapt to increased or reduced extra-cellular stiffness respectively[43] (termed outside-in signaling), but conversely may also refer to their ability to remodel the extra-cellular matrix and modify its mechanical properties in order to offset changes in intra-cellular tension (inside-out signaling). In a situation where sustained growth factor stimulation or oncogenic mutation causes increased mechanotransduction signaling such as via the Rho, YAP, β-catenin and/or MAP

kinase pathways, the combination of inside-out and outside-in signaling has the potential to establish a vicious cycle by which ever-escalating ECM stiffness causes uncontrolled cell proliferation. Indeed, this is observed in mechano-responsive cancers such as those of the skin, breast and intestine[14,44] and is characterized by persistent activation of the ROCK protein. We have shown that enhanced mechano-reciprocity of this kind promotes tumor progression[14]. Targeting runaway mechano-reciprocity is therefore a novel way in which diseases of cellular homeostasis characterized by increased ECM stiffness such as cancer or fibrosis may be treated.

Mechano-reciprocity in diseases of cellular homeostasis

It is becoming increasingly evident that persistent mechanical signaling can advance cancer progression. Overexpression of Rho GTPases has been associated with progression of disease in a number of malignancies, and Rho, Rac, Cdc42 and ROCK[45] have all been reported to be mutated in various cancers to confer tumor promoting functions and inhibit tumor suppressive functions (reviewed in [46]).

The role of mechanical signaling in driving tumorigenesis has been well characterized in the mammary gland. A known mechano-responsive tissue[44], the mammary epithelium is subjected to a multitude of external forces throughout development and cycling, such as ductal morphogenesis, lactation, and involution to remodel the gland and degrade the ECM[47]. A pliable ECM is best suited to these morphological changes. Compression analysis of normal and tumor tissue of murine mammary origin demonstrated that although normal mammary tissue was soft, tumor and peri-tumoral tissue was significantly stiffer[10]. Matrix stiffness is promoted by a greater amount of collagen cross-linking[48]. This ECM stiffening is sensed by cellular integrins, which in turn activate RhoA signaling, resulting in generation of

focal adhesions and influencing tumor cell invasion, cell proliferation, and changes in cytoskeletal organization. Thus, a persistent and high level of mechano-reciprocity is potentially able to create a feed forward mechanism, promoting further cancer development. This phenotype is often seen in patients with a high mammographic density and it is becoming clearer that mammographic density in the normal breast is an important prognostic marker for breast cancer, with a higher density indicating a four-to-six fold higher likelihood of developing breast cancer[49].

We have found that skin in which ROCK had been hyper-activated exhibited increased MLC2 phosphorylation and elevated ECM production resulting in greater tissue stiffness. Increased tissue stiffness gave rise to mechano-reciprocal activation of the PI 3-kinase signaling pathway which led to the stabilization of β-catenin and epidermal hyper-proliferation. Interestingly, in the context of the multi-step chemical carcinogenesis model, mechano-reciprocity cooperated with oncogenic transformation to promote tumor progression in a manner that was dependent on ROCK-induced tissue stiffness. Conversely, inhibition of ROCK signaling by treatment with the inhibitor Y-27632 lowered ECM stiffness and impeded tumor formation and growth[14,50]. These observations clearly show that tumor cells have a key role in establishing a stiff ECM that promotes their own proliferation and growth.

A number of parallels exist between normal and malignant tissue development and wound healing. Rapid cell proliferation and mechanisms mediating cytoskeletal changes during cell migration are similar in acute wound healing to those involved in tumor progression. At all stages of wound healing, including hemostasis, inflammation, proliferation and remodeling, examples of mechano-reciprocity exist between the ECM and a number of cell types including platelets, immune cells, fibroblasts, endothelial cells and keratinocytes[51]. We have recently reported that

enhanced signaling through ROCK, occurring during either cutaneous squamous cell carcinoma progression or acute skin wound healing and which causes increased ECM production in both contexts, is negatively regulated by the phospho-serine binding molecular adaptor protein 14-3-3ζ[39]. 14-3-3ζ bound to the ROCK antagonist MYPT1, promoting its MLC phosphatase function and antagonizing ROCK-mediated phosphorylation of MLC. Mice lacking 14-3-3ζ exhibited increased signal flux through ROCK leading to enhanced mechano-reciprocity, causing rapid wound healing. This observation was corroborated using a novel pharmacological inhibitor of 14-3-3ζ, which accelerated acute wound healing in wild-type mice[39].

Interestingly, this negative regulatory mechanism that moderated signaling through ROCK and thereby limited mechano-reciprocity, was lost in the majority of patient samples of cutaneous squamous cell carcinoma, which exhibited little or no 14-3-3ζ compared to normal skin. Accordingly, 14-3-3ζ-deficient mice on the multi-step chemical carcinogenesis protocol formed larger tumors than wild-type mice of the same strain background. Taken together, these observations lead us to the conclusion that 14-3-3ζ-mediated promotion of MLC dephosphorylation is a mechanism by which mechano-reciprocity is maintained within manageable limits such that normal wound healing is facilitated while tumor formation is prevented **(Figure 2)**.

We do not believe that 14-3-3ζ is the only negative regulator of mechano-reciprocity. Under steady-state conditions, 14-3-3ζ-deficient mice exhibit thinner skin than wild-type mice[39], suggesting that compensatory mechanisms as yet undiscovered and not involving the seven other 14-3-3 family members are capable of maintaining normal cellular homeostasis at near-physiological rates.

Targeting mechano-reciprocity and the extra-cellular matrix as therapy

Given the highly tumor-promoting environment caused by increased mechano-reciprocity, targeting the players that facilitate it (ROCK signaling, fibroblasts and their activation) and enhancing the activities of its negative regulators (14-3-3ζ) are potentially novel approaches to cancer therapy. However, an important consideration is the effect that inhibition of pleiotropic proteins like ROCK or 14-3-3ζ may have on cellular homeostasis and indeed normal development. For instance, tumor capillary endothelial cells subjected to stress exhibited cytoskeletal rearrangements, and exerted stronger Rho-ROCK-mediated traction compared to non-cancer cells. Pre-treatment with the ROCK inhibitor Y-27632 before the application of stress restored normal actin behavior in tumor cells, but caused significant cytoskeletal disruption in normal cells[52]. This indicates that normal and tumor cells differ in sensitivity to external mechanical force as governed by ROCK signaling and illustrates the challenges accompanying attempts to target tumors via the mechanotransduction machinery.

The most common current therapy regimes involve targeting tumor cells directly via inhibition of cell-intrinsic functions such as their proliferative capacity or their ability to evade apoptosis, whereas the therapies that offer the most promise, such as immunotherapy or hormone therapy, target non-intrinsic functions that require cell to cell communication. Their need for cell to cell communication could be viewed as a vulnerability of cancers. The cell to ECM communication mechanisms that enhance mechano-reciprocity are mediated by communication between cancer cells and normal (fibroblast) cells lacking oncogenic mutations, a particularly serious vulnerability. Normalizing the ECM by normalizing tumor-associated fibroblasts therefore holds out the tantalizing possibility of therapies that halt or even reverse

tumor progression. This approach may also provide new opportunities for combination therapies where the tumor and its ECM are both targeted concurrently.

Concluding Remarks

In conclusion, analyzing Rho-ROCK pathway activation in the in vivo context has revealed its function in augmenting mechano-reciprocity via enhanced ECM stiffness. These observations have also highlighted the importance of mechano-reciprocity in normal tissue homeostasis and demonstrated that negative regulators of this process have significant therapeutic utility.

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Figure Legends

Figure 1

Mechanosensing through engagement of integrins with the ECM causes mechanotransduction, by activation of Talin and autophosphorylation of FAK, which initiates several intra-cellular signal transduction pathways, including PI 3-kinase/Akt and ROCK signaling. Mechanotransduction gives rise to increased actin polymerization and actomyosin contractility, establishing mechano-reciprocity, which in turn leads to paracrine signaling between the parenchyma and the stroma, increasing ECM production and remodeling.

Figure 2

ROCK activation enhances intracellular tension in epidermal cells by activating MLC and LIM kinases. It also increases extracellular tension by elevating ECM production by dermal fibroblasts. ROCK activation in dermal fibroblasts promotes ECM remodeling by regulating focal adhesion dynamics and fibroblast migration. In both contexts, 14-3-3ζ limits signal flux through ROCK, thereby maintaining mechano-reciprocity between normal physiological boundaries, permitting normal wound healing and protecting against tumor formation. This figure has been adapted from [50].

Figure 1

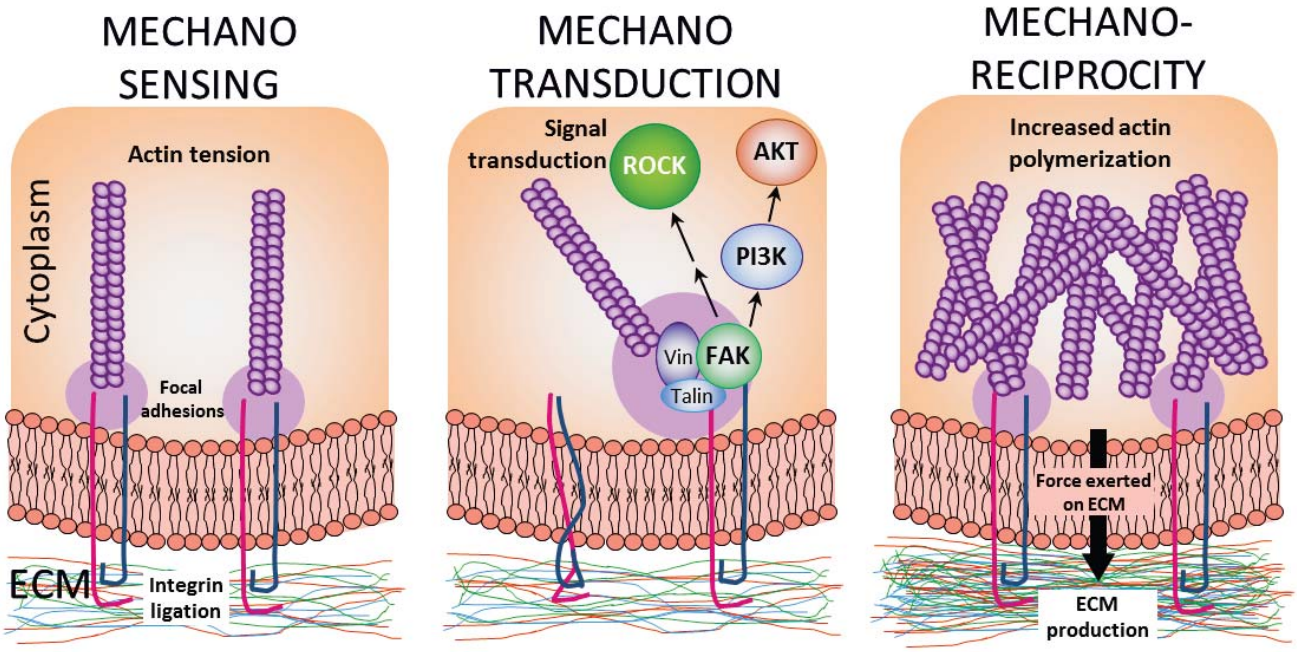
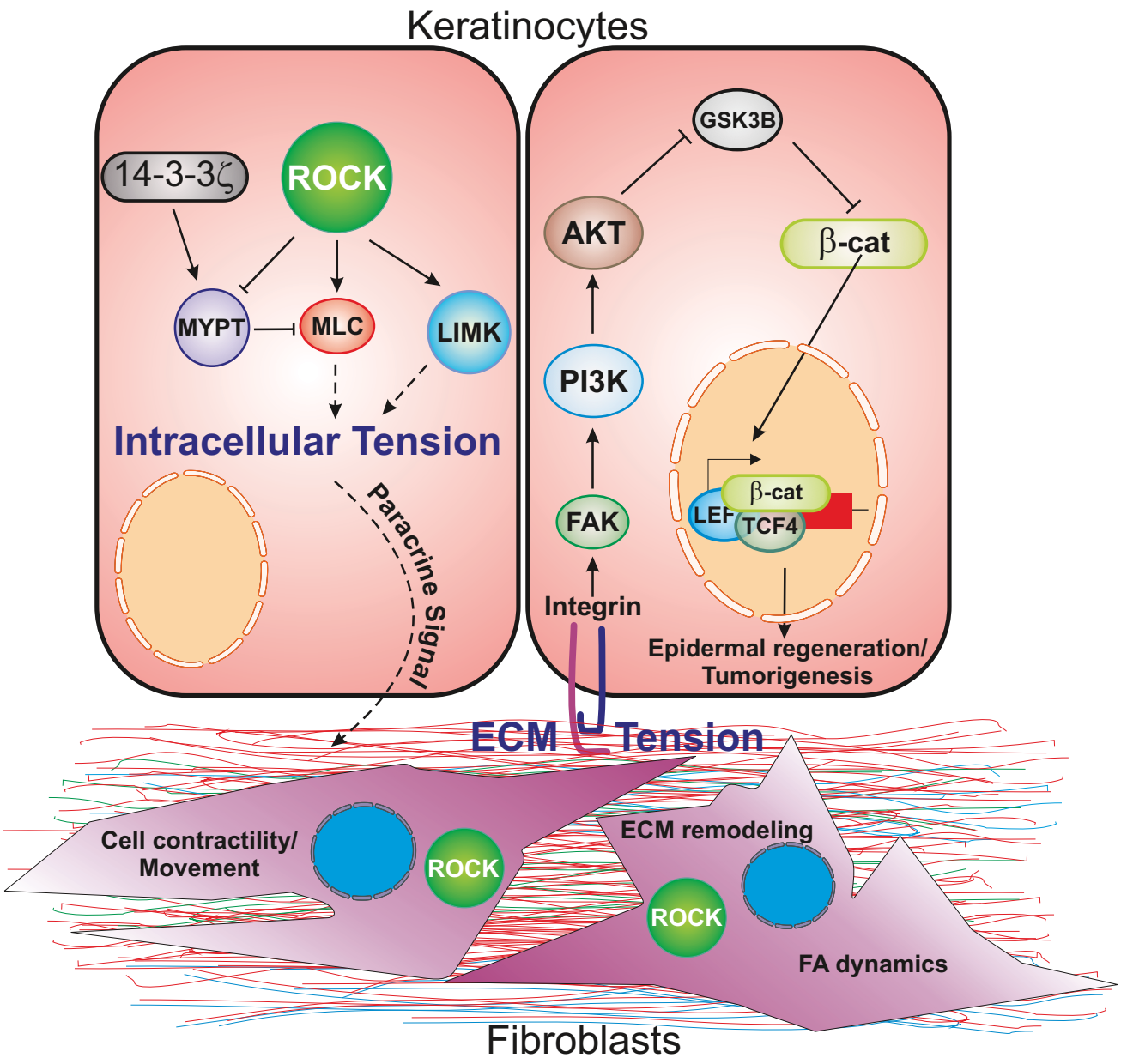

MECHANO SENSING
Actin tension
Cytoplasm
Focal adhesions
ECM
Integrin ligation
MECHANO TRANSDUCTION
Signal transduction
ROCK
AKT
PI3K
Vin
FAK
Talin
MECHANO-RECIPROCITY
Increased actin polymerization
Force exerted on ECM
ECM production

Figure 2


Keratinocytes
14-3-3ζ
ROCK
MYPT
MLC
LIMK
Intracellular Tension
Paracrine Signal
GSK3B
AKT
β-cat
PI3K
FAK
Integrin
β-cat
LEF
TCF4
Epidermal regeneration/
Tumorigenesis
ECM Tension
Cell contractility/
Movement
ROCK
ECM remodeling
ROCK
FA dynamics
Fibroblasts