Mechanotransduction in tumor progression: The dark side of the force

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Cancer has been characterized as a genetic disease, associated with mutations that cause pathological alterations of the cell cycle, adhesion, or invasive motility. Recently, the importance of the anomalous mechanical properties of tumor tissues, which activate tumorigenic biochemical pathways, has become apparent. This mechanical induction in tumors appears to consist of the destabilization of adult tissue homeostasis as a result of the reactivation of embryonic developmental mechanosensitive pathways in response to pathological mechanical strains. These strains occur in many forms, for example, hypervascularization in late tumors leads to high static hydrodynamic pressure that can promote malignant progression through hypoxia or anomalous interstitial liquid and blood flow. The high stiffness of tumors directly induces the mechanical activation of biochemical pathways enhancing the cell cycle, epithelial-mesenchymal transition, and cell motility. Furthermore, increases in solid-stress pressure associated with cell hyperproliferation activate tumorigenic pathways in the healthy epithelial cells compressed by the neighboring tumor. The underlying molecular mechanisms of the translation of a mechanical signal into a tumor inducing biochemical signal are based on mechanically induced protein conformational changes that activate classical tumorigenic signaling pathways. Understanding these mechanisms will be important for the development of innovative treatments to target such mechanical anomalies in cancer.

Introduction
The establishment of body shape in adult animals results from biochemical and biomechanical developmental patterning processes that regulate tissue differentiation and morphogenesis. Recently, it has become apparent that there is a reciprocal interplay between biochemical and biomechanical patterning throughout development. Although forces due to cell mitosis and morphogenetic activities are genetically regulated, developmental gene expression and protein activation are in turn mechanically regulated by the mechanical strains associated with cell and tissue morphological changes (Brouzés and Farge, 2004; Wozniak and Chen, 2009; Mammoto and Ingber, 2010; Chan et al., 2017). Indeed, studies ranging from cultured stem cells to in vivo investigations on early embryonic cells at gastrulation have revealed a role for forces, cell size, and substrate stiffness on cell fate and differentiation (Farge, 2003; McBeath et al., 2004; Engler et al., 2006). These studies demonstrated the existence of mechanotransductive feedback loops for the regulation of developmental morphogenesis and differentiation processes by the physical biomechanical phenotype (Desprat et al., 2008; Hamant et al., 2008; Fernandez-Gonzalez et al., 2009; Kahn et al., 2009; Pouille et al., 2009; Zhang et al., 2011; Brunet et al., 2013; Herszberg et al., 2013; Hiramatsu et al., 2013; Monier et al., 2015; Maître et al., 2016; Mitrossilis et al., 2017). This biochemical/biomechanical interplay regulates the integrative reciprocal trans-scale direct mechanical interaction between the macroscopic biomechanical structure of living tissues and the biochemical activities of its molecular components. By doing so, this interplay is proposed to robustly coordinate collective cell behaviors in tissues, as well as organism biochemical patterning with biomechanical patterning during development (Brunet et al., 2013; Chan et al., 2017; Mitrossilis et al., 2017). See the text box for the molecular mechanisms underlying mechanotransduction.

Because this developmental mechanobiochemical interplay physiologically regulates major traits of the shape of adult organisms, the homeostatic stability of the adult shape should logically also be regulated and maintained by a mechanobiochemical interplay. Correlatively, mutations that change the mechanical properties of adult tissues can cause an active biochemical response resulting in a pathological deregulation of the mechanobiochemical homeostatic equilibrium. Together with mutations that directly induce biochemical perturbations, this disturbs the biochemical and active biomechanical patterning of the tissue. From this perspective, the mechanosensitive pathways...
Mechanotransduction consists in the translation of mechanical cues, characteristic of cells and tissues, into specific intracellular biochemical cues. It is based on mechanically induced changes in protein conformation, or inhibition of signaling protein endocytosis, leading to junctional or cytoskeleton rearrangements, cell division modulation, or cell differentiation (Chen et al., 1997, Rauch et al., 2002, Engler et al., 2006, Sawada et al., 2006, Grashoff et al., 2010; Sinha et al., 2011). The characteristic energy of both a given protein conformation and the formation of an endocytic vesicle are on the order of a few 10 kT (i.e., 10 times the molecular Brownian energy kT; Jin and Nossal, 2000; Bruij et al., 2007). Thus, they are structured but can easily be deformed by soft physiological mechanical strains of biochemical energies of the same several-10-kT-magnitude order. This, for instance, can lead to the opening of phosphorylation sites to kinases. This is the case for p130Cas/BCAR1, in which tyrosines were found to be mechanically opened to phosphorylation by Src, thereby leading to the downstream activation of p38/MAPK, a tumorigenic signaling pathway (Sawada et al., 2006). Mechanical strains can also directly enhance protein binding affinities, such as interleukin-7-fibronectin interaction potentially trapping interleukin-7 in the ECM in a stress-dependent way (Ortiz Franyutti et al., 2018). Mechanically forced membrane flattening can induce inhibition of protein endocytosis and degradation, thereby enhancing or triggering the activation of downstream signaling pathways. This is the case for BMP2-dependent myoblast-osteoblast trans-differentiation, which can be enhanced, as well as triggered at an undercritical concentration of BMP2, by mechanical inhibition of BMP2 endocytosis (Rauch et al., 2002). The flattening of reservoirs of membranes stored in caveolae structures was also found in the process of the cell response to mechanical shocks preventing membrane rupture (Sinha et al., 2011). Ionic pores can also mechanically open in response to membrane tension in the processes of neuronal sensation (Rudnev et al., 1981; Chalif, 2009).

involved in embryonic development interestingly also comprise master pathways and transcription factors of tumorigenesis. This is the case for the cotranscription factor β-catenin (β-cat) of the Wnt pathway that vitally mediates the mechanical induction of the Drosophila melanogaster anterior midgut embryonic early differentiation and subsequent late functional differentiation in the larvae. This is the case also for Yap/Taz that maintains the global constitutive tissue tension ensuring the overall good shape of zebrafish early embryos, as well as for downstream genes (Desprat et al., 2008; Porazinski et al., 2015), which respectively enhance cell proliferation and invasiveness in tumor progression (Rosivatz et al., 2002; Barker et al., 2009; Zanconato et al., 2016). As pathological tumor progression leads to biomechanical anomalies in cells and tissues, the question can be asked whether the resulting anomalous mechanical strains can physiologically re-activate the physiological mechanosensitive developmental processes in healthy adult tissues, thereby participating in the breaking of adult tissue homeostatic equilibrium.

Indeed, mechanotransduction processes were initially discovered in examining how anomalous mechanical strains characteristic of pathological contexts can activate biochemical pathways and their role in the pathology development. Studies using aortic endothelial cells and explants demonstrated how endothelial cells react to hydrodynamic flow to maintain the homeostatic structure of the blood circulation network in resistance to the flow and how perturbations cause angiogenesis (Nakache and Gaub, 1988; Davies, 1995; Nakajima et al., 2017), whereas studies in human breast cancer epithelial cell lines examined how tumor cells react to the increase in stromal collagen density and stiffness due to fibrosis of advanced tumor progression states (Weaver et al., 1996; Bissell et al., 2005; Makale, 2007)

The molecular mechanisms of mechanotransduction

Here we will review evidence of the tumorigenic homeostasis-distabilizing role of the anomalous microenvironmental mechanical strains generated by tumor development, from anomalous hydrodynamic blood shear stress associated with tumor vascularization, the interstitial pressure of tumors and associated hydrodynamic flow, to the increase in fibrotic stiffness characteristic of advanced tumor stages and mitotic tumor growth solid-stress pressure present from the earliest tumor stages. In addition to describing mechanosensitive biochemical pathways involving junctions, the cytoskeleton, and membranes, we will focus on innovative, emergent evidence of the potential involvement of biomechanical structural nuclear factors and microRNAs as regulators of the tumorigenic response of cells to microenvironmental mechanical strain anomalies. We will also discuss the development of innovative in vitro and in vivo bio-physical tools to test for the tumorigenic involvement of anomalous mechanical properties of the tumor microenvironment within most physiologically relevant contexts.

Finally, similar to epidemiological studies in cancer that indicate the requirement of both genetic predisposition and environmental context in cancer occurrence (Jaffee et al., 2017), it is becoming apparent that both genetic and microenvironmental mechanical anomalies reveal to be synergistically required in the mechanotransductive induction or amplification of tumorigenesis.

The tumorigenic role of interstitial pressure and angiogenic blood flow shear stress in advanced tumor developmental stages

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demonstrated in a 3D in vitro invasion assay. This enhanced interstitial flow mechanotransducingly activates TGFβ1 and downstream collagenase-dependent fibroblast migration, thereby enhancing human melanoma cell tumor invasiveness in vitro (Shieh et al., 2011). Interestingly, interstitial flow was found to activate the N-methyl-d-aspartate receptor, stimulating malignancy in a mouse model of pancreatic neuroendocrine tumorigenesis in vivo (Li and Hanahan, 2013). In an in vitro 3D microfluidic assay, interstitial flow was able to remove the morphogenetic gradients of VEGF regulators of angiogenesis, thereby strongly stimulating angiogenesis processes in the opposite direction of the flow, namely in the direction of the growing tumor favoring the tumor vascularization process (Shirure et al., 2017).

The strong vascularization of advanced tumors is characterized by tortuous and disrupted vessel structures, leading to anomalously low-velocity blood flow (Goel et al., 2011). Although blood shear stress was proposed to induce the formation of stress fibers increasing the endothelial resistance to hydrodynamic flow, endothelial remodeling favoring angiogenesis was also suggested to be mechanically induced by anomalous hydrodynamic blood flow, likely involving the mechanical induction of the nuclear translocation of the NF-KB transcription factor leading to inhibition of tumor-expressed angiogenic genes such as MMP9 or ANGs at high levels (higher than 10 dyn/cm²). Although at low levels (lower than the 4-dyn/cm² physiological levels), it was not inhibitory but rather promoted angiogenic genes, thereby enhancing angiogenic vascularization in solid tumors that are characterized by low blood velocities (Tzima et al., 2005; Conway et al., 2013; Buchanan et al., 2014; Galie et al., 2014). The underlying molecular mechanism of the fluid flow–induced activation of angiogenesis involves PECAM-1, which mechanotransductively activates Src family kinases that transactivate VEGF receptors (VEGFRs) and phosphorylate Y658-VE-Cadherins at cell–cell junctions. Both activated VEGFRs and VE-Cadherins trigger multiple downstream pathways regulating endothelial cell alignment along the direction of the flow and flow dependent vascular remodeling, respectively (Conway et al., 2017).

The high fibrotic stiffness in tumors is also proposed to favor angiogenesis. Mice mammary tumor endothelial cells cultured on rigid substrates or submitted to stiffness increases in vivo show fibronectin overexpression as well as PKCβII and VEGF receptors (VEGFRs) and phosphorylate Y658-VE-Cadherins at cell–cell junctions. Both activated VEGFRs and VE-Cadherins trigger multiple downstream pathways regulating endothelial cell alignment along the direction of the flow and flow dependent vascular remodeling, respectively (Conway et al., 2017).

The high fibrotic stiffness in tumors is also proposed to favor angiogenesis. Mice mammary tumor endothelial cells cultured on rigid substrates or submitted to stiffness increases in vivo show fibronectin overexpression as well as PKCβII and VEGF 165β anti-angiogenic repression, possibly involving the phosphoinositol 3-kinase (PI3K)/Akt mechanosensitive pathway (Paszek et al., 2005; Ghajar and Bissell, 2008). In both cases, these investigations first focused on the mechanotransductive biochemical regulation of the downstream physical parameters of cells and tissues, namely their stiffness, shape, motility, and number (mitotic growth). Regarding tumor cells, the fibrosis-dependent stiffness increase was generally found to enhance cell proliferation, EMT, and motility involving the focal adhesion kinase FAK and PI3K/integrin signaling (Paszek et al., 2005; Mitra and Schlaepfer, 2006; Friedland et al., 2009; Levental et al., 2009; Samuel et al., 2011; Leight et al., 2012; Yuan et al., 2015). However, stiffness sensitivity of cancer cells appears to be context-dependent. For instance, NIH3T3 cells transformed with the H-ras oncogene are poorly sensitive to stiffness, whereas neuroblastoma cells proliferate better on softer substrates (Wang et al., 2000; Wong et al., 2015), and colon and prostate cancerous cells undergo EMT at low stiffness (Tang et al., 2014). Furthermore, human tongue squamous carcinoma cells are...
characterized by a softer stiffness than the healthy tissue, confirming that high stiffness is not always systematically associated with tumorigenic phenotypes (Runge et al., 2014).

The last decade has been prolific in discoveries leading to a deeper understanding of the biochemical pathways underlying the interplay between the stiffness of tumor microenvironment substrates and isolated cancer cells from a mechanobiological point of view (Kaushik et al., 2016). Indeed, cells were shown to adapt their cytoskeleton actomyosin-II contractibility to substrates and isolated cancer cells from a mechanobiological point of view (Kaushik et al., 2016). Indeed, cells were shown to adapt their cytoskeleton actomyosin-II contractibility to

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Relative to cancer, an increase in cell stiffness was found to enhance integrin/FAK signaling pathways leading to tumor hyperproliferation and increased motility (Paszek and Weaver, 2004; Provenzano et al., 2009; Kopanska et al., 2016). Here, high matrix stiffness promotes the activation of the FAK-Src complexes and consequently generates a Rho/ROCK-dependent myosin-mediated cellular contractile force, resulting in an invasive phenotype. The underlying associated transcriptional response is regulated by ERK phosphorylation that results in hyperactivation of the Ras-MAPK pathway, promoting a hyperproliferative state of mammary epithelial cells (Wozniak et al., 2003; Paszek et al., 2005; Provenzano et al., 2009). Changes in stiffness are also characterized by higher collective effects on multicellular clusters compared with individual cells through the long-range rigidification cohesive effects due to the activation of actomyosin motors (Sunyer et al., 2016). Such collective cell migration was additionally observed to be guided by fibronectin in head and neck squamous cell carcinomas invading the surrounding stroma (Gopal et al., 2017), as well as favored by low matrix degradability independently of stiffness (Trappmann et al., 2017).

In cell culture conditions the diversity involved in the underlying molecular mechanisms of cellular mechanosensing were found to be initiated by mechanically induced bond dissociation or changes in conformation of scaffold proteins. This is the case for integrin-ECM bond disruption involved in cell proliferation and survival through the induction of expression of CyclinD1 (Fournier et al., 2008; Friedland et al., 2009). This is also the case for p130Cas, for which conformational changes open its sites of phosphorylation by Src, thereby activating its functions involved in migration, transformation, and invasion (DeFilippi et al., 2006; Sawada et al., 2006), as well as for Talin conformational changes involved in cell adhesion and invasion (Del Rio et al., 2009; Margadant et al., 2011). In addition, in CV1 monkey kidney cell culture, a 12pN physiological force causes stretching of the Talin rod domain, exposing its Vinculin binding site thereby increasing Vinculin binding (Del Rio et al., 2009). Myosin contraction-dependent actin filament movement causes stochastically repeated stretching and relaxation cycles of Talin (50–350 nm for 6–16 s). In turn the head domain of the adhesion site protein Vinculin binds to stretched Talin and releases in multiple cycles, thereby transducing the mechanical signal into a biochemical signal (Margadant et al., 2011). Primary human dermal fibroblasts derived from foreskins also showed that traction forces unfold fibronectin modules, which strengthen their modular assembly with quaternary structure (Smith et al., 2007). Another step forward in understanding the molecular process by which cells sense changes in the physical properties of their microenvironment was established with the observation of a direct connection between matrix rigidity and force transmission and transduction into the cytoplasm, through the so-called actin-Talin-integrin-fibronectin clutch (Elosegui-Artola et al., 2016). Using mouse embryonic fibroblasts, a model was proposed in which a stiffness threshold of 5 kPa controls initiation of the mechanotransduction-triggering force for Talin unfolding. Above this threshold, Talin unfolds and binds to Vinculin, leading to growth of adhesions and to the translocation of the mitotic YAP factor to the nucleus through an unknown process (Fig. 1). Interestingly, the increase of rigidity associated with the actomyosin contractibility of tumorous individual cells was also shown to trigger their extrusion from epithelial layers. This was proposed to favor rigid tumor cell invasiveness in cancer in a human epithelial colorectal adenocarcinoma cell line (Wu et al., 2015). Here the process of apical cell extrusion was dependent on N-WASP, a modulator of junctional tension via stabilization of F-actin and Myo-II at cell lateral contacts.

In mouse and human breast cancer cells, high matrix stiffness causes an activation of mitotic YAP/TAZ signaling but also of integrins that, in turn, cause the phosphorylation of Twist, a protein involved in invasiveness, at Tyr 107 (Zhao et al., 2007, 2012; Dupont et al., 2011; Wei et al., 2015). Phosphorylated Twist is then released from its cytoplasmatic anchoring protein G3BP2 and translocated into the nucleus where it can activate the transcriptional events of EMT, Invasion, and metastasis in nontransformed and tumorous mouse cells and tumorous human cells, a new mechanism to be investigated in other cell types (Fig. 1).

Carcinoma-associated fibroblasts (CAFs) also play a critical role in stiffness-induced cancer progression. Activated by tumorigenic hypoxia, CAFs generate collagen reticulation that promotes cancer cell invasiveness, in addition to increased contractility and motility (Toulec et al., 2010; Pankova et al., 2016). Further enhanced by ECM stiffening due to TGF-β secretion by normal fibroblasts, CAF actomyosin contractility mechanotransductively leads to YAP nuclear translocation, which maintains the CAF activities of ECM remodeling and stiffening, thereby generating a positive tumorigenic feedback loop (Calvo et al., 2013; Liu et al., 2015). CAFs can in addition favor tumor cell invasion by directly pulling and stretching the basement membranes, thereby opening gaps in the membrane (Glentis et al., 2017). CAFs can also create cell-adhesion-mediated resistance to radiotherapeutic treatment in human breast cancer xenographs by deposition of ECM through β1-integrin binding, which was also suggested to be the case in BRAF mutated melanoma (Park et al., 2006; Picco et al., 2017).

In addition, positive feedback signaling between the glycoprotein ICAM-1, a member of the immunoglobulin superfamily, which promotes inflammation-dependent ECM contraction, and actomyosin contractility, which regulates expression of the membrane-bound ICAM-1, has been described in CAFs.
ICAM-1 is thus a crucial auto-enhancer of stromal pro-invasive matrix remodeling by CAFs, upstream of the Scr/RhoA/ROCK/MLC2-signaling pathway (Bonan et al., 2017). Furthermore, in both epithelial and stromal cells, caveolae depolymerization at the plasma membrane in response to an increase in membrane tension due to stiffness-induced actomyosin constriction is speculated to act as a mechanical trigger to mechanotransductively modulate tumor progression processes, for instance, by releasing caveolae associated signaling proteins into the cytosol under strain (Lamaze and Torrino, 2015).

In vivo experimental proof of stiffness having a direct inductive role in tumorigenesis consists of the enhancement of mouse mammary and skin carcinoma tumorigenesis and invasiveness, seen in response to a stromal stiffness increase by the amine oxidase cross-linking enzyme LOX and by a Rock-induced increase in the activity of actomyosin contractibility (Levental et al., 2009; Samuel et al., 2011). This observation is in line with the cell culture observations of EMT triggered by the stiffness increase of the substrate, combined with previously described cell culture findings based on actomyosin-mediated mechanotransductive responses of cells to the substrate stiffness (Weaver et al., 1996). Inhibition of endogenous LOX activity with BAPN treatment and inhibition of endogenous actomyosin contractibility by the ROCK inhibitor LIMKi and the Myo-II inhibitor Y27632, respectively, inhibited tumor progression by limiting the stiffness-induced response, thereby offering potential innovative therapeutic treatments (Bordeleau et al., 2017). Indeed, BAPN preventive treatment of a rat model of prostate tumorigenesis reduced tumor growth, and an enhancing effect was observed in a tentative of curative treatment (Nilsson et al., 2016). LIMKi...
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Mechanotransduction of gene expression, such as myc and twist, in the surrounding healthy tissue was first experimentally addressed ex vivo, on postoperative mouse colon tissues constrained by simple uniaxial deformation applied to the opened colonic tissue in culture medium at 37°C with the 1-kPa tumor growth pressure and in cell culture on kidney epithelial quiescent cells in response to silicon substrate deformation (Whitehead et al., 2008; Benham-Pyle et al., 2015). In the explants, there was mechanical induction of Y654-β-catenin phosphorylation and nuclear translocation of β-catenin (Whitehead et al., 2008). Interestingly, the β-catenin mechanosensitivity was also found to play an activating and inhibiting role in osteogenesis and adipogenesis in cell culture, respectively (Song et al., 2017). This mechanotransductive tumorigenic process was tested in vivo by the stabilization of ultramagnetic liposomes inside the conjunctive mesenchymal cells of the colon through which a 1-kPa magnetically induced pressure was applied (see Tools for testing the impact of mechanotransduction on tumor cell behavior). As a response, the healthy tissue activated the β-catenin pathway from the phosphorylation of Y654 to its nuclear translocation with the resulting activation of the expression of its myc, axin, and zeb-1 tumorigenic target genes, involved in both hyperproliferation and invasiveness (Fig. 2a). This led to hyperproliferation and to the formation of anomalous crypts. In the presence of one mutation on one copy of adenomatous polyposis coli (APC) only, an essential element of the cytoplasmic β-catenin degradation complex, for which mutation is involved in 90% of human colon cancer, the 1-kPa mechanical strain led to tumor formation (Fernández-Sánchez et al., 2015). The same result was confirmed in a genetic model of colon tumor growth in vivo, in which the 1-kPa strain was this time induced by mosaic domains of hyperproliferative crypts overexpressing Notch and applied to nongenetically transformed healthy neighboring crypts.

In vivo the mouse epidermis, the increase of stiffness due to actomyosin stimulation by transgenic overactivation of ROCK was also induced by the activation of the tumorigenic β-catenin pathway (Samuel et al., 2011; Fig. 2b). Here, the β-catenin pathway was activated but in a different way, depending on the pressure or stiffness-induced stimulation. Indeed, ROCK activation led to the integrin-dependent inhibition of GSK3β inactivation through Ser9, thereby enhancing the concentration of cytoplasmic and nuclear β-catenin. The mechanically induced phosphorylation of GSK3 Ser9 was also found to synergistically add to Y654-β-catenin phosphorylation, this time through a Ret kinase-dependent process, in response to tumor growth pressure (Fernández-Sánchez et al., 2015).

Therefore, the role of hyperproliferative solid-stress pressure adds to hydrodynamical and stiffness mechanotransductive tumorigenic effects characteristic of fibrotic advanced stages from the earliest stages of tumor development.

MicroRNA and chromatin: emerging actors in the interplay between cells and ECM stiffness

In addition to junctional, cytoskeleton, or membrane protein mechanical activation that have been found to mechanotransductively trigger tumorigenic biochemical pathways, accumulating evidence reports a striking effect of microRNA expression on the modulation of tumor progression and malignancy, through the mechanosensing of ECM stiffness. MicroRNAs are posttranscriptional regulators of gene expression and have been described as modulators of oncogenes and tumor suppressors (Zhang et al., 2007). In mouse and human breast tissues, high matrix stiffness has been reported to induce miR-18a and to promote PI3K-dependent malignant progression through integrin-dependent activation of β-catenin and Myc by targeting the tumor suppressor phosphatase and Tensin homologue (Mouw et al., 2014). In mouse mammary glands and human mammary gland cell lines, the increase in ECM density and stiffness has also been found to down-regulate miR-203 expression, thereby up-regulating the tumor suppressor Robo1 by targeting binding sites in its 3’ UTR, subsequently activating Rac and FAK to maintain cell shape and generate an antimetastasis barrier (Le et al., 2016). Note that, independently of tumorigenesis, miR-33-5p has been demonstrated to be expressed by fluid shear stress and to positively promote osteoblast differentiation by inhibiting its target HmgA2. Correspondingly, miR-33-5p expression decrease, leading to osteoblast differentiation decrease, was observed after treatment blocked xenograph pancreatic mouse tumor growth in vivo (Rak et al., 2014), and Y-27632 induced p53 expression and down-regulated VEGF expression, significantly inhibited cell proliferation, and induced cell apoptosis in hemangiomas endothelial cell–injected nude mice in vivo (Qiu et al., 2017).

Therefore, in addition to hydrodynamic effects, the increase of tissue stiffness characteristic of developed fibrotic tumors also acts as an enhancer of tumorigenesis though junctional mechanotransductive signals by Myo-II-dependent cell adhesive contractibility stiffness-induced effects on apoptosis, division, motility, and extrusion not only in epithelial cells but also in stromal CAFs.

From early to late tumor developmental stages: the tumorigenic role of solid-stress tumor growth pressure

Besides the increased stiffness characteristic of advanced fibrotic tumor development, hyperproliferation generates a tumor growth pressure that presents an additional mechanical factor from the onset of tumorigenesis to latest stages on both tumor cells and healthy surrounding epithelial tissues.

The role of the pressure produced by the surrounding tissue on the growing tumor itself, in resistance to the hyperproliferative growing tumor, was first noticed in cell culture. Indeed, 2D sheets of adherent cells were suggested to stop dividing at confluence, possibly because of mechanical feedback signaling from pressure-induced cell–cell contact or shape changes (Martz and Steinberg, 1972; Ukena et al., 1976). Such negative regulation of the mitotic activity of tumor cells was theoretically modeled (Shraiman, 2005; Basan et al., 2009) and found in 3D cell culture organoids through the mechanical induction of expression of the proliferation inhibitor p27Kip1 (Delarue et al., 2014).

In addition to controlling cell proliferation, pressure-induced mechanical cues were demonstrated to induce transformation of healthy cells into cancer cells through the mechanical activation of the β-catenin pathway, activated nuclear β-catenin being a diagnostic signature of tumors in many types of cancers. The role of the mechanical pressure developed by hyperproliferative tumor tissues in activating the tumorigenic β-catenin pathway and its tumorigenic target genes expression, such as myc and twist, in the surrounding healthy tissue was first experimentally addressed ex vivo, on postoperative mouse colon tissues constrained by simple uniaxial deformation applied to the opened colonic tissue in culture medium at 37°C with the 1-kPa tumor growth pressure and in cell culture on kidney epithelial quiescent cells in response to silicon substrate deformation (Whitehead et al., 2008; Benham-Pyle et al., 2015). In the explants, there was mechanical induction of Y654-β-catenin phosphorylation and nuclear translocation of β-catenin (Whitehead et al., 2008). Interestingly, the β-catenin mechanosensitivity was also found to play an activating and inhibiting role in osteogenesis and adipogenesis in cell culture, respectively (Song et al., 2017). This mechanotransductive tumorigenic process was tested in vivo by the stabilization of ultramagnetic liposomes inside the conjunctive mesenchymal cells of the colon through which a 1-kPa magnetically induced pressure was applied (see Tools for testing the impact of mechanotransduction on tumor cell behavior). As a response, the healthy tissue activated the β-catenin pathway from the phosphorylation of Y654 to its nuclear translocation with the resulting activation of the expression of its myc, axin, and zeb-1 tumorigenic target genes, involved in both hyperproliferation and invasiveness (Fig. 2a). This led to hyperproliferation and to the formation of anomalous crypts. In the presence of one mutation on one copy of adenomatous polyposis coli (APC) only, an essential element of the cytoplasmic β-catenin degradation complex, for which mutation is involved in 90% of human colon cancer, the 1-kPa mechanical strain led to tumor formation (Fernández-Sánchez et al., 2015). The same result was confirmed in a genetic model of colon tumor growth in vivo, in which the 1-kPa strain was this time induced by mosaic domains of hyperproliferative crypts overexpressing Notch and applied to nongenetically transformed healthy neighboring crypts.

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Clinorotation that mimics microgravity (a rotation of cell samples around the horizontal axis designed to nullify the integrated gravitational vectoral force by continuous averaging; Wang et al., 2016). It is therefore probable that ongoing experiments will elucidate additional multiple roles and underlying mechanisms of microRNAs in the mechanosensing of ECM stiffness, thereby contributing to deciphering the complexity of the molecular mechanisms involved in the mechanotransduction of tumor progression and expansion.

Mechanotransduction can also be transmitted directly into the nucleus, stimulated by the forces transmitted by the cytoskeleton from the ECM to chromatin (Swift and Discher, 2014). Indeed, external forces transmitted to the nucleus via the cytoskeleton result in nuclear deformation (Wang et al., 2009) and in the activation of signaling pathways into the nucleus (Belaadi et al., 2016). The molecular mechanisms of the ECM-nucleomechanotransduction processes are now beginning to be elucidated based on recent progress in the identification of the proteins linking the cytoskeleton to nuclear structures.

Although proteins connecting the cytoskeleton to integrins have been well characterized, those connecting the cytoskeleton to the nucleus were only recently identified and analyzed. Force transmission is mediated from the outside of the cell into the cytoplasm by adaptor proteins for integrins, such as Talin and Vinculin, located at cytoplasmic sites of focal adhesions, and from the cytoplasm to the nucleus at sites of cytoskeleton-nuclear membrane interaction via Nesprin1,2,3,4 that link actin filaments to the nuclear membrane (Fig. 3). Integrity of the nucleus is maintained from the inside by nuclear intermediate filament Lamin proteins and by the chromatin anchored to the inner surface with SUN proteins. SUN proteins bind to the inner lamina and other nucleoplasmic proteins while interacting with Klarsicht-ANC-Syne-homology (KASH) domain of Nesprin anchored at the outer nuclear membrane. Nesprin calponin homology domains at their N-terminal that bind the actin cytoskeleton and transmembrane KASH domains at their C-terminals that bind to the inner nuclear membrane SUN proteins in the luminal space between the outer and the inner membranes (Esra Demircioglu et al., 2016). Together, these linker-nesprins and SUN proteins are called linkers of nucleoskeleton and cytoskeleton (LINC) complexes and enable force transmission through the nuclear envelope to Lamins. The Lamin intermediate filaments form coiled-coil parallel dimers assembled into a highly ordered polymer network. Lamins are expressed from three genes: Lamin-A and -C are spliced products from the LMNA gene and referred as “A-type” Lamins and Lamin-B1 and -B2 are encoded by the LMNB1 and LMNB2 genes, respectively, and referred as “B-type” Lamins. Laminopathies, including muscular dystrophies and cardiopathies, are a result of defects in tissues in which A-type lamins are dominant. Micropipette aspiration experiments measuring the rate of deformation of nuclei under pressure could appreciate the contribution of A-type Lamins to viscosity and B-type Lamins to the elastic response (Swift and Discher, 2014). Lamins interact through extensive protein-protein interactions within the nucleus; in addition to binding directly to the DNA (for A-type Lamins), they also bind to nuclear actin polymers through their AB actin-binding sites,
avoiding spontaneous formation of F-actin in the nucleus, and to the nuclear membrane protein Emerin (Simon et al., 2010; Isermann and Lammerding, 2013).

Mechanical nuclear responses have been analyzed by various and complementary experimental techniques including (a) small deformation processes by using magnetic tweezers on beads attached to Nesprin where the force is applied via specific attachment, suggesting a role for Nesprin in nuclear stiffness, and optical tweezers revealing that chromatin attachment contributes to nuclear mechanical rigidity, (b) micropipette aspiration where the force is applied through nonspecific interactions suggesting a major role of Lamin-A in determining nuclear stiffness, and (c) atomic force microscopy (AFM) on nuclei, suggesting that both chromatin and Lamins were major contributors of nuclear mechanics (Lherbette et al., 2017). New recent studies on whole-nucleus extension revealed two regimes of nuclear mechanics: an initial short-extension regimen where chromatin dominates nuclear force response, followed by a second long-extension regimen with a 50% higher stiffness at 30% strain where the lamina dominates the mechanics. These new findings indicating that Lamins do not behave as rigid structures but instead as a bendable, elastic polymeric network are also supported by recent data showing that Lamin filaments are easily bent (Stephens et al., 2017).

Mechanical stress has been shown to trigger a dynamic remodeling of these nucleoskeleton structures. Mechanical
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To test the impact of mechanotransduction on tumor cell behavior

**Stretching cultured cells in 2D**

Most experiments on the role of stiffness or mechanical strains on the biochemical state of cells have been performed in 2D cell culture. Traction force microscopy is based on the deformation of a hydrogel substrate by cells or of a microcassette-compliant array substrate composed of soft polydimethylsiloxane micropillars individually deformed by cells, both allowing to measure from cells on the substrate and to modulate the stiffness of a cultured cell substrate in a controlled quantitative way (Dembo and Wang, 1999; Tan et al., 2003; Beussman et al., 2016). Cells can also be stretched through deformation of the overall substrate (Michael Delaine-Smith et al., 2015). Hydrodynamic flow applied directly on cultured cells has long been used to test for the mechanotransductive response of endothelial cells to blood flow constraints (Nakache and Gabu, 1988; Davies, 1995). Cells can also be individually mechanically deformed by using AFM, allowing both the probing of their mechanical properties and mechanical stimulation of the cell (Santos and Castanon, 2004; Tello et al., 2016). At the molecular level, functional magnetic nanobeads under magnetic fields are used to specifically address the mechanotransductive response of specific receptor in cellulo (Wang and Ingber, 1995; Marjoram et al., 2016). In addition to this method, optical tweezers are also used to address the question of functional protein conformation changes in response to strain in vitro (Biai et al., 2010; Siedlik et al., 2016) and in cellulo using fluorescence resonance energy transfer sensors to monitor conformation changes (Grashoff et al., 2010; Borghi et al., 2012).

**Stretching cultured cells in 3D**

From the tumor point of view, the behavior of cells cultured in two dimensions has recently been demonstrated to possibly differ from 3D culture conditions, which are closer to in vivo conditions. Indeed, the oxygen concentration gradient and hypoxia induced by the spatial constraints of the growing tumor mass are pivotal for oncogene expression, ECM remodeling, and angiogenic factor expression during carcinogenesis (Erler and Weaver, 2009). Practically, reconstructed 3D cultures better mimic endogenous tumor specificity by using the most abundant tumor cell populations isolated from tumor biopsies or adapted selected cancer cell lines, including the environment surrounding the tumor comprising other cellular components (fibroblasts and vascular and inflammatory cells), as well as the ECM. As a result of growing tumor cells in a 3D environment, their metabolism, proliferation, viability, and migration abilities are increased; thus, they are more similar to the cellular behavior observed in human tumors such as melanoma, head, neck, and breast cancers (Kim, 2005; Feder-Mengus et al., 2008; Erler and Weaver, 2009).

In addition to reducing the experimentation on animals for drug tests, the 3D model of tumor development is very useful to study the effects of physical constraints in carcinogenesis. Indeed, a large range of rheology and thus cancerous tissue types representing different tumor grades can be obtained because the spatial and mechanical properties of the engineered scaffolds that support cells can be precisely and turn. Stiffness and architecture can be modulated by modifying the molecular concentration, salt, and/or pH level, and/or by combining several components (Butcher et al., 2009). Scaffold structures thus allow the formation of gradients of external physical and biochemical stimuli that highly affect the genetic and epigenetic profile of the tumor in the course of tumor development, growth, and progression via the regulation of many transcription factors (API, p53, Myc, NF-kb, etc.) and expression and activity of chromatin-remodeling proteins (HDAC and MECP2; Gill and West, 2014). Indeed, lung organ on chips showed the recapitulation of both patient tumor growth and therapeutic response as modulated by breathing-associated mechanical strains (Hassell et al., 2017).

Interestingly, in addition to the development of spontaneous organoids generated from stem/niche cells in minimal conditions (Drost et al., 2016), recently developed innovative methods of 3D bioprinting that can reproduce high-resolution scaffold patterns of scales up to micrometers, based on several biomaterials contained in bio-ink (Fig. 4). Combined with stem cell reprogramming and gene editing, 3D bioprinting allows a precise spatial distribution of several cell types within the 3D scaffold, thereby ensuring the heterogeneity and the complexity of the tumor as well as the tumor microenvironment tissues (Knowlton et al., 2015; Driehuis and Clevers, 2017), Indeed, thanks to medical imaging, the heterogeneous structure of an endogenous tumor can be mapped and then reconstructed by 3D software to be reconstituted with 3D bioprinting with high fidelity and reproducibility. Such technology can also be used on endogenous decellularized tumor tissue to accurately mimic specific aspects of the native tumor tissue in a controlled way (Jung et al., 2016).

Thus, modulation of the substrate stiffness, or mechanical stress, will be applied to the reconstructed tumor, as begins to be the case for organoids (Gjoreski et al., 2016). Little is indeed known yet about the mechanical stiffness/stress effects on 3D engineered tumor tissue. Recent studies have suggested cell differentiation by low-rigidity substrates acts via mechanosensitive epigenetic processes involving H3K9 methylation (Tan et al., 2014) and that 3D reconstructed tumors cultured inside a compressed or tensed bioreactor have a higher metastatic potential (Caseraue et al., 2015). Mathematical models should additionally help predict and quantify tumor heterogeneities and effects of the scaffolds on these engineered tumor tissues (Soares et al., 2017). Thus, tumor tissue engineering is just starting as a promising cell culture methodology to better mimic the heterogeneous 3D structure of a developing tumor and its behavior as a function of stiffness and pressure, mechanical parameters which are inherent to tumor biology.

**Modulating stiffness and stretching cell tissues in vivo**

To modulate tissue stiffness in vivo, pharmacological approaches, such as BAPN treatment that inhibits collagen cross-link formation or Y27632 Rho/Rho kinase inhibitor treatments, inhibiting ECM cross-linking or contractile actomyosin activity and leading to the decrease in mice tumor stiffness, are used in addition to transgenic constitutively activated Rock (Levental et al., 2009; Samuel et al., 2011; Nilsson et al., 2016; Bordeleau et al., 2017). To apply solid-stress pressure in inhibitor treatments, inhibiting ECM cross-linking or contractile actomyosin activity and leading to the decrease in mice tumor stiffness, are used in addition to 3D bioreactors, such as the device described by transgenic constitutively activated Rock (Levental et al., 2009; Samuel et al., 2011; Nilsson et al., 2016; Bordeleau et al., 2017). To apply solid-stress pressure in vivo, intravenous injections of ultramagnetic liposomes were performed in mice previously implanted subcutaneously with a strong, small magnet in front of the organ (Fernández-Sánchez et al., 2015). After stabilization (from 30 min to 2 mo), the magnet served as a force generator, leading to a permanent pressure of 1 kPa for weeks to months, which corresponds to the colon tumor-growth pressure in mice, thereby quantitatively mimicking colon tumor-growth pressure. Interestingly, ultrasonic elastography showed tissue compression with no increase in the tissue stiffness resulting from the “magnetically induced mechanical treatment,” ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic treatment, ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic treatment, ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic treatment, ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic treatment, ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic treatment, ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic treatment, ensuring a pure pressure-induced effect independently of any stiffness effect. Such methodology might be generalized to the magnetization of any tissue vascularized enough to transport ultramagnetic liposomes and favor extravasation under local magnetic field gradients. The use of small-animal magnetic
nucleus in response to strain, whereas B-type Lamins are constitutively expressed (Swift et al., 2013). Indeed, in cancer cells, Lamins are often misexpressed or localized aberrantly and contribute to the multilobulated nuclear shape frequently observed (Foster et al., 2010).

Fluorescence resonance energy transfer analysis of Actin and Nesprin2 in NIH3T3 cells revealed their dynamic interaction in maintaining the cell nucleus in a prestressed state. Perturbation of the LINC complex by transfection of cells with dominant-negative KASH lacking the Actin-Nesprin2 binding domain causes the formation of actin stress fibers at the apical side of the nucleus. It also provokes an increase in nuclear height from 7 to 8 µm, which reflects a decrease of cross-linking nuclear structures possibly leading to chromatin decondensation effects putatively involved in downstream transcriptional effects (Kumar and Shivashankar, 2016).

Whether such nuclear mechanotransductive cues responsive to the fibrosis induced change of substrate stiffness are involved in the process of tumor progression is under investigation. On the other hand, at late tumor progression stages, tumors are characterized not only by a fibrosis-induced increase of ECM stiffness but also by cell motility and invasiveness. In such a scenario, individual tumor cells actively migrate inside a heterogeneous dense tissue composed of adherent cells and fibrotic stroma, leading to a strong deformation that can be transmitted to the nucleus by the cytoskeleton and can alternatively directly deform the nucleus (Harada et al., 2014; Krause and Wolf, 2015). A-type Lamin could have additional regulatory roles in preventing the deformation of nuclei at the entry of the dense tissue defect pores, with the nucleus acting as an anchor preventing cell movements through the stroma. Studies of MSC, U251 glioblastoma, and A549 lung carcinoma cell migration through narrow pores of 3 µm in diameter that mimic those of tumor tissues demonstrates that deformation of the nucleus depends on lamina composition. Lamin-A overexpression is correlated with a limited migration, whereas Lamin-A knock-down favors easier migration (Harada et al., 2014). In addition, nuclei with high A-type Lamin recover slowly after deformation and maintain an elongated morphology after emerging from the pores, whereas nuclei with dominant elastic B-type Lamin rapidly return to the more spheroid shape after deformation. Low lamin-A expression could thus represent a major actor in the development of metastasis, associated with EMT processes occurring during early development of metastasis (Wolf et al., 2013; Saarinen et al., 2015; Kaspi et al., 2017).

Indeed, variations in Lamin-A levels were reported in cancer and other pathologies, with low levels reported in malignant carcinomas and colorectal cancer (Foster et al., 2010; Sakthivel and Sehgal, 2016).

In addition, osteosarcoma and lung adenocarcinoma cancer cell migration across interstitial spaces under tension could lead to the deformation of the nucleus and the formation of new chromatin and lamina contacts influencing gene expression. Along with enhancing gene expression heterogeneity in cancer cells during migration, this results in the loss of the nuclear envelope integrity and DNA damage by the loss of DNA repair factors, thereby generating additional heritable mutations (Fig. 3, b and c; Denis et al., 2016; Irianto et al., 2017). Correspondingly, cancer genome mutations seem to increase with tissue stiffness: Stiff tissues such as bone, muscle, or skin produce cancer that exhibits more genomic changes than tumors originating from soft tissues (Pfeifer et al., 2017). DNA repair factors are now emerging as mechanosensitive factors that can alter the DNA sequence of the cells, leading to a new field of mechanogenomics to explore (Discher et al., 2017).

Nuclear mechanosensing is thus achieved through changes in protein conformation, translocation of transcription factors, chromosome conformation, and nuclear membrane dilatation or rupture. Omics-based metatranscriptome and proteome data analysis from diverse tissues revealed the potential importance of mechanosensory proteins in a broad range of species, not only in pathologies but also during embryonic development and aging (Cho et al., 2017).

Thus, because of the mechanical link between junctions, the plasma membrane, and the cytoskeleton with the nuclear membrane and chromatin, a wide variety of new mechanosensors should be discovered in the nucleus in the future, with their role in tumor development to be tested. This should increase the complexity and the subtlety of the mechanotransductive regulation processes, as will the new implications for microRNAs.

**Mechanotransduction in cancer: a mechanical reactivation of physiological developmental mechanotransductive cues in response to pathological mechanical strains?**

The mechanical strains generated by hydrodynamic blood flow during development are proposed to regulate blood vessel formation, or dorsal aorta reduction of vessel-diameter maturation in zebrafish, through a VEGFR2-dependent mechanosensitive pathway. This was suggested to be the result of a decrease in VE-Cadherin tension, as innovatively monitored in vertebrates with a VE-cadherin tension sensor. The pathway could then possibly down-regulate aorta junctions in response to blood flow shear stress, a process that would allow mechanical cell rearrangements in blood vessel formation and aorta maturation in response to stress (Grashoff et al., 2010; Sugden et al., 2017). These findings are reminiscent of hemodynamic regulation of angiogenesis in early chicken embryos by heart rate and cardiac output (Branum et al., 2013), or blood flow–induced mechanotransductive regulation of zebrafish heart valve formation through the endocardial flow-responsive gene klf2a (Hove et al., 2003; Steed et al., 2016). In addition, angiogenesis was also proposed to be favored in tumors by the high stiffness characterized by fibrotic tumor development via the p190RhoGAP-dependent mechanical activation of GATA nuclear translocation leading to angiogenic VEGFR2 expression, a stiffness-dependent process that could also be involved in neonatal mouse retinal angiogenesis development in vivo (Mammoto et al., 2009). It would thus be interesting to understand if, in adult tissues, pathological anomalous hydrodynamical and stiffness conditions in tumors reactivate pathways similar to physiological embryonic angiogenic mechanosensitive biochemical pathways that induce vessel formation.

Stiffness also plays an important role in the determination of embryonic biomechanical morphogenesis (Fleury et al., 2015; Serwane et al., 2017). For instance, in early Caenorhabditis elegans embryos, lateral stiffness anisotropies within the

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**References**

Discher DE, Hyland CE, Stroock AD, and Yarger JR (2017). Omics-based metatranscriptome and proteome data analysis from diverse tissues revealed the potential importance of mechanosensory proteins in a broad range of species, not only in pathologies but also during embryonic development and aging (Cho et al., 2017).

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fiber-reinforced dorsoventral epidermis are critical in driving an anisotropically shaping anteroposterior embryonic elongation in response to forces developed by Myo-II contractibility (Vuong-Brender et al., 2017). In addition, embryonic tissues were found to be reactive to shape change and stiffness increase by activating Myo-II in junctions as well as medio-apically (Fernández-Gonzalez et al., 2009; Pouille et al., 2009; Mitrossilis et al., 2017). The associated underlying molecular mechanisms consist of a direct process that potentially changes Myo-II affinity to actin in response to stress in junctions and a process of membrane flattening that inhibits endocytosis of the secreted factor Fog thereby enhancing the RhoGEF downstream pathway that stabilizes Myo-II medio-apically, respectively. Associated physiological functions consist of a reinforcement of tissue resistance in response to strains applied to the tissue or the trigger of major morphogenetic movements such as gastrulation in response to soft-cell deformation in the endomesoderm, respectively. It would thus be interesting to test if pathologically high stiffness/tension in tumors anomalously reactivate the β-cat embryonic developmental physiological pathway, thereby leading to the reactivation of an endomesoderm developmental process within the adult colonic tissue, that consequently destabilizes the homeostatic stability and participates in tumor progression by amplifying it though mechanotransduction.

Although such mechanical strain alone is sufficient to trigger hyperproliferation and anomalous crypt formation, the mutation of only one copy of APC is required to mechanically induce tumors (Fernández-Sánchez et al., 2015). This is because the APC/GSK3β complex, which sends cytoplasmic β-cat to degradation before nuclear translocation, is 50% less efficient in the APC\(^{+/−}\) genetic background. Therefore, the probability of nuclear translocation of the β-cat mechanically released from junctions by mechanically induced Y654 phosphorylation is enhanced from hyperproliferative doses to tumorigenic doses in the APC\(^{+/−}\) compared with the WT. Thus, in this case, both genetic and microenvironmental biomechanical anomalies are required together to trigger tumorigenesis with the mechanistic process underlying such synergy being understood.

This is conceptually reminiscent, at microenvironmental levels, of epidemiological studies currently revealing in a more general context the synergy between genetic predispositions and environmental contexts, the most evident being smoking.
fumes and nutrition (Jaffee et al., 2017). However, the advantage of external environmental parameters is that it is a priori possible to remove it to decrease cancer development frequency. In contrast, internal microenvironmental mechanical strains are in themselves much more delicate to inhibit. We saw that Y-27632 and BAPN treatments to down-regulate tumor tissue stiffness were successful in repressing mouse breast and prostate tumor progression (Levental et al., 2009; Samuel et al., 2011; Bordeleau et al., 2017) but might have undesired effects in other healthy tissues of the animal. Combining treatments that modulate the mechanical properties of tissues with efficient drug-targeting methodologies would certainly increase the probability of transferring such promising preclinical studies to human treatments.

Overall, the increasing evidence of the strong involvement of interstitial pressure, stiffness, or hyperproliferative pressure acting predominantly as an enhancer of tumor growth development, makes the investigation of new treatments based on the inhibition of such tumorigenic mechanical cues a potentially highly promising avenue for future innovative cures or synergic adjuvants to biochemically based current therapies and treatments.

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