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CELL SCIENCE AT A GLANCE

The myofibroblast at a glance

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ABSTRACT

In 1971, Gabbiani and co-workers discovered and characterized the "*modification of fibroblasts into cells which are capable of an active spasm*" (contraction) in rat wound granulation tissue and, accordingly, named these cells 'myofibroblasts'. Now, myofibroblasts are not only recognized for their physiological role in tissue repair but also as cells that are key in promoting the development of fibrosis in all organs. In this Cell Science at a Glance and the accompanying poster, we provide an overview of the current understanding of central aspects of myofibroblast biology, such as their definition, activation from different precursors, the involved signaling pathways and most widely used models to study their function. Myofibroblasts will be placed into context with their extracellular matrix and with other cell types communicating in the fibrotic environment. Furthermore, the challenges and strategies to target myofibroblasts in anti-fibrotic therapies are summarized to emphasize their crucial role in disease progression.

KEY WORDS: Fibrosis, Growth factor activation, Tissue repair, Wound healing

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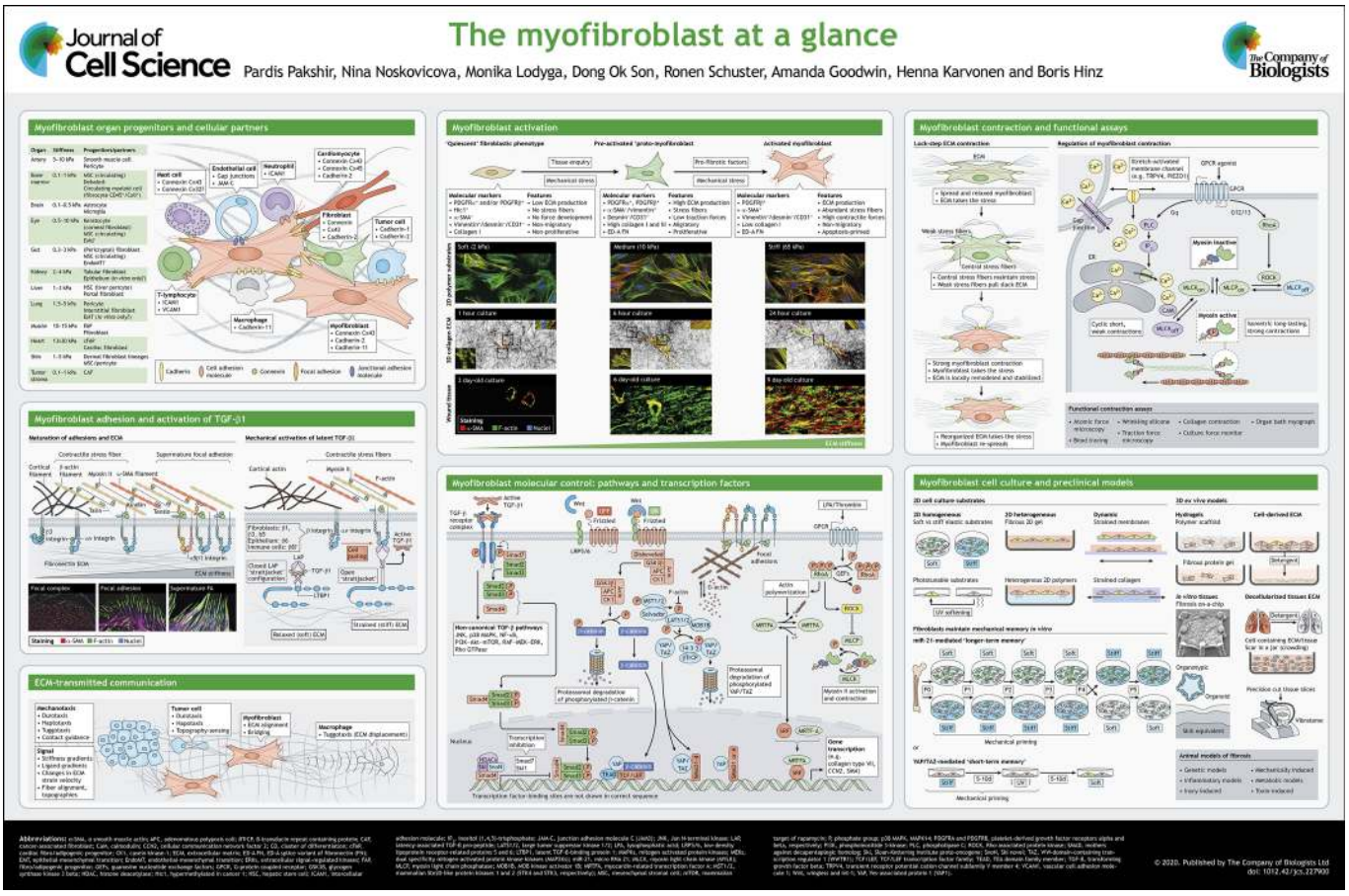
Introduction

Myofibroblasts were first described in healing rat wound granulation tissue by Gabbiani and co-workers as cells that share morphological features of "*conventional tissue fibroblasts*" and contractile smooth muscle cells by being "*capable of an active spasm*" (hence, the name – although 'spasmoblast' was discussed, too) (Gabbiani et al., 1971). This visionary two-page electron microscopy study already recognized numerous myofibroblast features that, five decades and >16,000 publications later, form our understanding of these intriguing – yet still enigmatic – cells. Myofibroblasts have crucial physiological roles in rapidly repairing



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injured tissues by laying down collagen scar, like in skin after trauma or heart after cardiac infarction. In addition, myofibroblasts are key effector cells in almost all organ fibrosis by persistently accumulating and contracting scar tissue beyond normal repair (Hinz, 2016a; Horowitz and Thannickal, 2019). In this short review, we discuss the myofibroblast in context with its extracellular matrix (ECM), other cells, and its roles in physiology and pathology. The accompanying poster summarizes central aspects of myofibroblast biology for newcomers but also aims to entertain those skilled in the myofibroblast art.

Organ progenitors and cellular partners

In his seminal work, Gabbiani proposed myofibroblasts as "*modified fibroblasts that have developed from the more conventional tissue fibroblasts*" (Gabbiani et al., 1971). This statement is still fundamentally true considering that, at the time, 'fibroblasts' denominated collagen-producing cells, which – we now know – comprise different mesenchymal cell populations. Cell-lineage tracing using fluorescent reporter mouse models and detection of various cellular markers in different species, including human, has further refined this population, and revealed additional contributors to the heterogenous myofibroblast community. 'Conventional fibroblasts' give rise to myofibroblasts in all injured or fibrotic organs, for instance, liver (portal fibroblasts; Karin et al., 2016), gut (Rieder et al., 2010), eye (keratocytes; Hinz, 2016a; Rocher et al., 2020; Wilson et al., 2017), heart (Tallquist and Molkentin, 2017), cancer-associated fibroblasts (CAFs) in the tumor environment (Sahai et al., 2020) and dermal fibroblast lineages in skin (Jiang and Rinkevich, 2020; Lynch and Watt, 2018) (see poster). Pericytes were identified as another main contributor to the myofibroblast pool in lung, kidney, skin, muscle and liver (where they are called hepatic stellate cells) (Affo et al., 2017; Di Carlo and Peduto, 2018). The markers and features of pericytes partly overlap with those of tissue-resident mesenchymal stromal cells (MSCs) (El Agha et al., 2017; Guimarães-Camboa et al., 2017; Lemos and Duffield, 2018; Zepp et al., 2017) and fibro/adipogenic-progenitors (FAPs) in cardiac (cFAP) and skeletal muscle (Scott et al., 2019; Soliman et al., 2020) – all multi-lineage-capable progenitor cells. The brain – as always – is special, with activated astrocytes and microglia contributing to scar formation in the central nervous system (Fernández-Klett and Priller, 2014).

Other myofibroblast precursors have had their prime times during the last 50 years but they appear to be very organ-specific, extremely scarce, mislabeled as myofibroblasts despite the absence of contractile features, ruled out with advanced technologies or *in vitro* artifacts. The list of debated precursors includes lung and kidney epithelial cells, and endothelial cells that, at least *in vitro*, can undergo epithelial–mesenchymal transition (EMT) and endothelial–mesenchymal transition (EndoMT), circulating fibrocytes, and bone marrow-derived MSCs. The debate is part of excellent reviews and still open (Chong et al., 2019; El Agha et al., 2017; Lemos and Duffield, 2018; Quaggin and Kapus, 2011; Zeisberg and Duffield, 2010) (see poster). Opportunistically (literally), we favor a concept where myofibroblasts can be generated from multiple precursors, depending on the organ, the insult, the timing and the nature of the activation signal. Extracellular signals that regulate myofibroblast activation and activity can derive from other cell types, and the ECM of healing and fibrotic environment (Box 1). Myofibroblast activation is jointly promoted by chemical and mechanical stimuli that establish positive feedback loops, which are central in the pathogenesis of fibrosis and discussed in more detail in the following sections.

Box 1. Myofibroblast partners and intercellular junctions

Myofibroblasts are physically affectionate, ready to form "*localized intercellular connections*" (Gabbiani et al., 1971) in addition to autocrine and paracrine exchange of soluble cytokines and growth factors, and production of exosomes and microvesicles (Bonnardel et al., 2015; Laberge et al., 2018; Lemoinne et al., 2015; Zanotti et al., 2018). Gap junctions allow the exchange of ions and small molecules between myofibroblasts and fibroblasts (Follonier et al., 2008), cardiomyocytes (Miragoli et al., 2006), mast cells (Termei et al., 2013) and, possibly, the endothelium (Lemoinne et al., 2015; see poster). Various cadherins are expressed in myofibroblast adherens junctions, of which cadherin-11 (also known as OB-cadherin) is most prominent and co-regulated with the activation of myofibroblasts (Hinz et al., 2004; Thedieck et al., 2007; Valletta et al., 2012). Cadherin-11 mediates mechanically resistant junctions between myofibroblasts (Black et al., 2018; Pittet et al., 2008) and direct communication with macrophages (Lodyga et al., 2019; To and Agarwal, 2019). Cadherin-2 (also known as N-cadherin) transmits contractile force between myofibroblasts and cardiomyocytes (Thompson et al., 2014), and heterotypic junctions have been described between cadherin-1 (also known as E-cadherin)-expressing cancer cells and cadherin-2-expressing CAFs (Labernadie et al., 2017). Furthermore, junctional adhesion molecules (JAMs) exist between myofibroblasts and endothelial cells (Hintermann et al., 2016), together with the zonula occludens protein 1 (TJP1) in dermal fibroblasts (Morris et al., 2006). Finally, the cell-adhesion molecules (CAMs) ICAM1 and VCAM1 are expressed in subtypes of myofibroblast (Fontani et al., 2016; Hellerbrand et al., 1996), where they promote communication with immune cells, including T-cells (Bombara et al., 1993) and neutrophils (Clayton et al., 1997; Domazetovic et al., 2019). In addition to direct intercellular communication, myofibroblast transmission of force to fibrous ECM generates mechanical signals (see poster). Other cells, such as macrophages, follow changes in ECM strain velocity, i.e. 'tugging' events, created by contracting myofibroblasts (Pakshir et al., 2019), and cancer cells have been shown to follow fibroblast-aligned ECM cues (Erdogan et al., 2017). Such ECM signals are typically sensed via integrins or other collagen receptors, such as discoidin domain receptor 1 (DDR1), and involve mechanosensitive channels that are yet to be identified in the mechano-responding cells (Pakshir and Hinz, 2018; Te Boekhorst et al., 2016).

Activation states and features

Myofibroblasts are gradually activated from their precursors in different stages, which has already been described in the seminal discovery. Early during healing, myofibroblasts "*showed the cytologic structure regarded as 'typical'* […] (i.e. *numerous cisternae of rough endoplasmic reticulum and many mitochondria*)" and later exhibited "*bundles of packed fibrils resembling those of smooth muscle*" (Gabbiani et al., 1971). Most experts agree on few minimal requirements to define myofibroblast precursors across different organs, i.e. protein expression of collagen type I (Kisseleva et al., 2012), the platelet-derived growth factor (PDGF) receptor alpha (PDGFRA) (Iwayama et al., 2015; Li et al., 2018) and/or receptor beta (PDGFRB) (Henderson et al., 2013) and the novel marker for mesenchymal lineage hypermethylated in cancer 1 (Hic1; Scott et al., 2019) (see poster). Other tissue-specific myofibroblast precursor lineage markers exist (Hinz, 2016a) but are not discussed here.

Cells in tissues are exposed to mechanical conditions such as the 'stiffness' of the organ, measured as elastic modulus in kPa (Achterberg et al., 2014; Chevalier et al., 2016; Hinz, 2016a; Stewart et al., 2018; Wyss et al., 2010) (see poster). Upon tissue injury, exposure to high strain or stiffening of the ECM activates myofibroblast precursors by inducing the formation of stress fibers that allow to exert traction forces and migrate (Hinz et al., 2001b). The acquisition of contractile features by reorganization of the

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existing actin cytoskeleton has been defined as the 'proto-myofibroblast' stage (Tomasek et al., 2002). Under continued mechanical stress and addition of pro-fibrotic factors, such as transforming growth factor beta-1 (TGFB1, hereafter TGF-β1) and the extradomain-A (ED-A) splice variant of fibronectin (FN) ED-A FN, myofibroblasts fully activate by neo-expressing α-smooth muscle actin (α-SMA). Expression of α-SMA confers high contractile force to stress fibers and is the most frequently used myofibroblast marker (Hinz et al., 2001a) (see poster). It is important to understand myofibroblast activation as a gradual maturation process, and not to expect all features and functions within one single cell at the same time. For instance, high proliferation and migration are occasionally used to claim myofibroblast activation; however, these early fibroblast activation features seem incompatible with the later occurring highly contractile phenotype (Hinz et al., 2019; Rønnov-Jessen and Petersen, 1996).

The sequential development of mutually exclusive features and functions during myofibroblast maturation can explain seemingly perplexing findings, such as low or absent α-SMA expression in cells that express high levels of collagen during stages of fibrosis in mouse and human (Tsukui et al., 2020). Considering a spectrum of plastic myofibroblast phenotypes also offers a different view on single-cell RNA sequencing (scRNAseq) studies, defining numerous new 'types' or 'classes' of myofibroblast almost every month (Bartoschek et al., 2018; Croft et al., 2019; Guerrero-Juarez et al., 2019; Park et al., 2018; Philippeos et al., 2018; Ramachandran et al., 2019; Reyfman et al., 2019; Skelly et al., 2018; Soliman et al., 2020; Tabib et al., 2018; Xie et al., 2018). On the basis of variable expression of α-SMA, it has long been proposed and documented that 'the myofibroblast', in fact, comprises a heterogeneous population of cells in different states of activity that depend on their origin and microenvironment (Dugina et al., 1998; Schmitt-Gräff et al., 1994). It is, thus, not surprising that scRNAseq data confirm the existence of fibroblast populations with different gene expression patterns, such as low and high α-SMA-expressing CAFs with several different stemness potentials and cytokine profiles (Patel et al., 2018; Saraswati et al., 2019), or myofibroblasts with similar origin but different RNA expression patterns in lung and liver fibrosis (Krenkel et al., 2019; Peyser et al., 2019; Tsukui et al., 2020; Valenzi et al., 2019). Longitudinal scRNAseq studies and meta-analysis over multiple independent studies will be required to decide whether computed similarity clusters reveal distinct fibroblast classes or represent transition stages.

All these considerations also relate to the question of myofibroblast fate. Although originally considered being terminally differentiated, loss of myofibroblast contractile features has since been demonstrated in cell culture, several animal models and fibrotic human liver (Horowitz and Thannickal, 2019; Jun and Lau, 2018; Kisseleva et al., 2012). It is unknown whether de-activated myofibroblasts return to their respective precursor state, e.g. reacquire pericyte, FAP or MSC characteristics, or whether they lose contractile features and end up in some cellular twilight zone. Alternative myofibroblast fates that contribute to their resolution during normal tissue repair but are dysregulated in fibrosis are senescence (Demaria et al., 2014; Merkt et al., 2020) or suicide by entering intrinsic or extrinsic apoptosis pathways (Hinz et al., 2019).

Contraction and functional assays

The ultimate defining feature and function of myofibroblasts is contraction: they "*play a role in granulation tissue contraction*" using "*a well-developed cytoplasmic fibrillar system*" of actomyosin stress fibers (Gabbiani et al., 1971). Similar but not identical to smooth muscle, tissue myofibroblasts produce an incremental contractile tone and can be stimulated to contract acutely (Hinz et al., 2001b; Majno et al., 1971). *In vitro* studies have revealed a lock-step mechanism that allows myofibroblasts to produce collagen contractures over several days during normal wound healing, and up to years in scar tissue (Tomasek et al., 2002). Strong (μN-range) and long-lasting (hours) isometric contractions of myofibroblasts are proposed to reduce stress on single collagen fibrils in a tense ECM environment (Follonier Castella et al., 2010a; Hinz et al., 2019; see poster). Relaxed collagen fibers are then amenable to proteolytic processing and/or remodeled by periodic (minutes), short-range (∼400 nm) and weak (∼100 pN) contractile events that establish a new and shorter ECM, which takes over the mechanical load to allow myofibroblast re-spreading (Follonier Castella et al., 2010a; Hinz et al., 2019).

Strong isometric tension is produced through the activation of Rho-associated protein kinase (ROCK) pathway, downstream of G protein-coupled receptors (GPCR), such as the lysophosphatic acid receptor 1 (LPAR1) and proteinase-activated receptor 1 (F2R, hereafter PAR-1) (Sakai et al., 2017; Ungefroren et al., 2018). Active GTP-bound RhoA activates ROCK that, in turn, phosphorylates myosin light chain phosphatases (MLCPs), inhibiting their ability to dephosphorylate and, thereby, also inactivating myosin light chains (MLCs) (see poster). Persistently phosphorylated active MLCs mediates long-lasting actomyosin stress fiber contraction (Follonier Castella et al., 2010b). By contrast, periodic local contractions are regulated by transients in cytosolic Ca^{2+} through binding of calmodulin (CaM), which activates the MLC kinase (MLCK). Transient cytosolic Ca^{2+} is released from the endoplasmic reticulum through channels that are sensitive to inositol (1,4,5)-trisphosphate (IP_3) generated by phospholipase C (PLC), also downstream of GPCR signaling (Gopal et al., 2020; Janssen et al., 2015). Other Ca^{2+} sources are cells connecting via gap junctions or entry through stress-sensitive plasma membrane channels (Follonier et al., 2008), such as transient receptor potential cation channel subfamily V member 4 (TRPV4) (Arora et al., 2017; Zhan and Li, 2018) or PIEZO1 (Blythe et al., 2019) (see poster).

Local contractions at the sub-micron scale can be detected by using atomic force microscopy and by optically tracing the displacement of marker beads on the surface of myofibroblasts or in soft collagen gels (Follonier Castella et al., 2010a). Isometric contraction of single myofibroblasts is best quantified by using two-dimensional elastomer substrates, either wrinkling under cell force (Harris et al., 1980) or amenable to traction force microscopy with embedded surface markers (Schwarz and Soiné, 2015). Contraction of (myo)fibroblast populations is often assessed by measuring the diameter reduction of three-dimensional collagen gels. Of note, only restrained collagen-containing ECM but not free-floating gels provide the mechanical resistance required for myofibroblast activation (Grinnell and Petroll, 2010; Tomasek et al., 2002). True force measurement of (myo)fibroblast-populated collagens is possible by using the culture force monitor (Eastwood et al., 1994), an *in vitro* version of the conventional organ myographs that were originally used to demonstrate contraction of wound tissue (Gabbiani et al., 1972).

Adhesion, ECM and integrin-mediated mechanical activation of latent TGF-β1

To transmit stress fiber contractile force to the ECM, myofibroblasts *in vivo* develop "*peripheral attachment sites*" (Gabbiani et al., 1971), later called 'fibronexus' (Singer, 1979). The *in vitro*

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equivalents are integrin-containing focal adhesions (Burridge, 2017). Maturation of cell–ECM attachment sites from heterodimeric inactive and subsequently activated integrins, to nascent focal complexes, to larger focal adhesions and, eventually, 'supermature' focal adhesions is controlled by intracellular and extracellular stress (Livne and Geiger, 2016; Miller et al., 2020; Sun et al., 2019; see poster). Supermature adhesions are characterized by co-expression of αvβ3 integrin, α5β1 integrin and tensin (Dugina et al., 2001; Hinz et al., 2003; see Table S1 for a summary of adhesion receptors expressed in myofibroblasts). By communicating changes in the mechanical status of the ECM to the actin cytoskeleton, adhesion maturation is closely associated with – and, in fact, the prerequisite of – myofibroblast activation (Goffin et al., 2006; Hinz, 2006). Transmission of force by myofibroblast adhesion to fibrous ECM also generates far-reaching mechanical signals, such as gradients of stiffness and adhesion receptor-binding ligands, ECM alignment and ECM tunnels that can be perceived by neighboring myofibroblasts and other cells as sharing the same ECM (see poster and Box 1). Moreover, different transmembrane adhesion receptors convey specific information that is coded in the molecular composition of the ECM. In addition to collagen type I, other ECM components are frequently associated with myofibroblasts and, sometimes, used as molecular markers – including ED-A FN, tenascin C, cellular communication network factor 2 (CCN2; also known as CTGF) and periostin (Herrera et al., 2018; Klingberg et al., 2013; Schiller et al., 2015; Taha and Naba, 2019).

One of the most unusual but impactful ECM ligands for myofibroblast integrins is TGF-β1, the first fully cloned member of the TGF-β superfamily, comprising 33 different genes in mammals (Moses et al., 2016). TGF-β1 is always secreted as a latent protein complex consisting of the active cytokine and the non-covalently bound latency-associated TGF-β pro-peptide (LAP) (Kim et al., 2018; Lodyga et al., 2019; see poster). The RGD motif within LAP of TGF-β1 and TGF-β3 is a bona fide binding site for all αv integrins (Henderson et al., 2013; Mu et al., 2002; Munger et al., 1999; Sarrazy et al., 2014), with αvβ1 integrin being the strongest latent TGF-β1-binding receptor expressed in (myo)fibroblasts (Reed et al., 2015). Because the opposite end of LAP is covalently linked to the latent TGF-β-binding protein 1 (LTBP1) in the ECM, integrin-mediated fibroblast pulling can mechanically activate TGF-β1 by inducing a conformational change in the LAP–TGF-β1 'straitjacket' configuration (Buscemi et al., 2011; Dong et al., 2017; Wipff et al., 2007; see poster). Consequently, the levels of ECM resistance and pre-strain dictate the efficacy of latent TGF-β1 activation and, thus, link the progress of tissue remodeling to growth factor signaling (Klingberg et al., 2014).

Molecular control–pathways and transcription factors

Active, but not latent TGF-β1, binds to and assembles the TGF-β receptor complex to promote activation of canonical mothers against decapentaplegic homolog (Smad) proteins (Derynck and Budi, 2019; Lodyga et al., 2019; see poster). In Smad-dependent TGF-β signaling, Smad2 and Smad3 are recruited and, subsequently, phosphorylated by the TGF-β type I receptor. The activated Smad2–Smad3 complex assembles with Smad4 and translocates to the nucleus, where it binds to Smad-binding elements within promoters and enhancers of genes that encode fibrogenic proteins, such as α-SMA, but also regulate other biological processes (David and Massague, 2018). Inhibitory Smad7 antagonizes TGF-β signaling by inhibiting Smad-binding sites on the receptor (Miyazawa and Miyazono, 2017). Both Sloan-Kettering Institute (Ski) proto oncogene and Ski novel (SnoN) negatively regulate the TGF-β1 signaling pathway by interacting with Smad4 and recruiting corepressors, such as histone deacetylases (HDACs), to repress the expression of TGF-β target genes, including *Smad7* and Ski1 (officially known as *MBTPS1*), another transcription factor that has been specifically associated with myofibroblast repression (Landry et al., 2018; Tecalco-Cruz et al., 2018).

In addition to canonical Smad signaling, TGF-β1 can also activate various other signaling pathways, for example, Jun N-terminal kinases (JNKs), p38 MAPK (officially known as MAPK14), extracellular signal-regulated kinases (ERKs) – all of which are mitogen activated protein kinases (MAPKs) – and Rho-ROCKs, which regulate various cellular functions, such as actin cytoskeleton changes, proliferation and transcriptional regulation (Derynck and Budi, 2019; Heldin and Moustakas, 2016; see poster). Another main signaling pathway that controls myofibroblast biology is the Wnt cascade, which regulates the localization of β-catenin between intercellular cell junctions at the plasma membrane, proteasomal degradation complexes and the nucleus (Distler et al., 2019). In the nucleus, β-catenin associates with transcription factors that stimulate the transcription of WNT target genes, such as cycline D1 (*CCND1*), *Myc* and *Axin2* (Burgy and Königshoff, 2018).

Biochemical and biomechanical myofibroblast signaling pathways converge at multiple intersection points (Bialik et al., 2019; Haak et al., 2018; Hinz et al., 2019; Piersma et al., 2015; Santos and Lagares, 2018; Tschumperlin et al., 2018). For instance, RhoA-mediated ROCK activation drives polymerization of G-actin into F-actin downstream of GPCRs and integrin-mediated mechanosignaling from a stressed ECM (Dooling and Discher, 2019; Knipe et al., 2018; see poster). The shift in G- to F-actin ratio liberates myocardin-related transcription factor A (MRTF-A) from its inhibitory complex with G-actin in the cytoplasm and allows its translocation to the nucleus. Together with serum response factor (SRF), MRTF-A drives the transcription of profibrotic gene products, such as CCN2 and α-SMA (Speight et al., 2016; Varney et al., 2016). ROCK further regulates gene expression through the transcriptional coactivators Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which are downstream effectors of the Hippo pathway (Dasgupta and McCollum, 2019; Rausch and Hansen, 2020). YAP and TAZ can then translocate to the nucleus where they associate with transcription factors, such as TEA-domain family member (TEAD), to modulate transcription of genes that promote cell proliferation and fibrogenesis, including *CCN2* and the microRNA 21 (miR-21, *MIR21*) (Kofler et al., 2018; Noguchi et al., 2018; Piersma et al., 2015). Notably, the activity of individual and networks of pathways is likely to depend on the activation state and cell origin of the myofibroblast, and have been discussed earlier. For instance, scRNAseq analysis shows poor correlation between the expression of TGF-β1-pathway components and myofibroblast transition in intermediate stage mouse fibrosis (Peyser et al., 2019). The complexity of pathway interactions has been well-studied for EMT in the context of kidney fibrosis (Bialik et al., 2019; Quaggin and Kapus, 2011; Speight et al., 2016) – yielding insight that would not have been possible without elaborate experimental systems.

Cell culture and pre-clinical models

To study molecular mechanisms of fibrosis relevant to the human disease, various *ex vivo* tissue and *in vitro* cell culture models are available to the myofibroblast researcher (see Box 2 and poster). Whereas cell culture models aid mechanistic studies, animal models

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Box 2. Cell culture models to study myofibroblast biology

The simplest way to induce myofibroblast activation is to culture fibroblasts on conventional tissue culture plastic in the presence of serum. Exposure to rigid surfaces always induces formation of stress fibers – which already satisfies the (proto-) myofibroblast criterion – as well as expression of α-SMA in a fraction of cells that is characteristic for the cell source. However, plastic is 'infinitely' more stiff (GPa modulus), even compared with fibrotic tissue (20-100 kPa), and overwhelms cell mechanosensation (Hinz et al., 2019). By contrast, silicone and hydrogel substrates, such as polyacrylamide (PAG) and polyethylene glycol (PEG), can be tuned in a pathophysiological stiffness range (0.1–100 kPa) by modulating chemical crosslinker ratios or using photochemistry (see poster). The power of two-dimensional (2D) systems to control mechanical myofibroblast activation and to generate longer-lasting 'mechanical memory' has been shown and reviewed elsewhere (Balestrini et al., 2012; Li et al., 2017; Santos and Lagares, 2018; Tschumperlin et al., 2018; Yang et al., 2014; Zhou et al., 2018).

In contrast to tissues, polymer materials for cell culture are often 'ideally elastic', homogenous and less susceptible to degradation without chemical functionalization. Alternatively, cells can be cultured on fibrous ECM, such as collagen or fibrin or by using controllable heterogenous substrates with stiff foci in a soft environment (Dingal et al., 2015; see poster). Variations and miniature versions of 2D substrates with and without externally applied strain are also available in three dimensions (3D) (Asmani et al., 2018; MacQueen et al., 2013; Mazza et al., 2017). A more-complex *in vitro* environment, with respect to production and interpretation, can be generated by using myofibroblast-derived ECM, either with cells still present or removed with detergents (Benny and Raghunath, 2017; Franco-Barraza et al., 2016). 'High-end' versions are organotypic cultures and stem cell-derived organoids with multiple levels of complexity and cell types involved (Moulin, 2013; Sobral-Reyes and Lemos, 2020; Sundarakrishnan et al., 2018). If human tissue is available for experimental studies, myofibroblast cultures can be established and studied on living-tissue slices that have been precision-cut with a vibratome, or by using ECM directly derived from decellularized organs (Clouzeau-Girard et al., 2006; Paish et al., 2019; Parker et al., 2014; Uhl et al., 2017).

are often pivotal to understand mechanisms of myofibroblast activation at the organism level. Full-thickness rat skin wounds produced by "*removal from the chest of a square of skin (with the cutaneous muscle)*" were the prototype animal model to study myofibroblasts (Gabbiani et al., 1971). The seminal study further included fibrosis models such as "*scarring of a small area of the liver capsule*" and "*subcutaneous injection of 20 ml of air and 1 ml of 1% croton oil*", where phorbol esters generate an exacerbated inflammatory reaction (Gabbiani et al., 1971). Animal models of enhanced myofibroblast activation can be broadly classified into (i) tissue injuries by physical interventions, including surgery, foreign-body reactions to implanted materials, burns and gamma irradiation, (ii) mechanically induced organ damage or chronic overload and, (iii) administration of toxins, infectious microorganisms and parasites (see poster). Most of these interventions are causing severe or chronic damages and/or chronic inflammation as one prerequisite for myofibroblast development and fibrosis (Table S2). Fibrosis models can be used in different species but mice are predominant study objects because of low cost, short timelines and amenability to genetic manipulation.

However, there are a few fibrotic diseases without an equivalent in mouse, such as formation of keloids, i.e. overgrowing skin scars, and contractures of palmar fascia called Dupuytren's disease. These human-specific conditions typically require direct sampling of tissue from humans and/or cell culture models. As another limitation of mouse models, fibrosis often reverses spontaneously once the insult is removed, such as in bleomycin-induced lung fibrosis (Jenkins et al., 2017). By contrast, human lung fibrosis is considered terminal at the advanced stages that typically present at first diagnosis (Jenkins et al., 2017). To overcome these limitations, genetic and dietary and/or metabolic 'lifestyle' mouse models have been developed to cause spontaneous fibrosis and/or sensitize the animal to experimentally induced fibrosis, which have different strengths and weaknesses (summarized in Table S2). Even though animal models and cell culture systems are limited in reproducing human fibrosis, pre-clinical assessment is essential to discover and develop promising candidates of anti-fibrosis drugs (Jenkins et al., 2017).

Conclusions and perspectives

As key effector cells in fibrosis, myofibroblasts are attractive targets for antifibrotic therapies. However, the development of myofibroblast-targeted therapeutic strategies faces several challenges (Walraven and Hinz, 2018; Yazdani et al., 2017). First, efficient and specific drug actions depend on myofibroblast-specific targets, ideally on the cell surface for better accessibility. Because myofibroblasts share features with other cell types (e.g. smooth muscle, fibroblasts and pericytes), these features are often not amenable to therapy and several promising drug candidates failed in safety assessment or at later stages of clinical trials due to off-target effects (Goodwin and Jenkins, 2016). Second, the physical barrier formed by stiff and dense ECM of fibrotic lesions, and poor vascularization of scar tissue often impede efficient systemic or even local drug delivery. Other inherent properties of fibrotic diseases, such as their vast heterogeneity, redundancies in the myofibroblast activation programs and variable rates of disease progression, all complicate the definition of clinical trial endpoints and add to the difficulties (Goodwin and Jenkins, 2016; Montesi et al., 2019).

In principle, all effectors of myofibroblast activation, deactivation and fate programs have therapeutic potential (Allinovi et al., 2018; Franco-Barraza et al., 2016; Friedman et al., 2013; Hinz, 2016b; Sato et al., 2019; Schuppan et al., 2018; Volkmann and Varga, 2019). The list of molecules that have advanced into clinical assessment includes 'conventional' extracellular signaling molecules, such as IL4, IL6, IL13, the peptide hormone angiotensin II, ET-1 (EDN1), PDGF, their respective receptors, as well as LPAR1 and TLR4 (Allanore et al., 2018; Bhattacharyya et al., 2018; Frangogiannis, 2019; Hinz and Suki, 2016; Hsu et al., 2018). Receptors, cytoplasmic kinases and intracellular signaling molecules – such as those of the Wnt, PI3K/Akt or FAK (PTK2) pathways – are more difficult to target because of their multiple roles within myofibroblasts and in other cells (Hinz et al., 2019; Kimura et al., 2017). Side effects are also a main limitation for therapies that target TGF-β1 or TGF-β1 receptor components, due to the pleiotropic character of the growth factor. More recent strategies tackle different components of the TGF-β1 activation machinery, which provide cell- and context-specificity (Akhurst, 2017; de Gramont et al., 2017; Friedman et al., 2013; Ganesh and Massague, 2018; Hinz, 2016c; Huynh et al., 2019; Lodyga and Hinz, 2019; Varga and Pasche, 2009). However, broader drug actions may also be beneficial, as in the case of the two only licensed antifibrotic drugs Nintedanib and Pirfenidone, of which Nintedanib targets multiple kinases involved in key profibrotic signaling pathways (Richeldi et al., 2014; Sun et al., 2018). Although these drugs represent a great advance in the treatment of pulmonary fibrosis, neither can reverse nor halt disease progression and both have significant side effects (King et al., 2014; Richeldi et al., 2014).

Inhibition of myofibroblast intracellular (contraction) or extracellular (stiffness and strain) stress is another promising approach to suppress myofibroblast activation or to drive myofibroblasts into controlled suicide (Haak et al., 2019; Hinz et al., 2019; Kuehl and Lagares, 2018; Merkt et al., 2020). ECM-remodeling and crosslinking factors, such as matrix metalloproteases, lysyl oxidases (LOXs), LOX-like enzymes, tissue transglutaminases, advanced glycation end products and lysyl hydroxylases, can be targeted to change ECM mechanics (Afratis et al., 2018; Theocharis et al., 2019; Walraven and Hinz, 2018; Wei et al., 2017; Yamauchi et al., 2018). Mechanical offloading through physical intervention is used to reduce the formation of topical skin scars (Barnes et al., 2018) but is difficult to achieve in mechanically active internal organs, such as heart and lung (Hinz and Suki, 2016). Effectors targeting the myofibroblast contractile machinery bear the risk to exert side-effects on other contractile cells, such as smooth muscle or cardiomyocytes in the heart. Off-target actions are probably the largest obstacle to overcome in the future if anti-myofibroblast and, thus, anti-fibrosis therapies are to be successful.

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Supplementary information

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Cell science at a glance

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