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Mechanotransduction in stem cells

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ABSTRACT

Nowadays, it is an established concept that the capability to reach a specialised cell identity via differentiation, as in the case of multi- and pluripotent stem cells, is not only determined by biochemical factors, but that also physical aspects of the microenvironment play a key role; interpreted by the cell through a force-based signalling pathway called mechanotransduction. However, the intricate ties between the elements involved in mechanotransduction, such as the extracellular matrix, the glycocalyx, the cell membrane, Integrin adhesion complexes, Cadherin-mediated cell/cell adhesion, the cytoskeleton, and the nucleus, are still far from being understood in detail. Here we report what is currently known about these elements in general and their specific interplay in the context of multi- and pluripotent stem cells. We furthermore merge this overview to a more comprehensive picture, that aims to cover the whole mechanotransductive pathway from the cell/microenvironment interface to the regulation of the chromatin structure in the nucleus. Ultimately, with this review we outline the current picture of the interplay between mechanotransductive cues and epigenetic regulation and how these processes might contribute to stem cell dynamics and fate.

1. Introduction

One of the basic principles enabling multicellular life is the differentiation of stem cells (SCs), into specialised cells with a defined identity, shape, and function (McBeath et al., 2004) (Tewary et al., 2018). SCs are defined as cells that are clonogenic, which defines their capability of both, self-renewing and retaining multilineage differentiation potency. In vertebrates, the Zygote represents the earliest stem cell (totipotent) in ontogeny, from which originate the pluripotent stem cells (PSCs) of the inner cell mass of the blastocyst (Dean et al., 2003; Smith, 2017). Soon thereafter, PSCs continue to expand during embryo development becoming progressively committed in their cell fate and acquiring a more restricted potency (Dean et al., 2003; Osorno et al., 2012; Smith, 2017). Even at the end of embryogenesis, when the organism is fully formed, and its organs are composed of functionally mature cells, some SCs are still present. These cells, which possess a more limited differentiating potential (multipotent), in turn, give rise to a progeny of precursor cells and, finally, to functionally mature cells. Some examples are represented by the hematopoietic SCs in the bone marrow, the intestinal SCs in the gut's crypts, the epithelial SCs in the epidermis or the mesenchymal stem cells (MSCs) which play an important role in the maintenance and regeneration of bones and cartilage (Barker, 2014; Evans et al., 2013; Guan et al., 2012; Tewary et al., 2018; Watt, 2002; Weissman, 2000).

The inherent high level of plasticity characterising SCs is at the base of their capability to respond precisely to appropriate stimuli with differentiative behaviour. Understanding this essential principle of multicellular life; *i.e.,* the regulation of cell identity and fate, is one of the most important fields of research in modern cell biology, primarily due to its impact on the development of new therapeutic approaches in regenerative medicine. Such knowledge can directly benefit stem cell bioengineering by leveraging the SC plasticity and control over SC behaviour for the application in tissue engineering, patient-specific disease modelling and cell therapies (Tewary et al., 2018).

In recent years, it emerged that the stem cell fate, apart from biochemical regulation, is also strongly influenced by mechanobiological aspects of the extracellular matrix (ECM) and the cells themselves, implemented by a pathway called mechanotransduction. Even though we still lack a thorough and systematic data collection which precisely characterises the physical microenvironment of SCs niches *in vivo*, we now understand that the cells can perceive biophysical cues of their microenvironment by mechanosensing (such as stiffness/(visco)

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elasticity and the spatial organisation and dimensionality of adhesion sites in terms of topography and geometry). The cells then convert the information by mechanotransductive processes. Mechanotransduction essentially is a force-based bidirectional dialogue between the ECM and cells. Mechanotransductive structures and signalling physically and functionally connect the microenvironment, the cell surface, the cytoskeleton, and the nucleus (Fig. 1) in a way that governs also stem cell behaviour and development (Kim et al., 2018; Lenzini et al., 2019; Walma and Yamada, 2020; Watt and Huck, 2013). Three seminal works that were performed with the multipotent human MSCs (Dalby et al., 2007; Engler et al., 2006; McBeath et al., 2004) put these mechanobiological facets into the spotlight and gave a major boost to the recognition of the importance of mechanotransduction in the stem cell field. These studies highlighted that the cell shape and mechanobiology (regulated, in particular, by RhoGTPase signalling and actomyosin-generated tension), as well as the geometry, stiffness/elasticity and nanotopography of the cellular microenvironment, play a crucial role in stem cell lineage commitment (Dalby et al., 2007; Engler et al., 2006; McBeath et al., 2004).

In general, micro- and nanometric biophysical features of the ECM are capable of modulating principal adhesive structures in the cell/microenvironment interface, such as Integrin adhesion complexes (IAC). Consequentially, a remodelling of the cytoskeletal configuration takes place (Changede and Sheetz, 2017; Chighizola et al., 2019; Kechagia et al., 2019; Schulte, 2023) (Fig. 2) which affects the mechanical properties of the cell, the nuclear shape (via the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex) and the activity of mechanosensitive transcription factors (such YAP/TAZ and MRTF). Together, these actions trigger epigenetic regulation and chromatin organisation, which in turn modulate gene expression patterns, and ultimately the cellular identity (Uhler and Shivashankar, 2017a) (Fig. 1). Human embryonic stem cells (hESCs), *e.g.*, undergo morphological changes during the exit from pluripotency and commitment to specific cell lineages (Chalut and Paluch, 2016; McBeath et al., 2004), accompanied by intricate remodelling events affecting the mechanotransductive machinery and the chromatin which controls gene expression programs in time and space (Crowder et al., 2016; Hamouda et al., 2020; Maurer and Lammerding, 2019).

However, despite the growing consensus for the relevance of such mechanobiological aspects controlling multi- and pluripotent stem cell state and (epigenetic) gene regulation, this phenomenon still represents a developing frontier of life sciences. Many particulars of how mechanotransduction-mediated microenvironment/genome crosstalk determines SCs plasticity remain elusive, because of the intricacy of the involved structures and processes.

In this review, we provide an updated overview of this rapidly evolving field. In particular, we will highlight what is known about the mechanotransductive machinery of multi- and pluripotent SCs and assess how mechanotransduction might be linked to epigenetic regulation during cell fate transition from pluripotency to (early) differentiation.

2. Stem cell microenvironment

In their *in vivo* environment, cells (except for floating blood cells) reside in or on a three-dimensional (3D) network of macromolecules, the extracellular matrix (ECM). This structure is built up of multiple components, *i.e.* proteins and polysaccharides. These constituents strongly interact and crosslink, thereby generating an intricate meshwork with fibrils, pores and asperities that define the stiffness/(visco)elasticity, nanotopography and geometry of the ECM. Cell surface adhesion receptors, such as integrins, can detect ligands that are present in the ECM. However, apart from being an anchoring point for the cells through ligands, the ECM provides biochemical and biophysical signals that guide cell behaviour and fate (Gasiorowski et al., 2013; Walma and Yamada, 2020; Watt and Huck, 2013; Young et al., 2016) (these processes are discussed in **chapter 3, respectively 4**).

During early embryonic development and in stem cell niches, specific ECM structures, called basement membranes, are crucial. Basement membranes are thin, lattice-like, specialised ECMs which, as principal

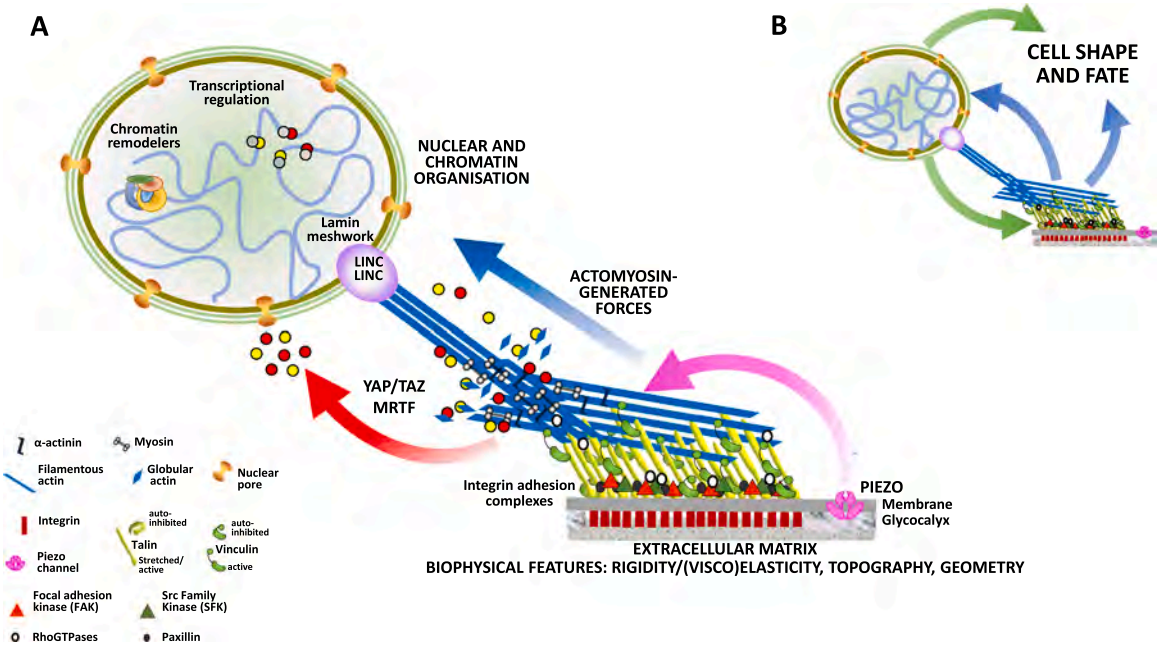

Fig. 1). Integrin-mediated mechanotransductive structures and signalling sequence. (A) The scheme outlines the principal components and processes of the Integrin-mediated mechanotransductive pathway and the involved cellular structures that will be detailed more specifically throughout this review. **(B)** The force-based mechanotransductive dialogue is bidirectional, i.e., from the cell/microenvironment interface to the nucleus as visualised in (A), but then the nuclear events also feedback on the composition of the structures that are involved in mechanotransduction, such as the extracellular matrix, the glycocalyx, the Integrin adhesion complexes and the cytoskeleton. Together, these processes define the cell shape and fate which will be a particular focus of this review. The figure contains adapted elements from Schulte (Schulte, 2023) with permission, Copyright (2023) de Gruyter.

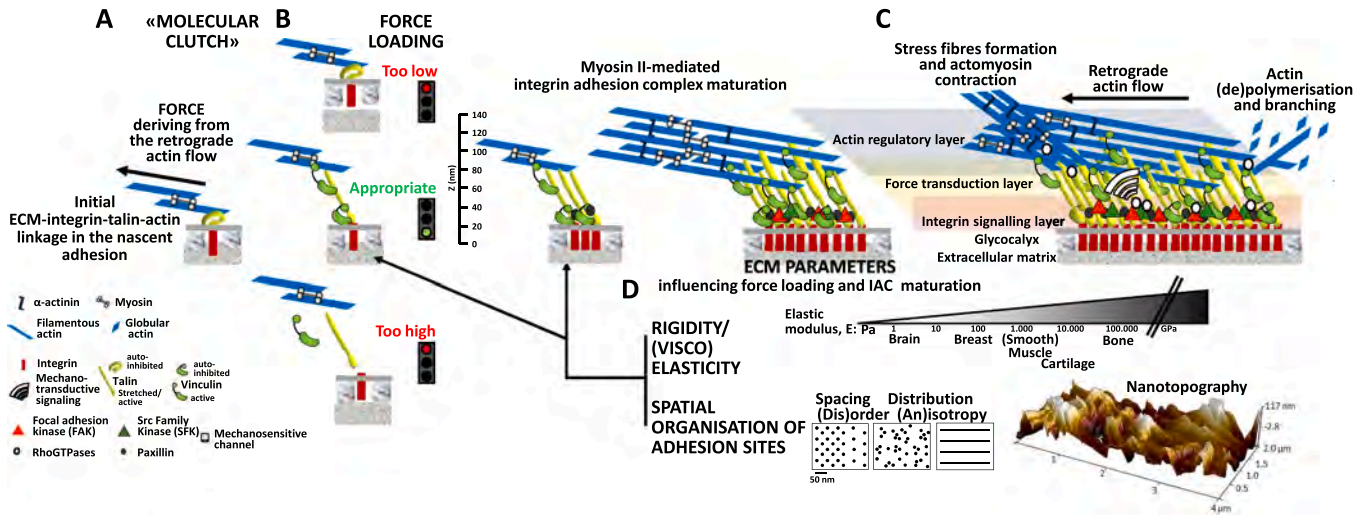

Fig. 2). Integrin-mediated mechanosensing and maturation of Integrin adhesion complexes in dependency of biophysical extracellular matrix parameters. (A) The scheme shows the initial ECM-Integrin-Talin-Actin linkage in nascent adhesions, the so-called "molecular clutch", which connect the cellular microenvironment to the forces deriving from the retrograde Actin flow. (A,B) Whether the nascent adhesions reinforce and mature into (C) Integrin adhesion complexes (IAC) with a particular layered nanoarchitecture depends on the force loading within the molecular clutch (D) which is determined by certain indicated biophysical parameters of the ECM, such as the rigidity (underneath the scale bar some examples are given where on this scale certain tissue ECMs fall) and the spatial organisation and dimensionality of adhesion sites defined by the nanotopography (the nanotopography is represented by an AFM recording of a decellularised bladder ECM, by courtesy of Prof. Alessandro Podestà, University of Milan). Details on the various steps can be found in the **chapter 3**. The figure and caption (with minor adaptions) are from Schulte (Schulte, 2023) with permission, Copyright (2023) de Gruyter.

constituents, contain Laminins, type IV Collagen, Perlecan, Nidogens, Agrins, and other macromolecules (Gattazzo et al., 2014; O'Connor et al., 2020; Walma and Yamada, 2020; Watt and Huck, 2013; Yurchenco et al., 2004). Laminin-111 (Miner et al., 2004; Smyth et al., 1999), β1-Integrin (Molè et al., 2021; Stephens et al., 1995), and downstream integrin signalling factors (Li et al., 2005; Sakai et al., 2003) have thus been demonstrated to be critical for embryonic peri-implantation and survival.

Congruently, when basement membrane components were presented as substrates in *in vitro* experimentation, they decisively impacted ESC behaviour and fate; albeit the individual effects of the components can be rather versatile. The first fibroblast feeder-free cultures of undifferentiated hESCs were achieved on matrigel® (which mainly contains Laminin-111), or Laminin substrates, whereas Fibronectin did not have the same effect (Xu et al., 2001). Recombinant Laminin-111, −332, and −511 substrates support hESC expansion, maintaining their pluripotency (Miyazaki et al., 2008). Laminin-511 and Nidogen-1 were identified as essential ECM substrates for hESC assembly to embryoid bodies that can differentiate into endodermal, ectodermal, and mesodermal derivatives (Evseenko et al., 2009). In mESCs, Laminin and Fibronectin were shown to activate β1 integrin-dependent signalling and differentiation with corresponding changes in morphology and gene expression, while Collagen type I and IV instead promoted the undifferentiated mESC state. This effect was found to rely on the fact that mESCs did not express Collagen-binding Integrins, as overexpression of collagen-binding integrin subunits in these cells led to the induction of differentiation on Collagen type I (Hayashi et al., 2007).

3. General mechanisms of integrin-mediated mechanosensing and mechanotransductive processes

In recent years, it has become increasingly evident that integrin-mediated cell/microenvironment interaction and subsequent mechanotransductive processes are involved in regulating multi- and pluripotent stem cell behaviour and fate. Before referring specifically to what is known in this context with respect to multi- and pluripotent SCs, we feel that it is important to introduce some general principles of the integrin-mediated mechanosensing and mechanotransduction for the reader.

3.1. Integrin adhesion complexes

Integrin adhesion complexes (IAC) are key mediators of cellular interaction with the microenvironment. They are highly sophisticated cellular adhesive structures and signalling hubs that connect the ECM with the actin cytoskeleton in a bidirectional force-based dialogue. Their spatiotemporal dynamics and composition (potentially >200 different proteins) are intricate and directly modulate mechanotransductive signalling and cytoskeletal configuration. The nature of IACs is determined by the extent to which nascent adhesions mature into bigger structures (such as focal complexes and focal adhesions), defined by the forces that develop within IACs (see **3.1.1**) which, in turn, depends on the configuration of local biophysical and structural cues in the microenvironment (see **3.1.2**) (Kechagia et al., 2019; Schulte, 2023).

3.1.1. Regulation of initial IAC formation by force loading within nascent adhesions

The eponymous Integrins of the IACs are heterodimeric transmembrane surface adhesion receptors (composed of one α- and one β-subunit) whose extracellular domains can bind ECM ligands after integrin activation, *i.e.* a change of the Integrin conformation from inactive, bent and closed to activated and extended with separated cytoplasmic tails leading to a high-affinity state for the ligand. This Integrin activation occurs either by the Integrin/ligand binding itself (outside-in signalling) and subsequent recruitment of Talin and Kindlin to the cytoplasmic tail of the β-subunit, or from inside induced by Talin and Kindlin binding (inside-out signalling) (Horton et al., 2016, 2015; Kechagia et al., 2019; Schulte, 2023).

The initial phase of the formation of Integrin nanoclusters (with a dimension of ~100 nm, containing 20–50 integrins) is independent of Myosin II (Changede et al., 2015; Changede and Sheetz, 2017), but further maturation into micrometric IAC structures (such as focal adhesions) by the loose aggregation of numerous Integrin nanoclusters requires Acto-Myosin contraction (Changede and Sheetz, 2017).

Inside the nascent adhesions so-called molecular clutches form (a concept first introduced by Chan and Odde in 2008 (Chan and Odde,

2008)). These molecular clutches are the initial linkage between the ligands in the ECM to the Actin cytoskeleton through the activated Integrins and Talin (Case and Waterman, 2015; Changede and Sheetz, 2017; Kechagia et al., 2019; Schulte, 2023) (Fig. 2**A**). However, these early structures are rather instable and disassemble quickly (within a few seconds) if they are not reinforced. The reinforcement requires exposure of the molecular clutches to forces that derive from the retrograde actin flow, which is generated by the combination of polymerisation and contraction (via Myosin II) of Actin filaments (f-Actin). The binding of Talin to f-Actin allows the transmission of actomyosin-generated retrograde flow forces through the molecular clutch, leading to a force loading (Kechagia et al., 2019; Oria et al., 2017; Schulte, 2023; Sun et al., 2019). Surpassing specific force loading thresholds can then enable reinforcing events, such as keeping the Integrin in the activated, extended conformation and catch bond formation between Integrin and ligand which necessitates a few piconewton (pN) (Li and Springer, 2017; Strohmeyer et al., 2017), respectively tens of pN (Chen et al., 2017; Kong et al., 2009), of force. Moreover, appropriate force (>5 – 25 pN) leads to the stretching and extension of the Talin rod which sequentially uncovers cryptic Vinculin binding sites (Fig. 2**B**). Vinculin is then recruited to these binding sites (first near the Integrins, then near f-Actin) and creates additional linkage to the f-Actin, which progressively stabilises the molecular clutch and increases its lifetime (Chighizola et al., 2019; Kechagia et al., 2019; Schulte, 2023; Sun et al., 2019).

The stabilisation of nascent adhesions by Vinculin, in turn, enables the recruitment of additional adaptor, signalling, and crosslinking components (e.g., focal adhesion kinase (FAK), Src, RhoGTPases, LIM domain-containing proteins (such as Zyxin and LIM-domain kinases, LIMK), α-Actinin, and Erk) to the structures and the IACs mature. The IACs are thereby transforming into signalling hubs (Carisey et al., 2013; Case et al., 2015; Ciobanasu et al., 2014; del Rio et al., 2009; Goult et al., 2021; Kechagia et al., 2019; Schulte, 2023; Sun et al., 2019; Yao et al., 2016, 2014) (further details in **3.2**). In addition, during the course of IAC maturation, f-Actin bundle and crosslink, in a Rho A-dependent manner, into stress fibres (Chrzanowska-Wodnicka and Burridge, 1996; Endlich et al., 2007) that are embedded in the contractile cortical actin network (Vignaud et al., 2021). The mature IACs become multiprotein assemblies with a particular nanoscale architecture composed of Integrin signalling, force transduction and Actin regulatory layers (Case et al., 2015; Kanchanawong et al., 2010) (Fig. 2**C**).

3.1.2. Impact of microenvironmental biophysical cues on force loading in molecular clutches

The biophysical and structural characteristics of the cellular microenvironment (in particular, stiffness/(visco)elasticity and the spatial organisation of adhesion sites, *i.e.* nanotopographical features) define whether the force loading within the molecular clutches will be appropriate for the mentioned reinforcing and maturation events to happen (Chighizola et al., 2019; Kechagia et al., 2019; Oria et al., 2017; Schulte, 2023) (Fig. 2**D**).

In general, a rigid substrate (from few tens of kPa upwards, mimicking the mechanical properties of muscle, cartilage, or bone ECM, see also Fig. 2 **D**) enables a fast and strong force loading inside the molecular clutches favouring the formation of focal adhesions, whereas the force loading on soft substrates (below a few kPa, mimicking the mechanical properties of brain ECM) is weaker and slower, often leading to a disengagement of the molecular clutches before the reinforcing actions can take place. In this latter case, mature IAC structures thus cannot form or remain of smaller size (focal complexes or, in the neuronal context, point contacts) (Elosegui-Artola et al., 2016; Oria et al., 2017). Analogous effects have been shown for substrates with high, respectively low viscosity (Bennett et al., 2018).

However, the spatial organisation and dimensionality of adhesion sites is another key factor in the mechanotransductive setting. In the case of rigid substrates, the ligand spacing and density has to be suitable to permit Integrin clustering and IAC maturation. A critical ligand spacing threshold (>60–70 nm) has been determined above which focal adhesion formation is abolished because the single molecular clutches are exposed to too strong force loading which leads to their break-up as the force cannot be distributed sufficiently among the integrins (Arnold et al., 2004; Cavalcanti-Adam et al., 2006; Liu et al., 2014; Oria et al., 2017). This situation changes again, when you introduce disorder into the ligand spacing. Even if the average general average ligand spacing is above the critical threshold, local occurrence of ligand spacing beneath the threshold in the case of appropriate disordered ligand distributions can provide the necessary support for Integrin clustering and IAC maturation (Huang et al., 2009) (Fig. 2**D**). Indeed, the presence of a certain number of Integrin binding sites (≥4) within the ligand spacing threshold enables the establishment of a minimal adhesion units that promotes focal adhesion formation (Schvartzman et al., 2011). It has furthermore been shown that Integrin nanoclusters can bridge tens of nanometers of distance between thin, fibrous adhesion sites (that alone do not support IAC formation) by recruitment of unliganded Integrins (Changede et al., 2019). On soft substrates, instead, there is the counter-intuitive effect that higher ligand spacing can increase the force loading per single molecular clutch to a point that permits to surpass the force thresholds necessary for molecular clutch reinforcement, enabling IAC maturation (Kechagia et al., 2019; Oria et al., 2017; Schulte, 2023).

Altogether, this importance of the precise spatial organisation and dimensionality of adhesion sites explains the complexity and versatility of the impact of different nanotopographies (Fig. 2**D**) on IAC maturation and cell behaviour. Even relatively small local variations in nanotopographical features can change mechanotransductive processes and signalling decisively. In addition, recently it has been shown that it is crucial how the cell actually perceives and reads out the 3D cues of nanotopographical substrates. This perception can be influenced by the cortical stiffness of the actin cytoskeleton, the availability of activated integrins and the configuration of the glycocalyx. The same nanotopography might provide completely different cues to cells depending on these latter parameters (Chighizola et al., 2022, 2020; Park et al., 2016). Moreover, especially in 3D environments, also substrate properties that allow a remodelling of the spatial organisation of adhesion sites upon cell traction, such as softness, degradability, low viscosity, can affect the final cellular mechanotransductive perception of the substrate (Saraswathibhatla et al., 2023) (further details on the stem cell context can be found in **4.1**).

The combined effect that these microenvironmental biophysical and three-dimensional cues have on the molecular clutch force loading represents thus the decisive factor and eventual universal mechanism of mechanosensing (Kechagia et al., 2019; Oria et al., 2017; Schulte, 2023) (Figs. 2**B and** 2**D**). In this context, there is a fascinating, new MeshCODE theory (Barnett and Goult, 2022; Goult, 2021; Goult et al., 2021) that discusses the possibility of encoding information from the cell microenvironment in the Talin configuration, induced by force-dependent binary switches in Talin domains that occur during Talin extension and disclose cryptic binding sites for IAC proteins (in particular Vinculin). The MeshCODE theory has been discussed in a neuronal context, but it is intriguing to think of such a possibility also in the stem cell framework.

3.2. Mechanotransductive signalling

During IAC maturation, numerous signalling components are recruited to these structures that can activate various Integrin downstream signalling pathways and events (Fig. 1).

FAK plays a key role therein. After its recruitment to the developing IACs, it autophosporylates at Tyr397 which induces src family kinase (SFK) binding, leading to further phosphorylation events that activate the FAK/SFK complex. This activated complex then serves as a core for the recruitment of further proteins and the initiation of intricate signalling cascades that eventually often result in the in/activation of

proteins or multi-protein complexes in control of the cytoskeletal organisation, such as RhoGTPases (*e.g.*, Rac1, Cdc42, RhoA) and their regulators (*e.g.*, RhoGEFs, -GAPs, -GDIs), ROCK, LIMK, as well as WAVE, WASp, ADF/Cofilin, and Arp2/3. Also, other major pathways can be affected by the Integrin downstream signalling through crosstalk, such as MAPK/Erk, PI3K-ILK/Akt, or Jnk, which influences gene expression patterns and cell behaviour (Chighizola et al., 2019; Lawson and Ridley, 2018; Mitra and Schlaepfer, 2006; Schulte, 2023).

Further details on these integrin downstream signalling cascades can be found in Huveneers and Danen (Huveneers and Danen, 2009), Cooper and Giancotti (Cooper and Giancotti, 2019), and Schulte (Schulte, 2023).

Furthermore, there are mechanosensitive transcription factors that react to IAC-mediated remodelling of the Actin cytoskeleton (Fig. 1). MRTF (myocardin-related transcription factor), *e.g.*, binds globular (g)-Actin and is thus sensitive to the level of free, cytoplasmic g-Actin. At a high pool of free g-Actin MRTF remains predominantly in the cytoplasm, but if the g-Actin pool decreases (when f-Actin polymerises), MRTF enters the nucleus, binds SRF, activating thereby MRTF/SRF-dependent gene expression. In a seminal work, (Dupont et al., 2011) have shown that YAP/TAZ activity responds to substrate stiffness and cell shape in hMSCs and is necessary for their differentiation. On soft substrates YAP was located in the cytoplasm, whereas on rigid substrates it had a mostly nuclear localisation. IAC maturation, abovementioned FAK/SFK-dependent integrin signalling, and stress fibre formation lead to the inactivation of Lats 1/2 kinases and consequentially to a decrease of YAP (yes-associated protein) phosphorylation level and a release of YAP from 14 to 3–3 protein. The released cytosolic YAP translocates to the nucleus and induces, together with co-transcription factors (such as TEAD1, Runx, Smad, REST), modulations of the gene expression (often also target genes related to mechanotransduction) (Wada et al., 2011). In addition, the nuclear import of these mechanosensitive transcription factors also depends on IAC-mediated force transmission. The force transmission (*e.g.*, on rigid substrates) causes a flattening of the nucleus and a stretch of nuclear pores, by increasing nuclear membrane curvature, and the exposure of nuclear pores to the cytosol (versus nucleus). It reduces the mechanical resistance of the pore's disorganised flexible meshwork which otherwise impedes free diffusion of proteins into the nucleus. Altogether, these effects then promote nuclear import versus export; especially for mechanically instable proteins and passively diffusing small molecular weight proteins (Andreu et al., 2022; Elosegui-Artola et al., 2017; Infante et al., 2019).

The Actin cytoskeleton is furthermore connected to the nucleus via LINC. It can thereby influence the configuration of the nuclear envelope, chromatin configuration and gene expression patterns (Fig. 1 and **chapter 5**).

3.3. Further elements with relevance for mechanotransduction in the cell/microenvironment interface

3.3.1. Mechanosensitive channels

Mechanosensitive channels, such as Piezo or TRP channels, represent another important element of the mechanotransductive sequence that is highly susceptible to changes in (extra)cellular forces because they can alter the channels' opening via a mechanogating mechanism. These channels have a strong bidirectional crosstalk with the ECM and the IAC and react to variations of cytoskeletal contractility and force loading-related events, because the channels' activation status can be affected by modulations of membrane tension, membrane bending/curvature, osmolarity and shear stress. The activation of these channels, in turn, can also feedback on IAC by influencing their turnover rate (Cheng et al., 2023; Jiao et al., 2017; Kanoldt et al., 2019; Nourse and Pathak, 2017; Zhao et al., 2018). Piezo1, *e.g.*, can often be found enriched in focal adhesions in an actomyosin contraction-dependent manner. A force-dependent opening of these channels then leads to a Ca^{2+}-influx that modifies the activity of focal adhesion proteins that are influenced by the Ca^{2+} concentration, such as calpain, and consequentially alter focal adhesion dynamics and turnover (Yao et al., 2022).

In human neural stem cells, Piezo1 activity influences whether the cells commit to neuronal or astrocyte lineage (Pathak et al., 2014). Various functional effects of the activation of Piezo channels by mechanical stimuli have been found in MSCs, such as changes in proliferation and differentiation (*e.g.*, favouring osteogenesis versus adipogenesis, and migration) (Huang et al., 2023). However, in mESCs, despite being able to modulate their rate of proliferation, Piezo1 activation does not affect their pluripotency, early differentiation, and substrate stiffness response (Del Mármol et al., 2018). In general, there are still a lot of open questions regarding the function of Piezo channels in pluripotent SCs.

3.3.2. Glycocalyx

Increasing evidence demonstrates a strong involvement of the glycocalyx, a peri-cellular sugar coat attached to proteoglycans, glycoproteins and glycolipids, in the mechanosensing of the ECM, Integrin clustering, and mechanotransductive processes (Chighizola et al., 2022; Paszek et al., 2014).

Initially, this structure represents a steric barrier for adhesion, as the sugar chains are often much longer than the extracellular integrin domains. Once a first integrin/ligand foothold has been established, however, a "kinetic trap" is formed. The compressed glycocalyx close to the foothold favours the clustering of activated integrins in this zone, by keeping the Integrins in their extended form (due to the mechanical loading of the membrane caused by the glycocalyx compression) and by impeding lateral diffusion of Integrins outside the Integrin/ligand contact area (Paszek et al., 2014). The glycocalyx configuration furthermore impacts on membrane bending/shape (Lu et al., 2022; Shurer et al., 2019) and affects how the cell interacts with nanotopographical features (Chighizola et al., 2022; Lu et al., 2022) and molecular clutch force loading (Chighizola et al., 2022).

It has been shown that the glycosylation and glycome profile of ESCs change during differentiation (Satomaa et al., 2009; Venable et al., 2005; Wearne et al., 2008) and that glycosaminoglycans (a major glycocalyx component) contribute to the regulation of stem cell differentiation (Holley et al., 2014; Smith et al., 2011). However, very little is yet known about whether or how this relates to mechanotransductive processes in stem cells.

3.3.3. Cadherin-mediated cell/cell interaction

Cadherin-mediated cell/cell interaction by adherens junctions plays a vital role in mechanotransduction (Weber et al., 2011). Indeed, these two essential elements of mechanotransduction share several components (*e.g.*, Vinculin, Rac1, Zyxin) and are connected through the Actin cytoskeleton. Moreover, Cadherins link the Actin cytoskeleton of different cells. Through the Cadherin/F-Actin interface and constituents, such as α-Catenin and Vinculin, adherens junctions are also mechanosensitive. The adhesive crosstalk between Integrins and Cadherins defines the mechanical landscape of cell clusters and tissues, regulating thereby multicellular processes, such as collective cell migration, developmental morphogenesis and tissue patterning. The balance between Integrin-mediated cell/ECM interaction and cell/cell contacts dependent on E-Cadherin represents an essential factor in epithelial-mesenchymal transition events that have a significant relevance during embryogenesis (see also **4.4**) (Barcelona-Estaje et al., 2021; Mui et al., 2016; Weber et al., 2011).

The interested reader can find further details on how Cadherin-mediated structures, and their crosstalk with Integrin-mediated complexes, contribute to mechanotransduction in other reviews (Barcelona-Estaje et al., 2021; Mui et al., 2016; Weber et al., 2011).

4. Mechanobiology and mechanotransductive processes in multi- and pluripotent stem cells

It is long-known that Integrins and their signalling are crucial in SC biology (Hayashi et al., 2007). Despite many details remain still to be resolved, recent studies provided a clearer picture of the (extra)cellular structures and processes involved in mechanotransduction in multi- and pluripotent SCs, such as biophysical microenvironmental cues, IACs, the actin cytoskeletal dynamics, cell/cell adhesions mediated by E-Cadherin, membrane tension, and mechanosensitive transcription factors.

4.1. Biophysical cues of the microenvironment modulating multi- and pluripotent stem cell behaviour

Apart from the biochemical composition, also biophysical cues of the microenvironment (such as mechanical properties, (nano)topography, and geometry) have the capacity to modulate stem cell fate (Abagnale et al., 2015; Crowder et al., 2016; Dalby et al., 2014; Donnelly et al., 2018; Gasiorowski et al., 2013; Kim et al., 2018; Kumari et al., 2018; Lenzini et al., 2019; Li et al., 2018; Loye et al., 2018; Lü et al., 2014; Vining and Mooney, 2017).

4.1.1. Stiffness/(Visco)elasticity

A seminal work using naïve hMSCs demonstrated that presenting substrates mimicking certain *in vivo* tissue matrix elasticities (such as soft brain or rigid bone) can direct the cells towards a tissue-specific lineage commitment (Engler et al., 2006). The study opened up the research in the stem cell field to the concept that biomechanical aspects of the microenvironment also play an essential role during SCs differentiation. Brain-like stiffness induced neuronal lineage specification in hMSCs, muscle-like stiffness committed the cells towards myogenic differentiation, and bone-like stiffness promoted an osteogenic phenotype and gene expression (Engler et al., 2006). These mechanosensitive differentiation events were furthermore found to be dependent on the regulation of Acto-Myosin contraction. The impact of substrate rigidity and appropriate cell traction forces on mMSCs fate was also confirmed for 3D matrices, but here no correlation with the cell morphology was found (Huebsch et al., 2010).

There is furthermore a complex contribution of additional mechanical features, especially in 3D, such as viscoelasticity, degradability, stress relaxation (Chaudhuri et al., 2016; Huebsch et al., 2010; Khetan et al., 2013), and local stress stiffening of the substrates (Das et al., 2016). 3D substrates with intermediate rigidity that allow an Acto-Myosin contraction-dependent reorganisation of ligands, and consequentially an increased ligand and integrin clustering, favoured osteogenic differentiation of MSCs, comparable to stiffer substrates (Chaudhuri et al., 2016; Huebsch et al., 2010; Khetan et al., 2013). If soft 3D substrates are engineered in a way that local stress stiffening is possible, this can enable a switch from adipo- to osteogenesis (Das et al., 2016).

Similar effects of mechanical cues have since been reproduced in different stem cell contexts (*e.g.*, ESCs and iPSCs) and extended to a variety of pluripotency exit and/or differentiation scenarios by using various substrate materials that enable a tuning of the mechanical properties (Cao et al., 2022; Darnell et al., 2018; Evans et al., 2009; Gerardo et al., 2019; Indana et al., 2021; Keung et al., 2012; Macrí--Pellizzeri et al., 2015; Przybyla et al., 2016).

4.1.2. Topography

Another important study showed that topographical substrate cues also represent an important microenvironmental parameter which can control stem cell fate (Dalby et al., 2007). They discovered that specific nanoscale substrate features, *i.e.* nanopits of 120 nm width and 100 nm depth (produced by E-beam lithography), promote hMSC osteogenic differentiation even in the absence of osteogenic medium, when the features are arranged in a disordered manner (with ±20 or 50 nm deviations from the average 300 nm centre-to-centre spacing). Congruent results were found for titanium oxide surface structures with disordered 70–100 nm wide nanotubes (Oh et al., 2009). Certain roughness parameter (R_q = 20 nm) of zirconia cluster-assembled substrates favour osteogenesis in hMSC (compared to flat zirconia substrates) which is accompanied by cytoskeletal rearrangements (Castiglioni et al., 2023). On PDMS nanogratings with a 350 nm width, hMSCs (in the presence of neuronal induction medium) obtained an elongated morphology and an upregulation of neuronal markers (such as MAP2), differently from what was seen in the cells grown on unpatterned substrates (Yim et al., 2007).

Furthermore, an impact of the disordered nanotopographical features on hESCs was demonstrated that was akin to the abovementioned hMSC effects, *i.e.* an induction of osteogenic differentiation (Kingham et al., 2013). Additionally, also many other studies showed that the interaction of ESCs with various nanotopographical substrates clearly affects the ESC pluripotency and differentiation status in a different manner than the interaction with corresponding flat substrates. However, there was a certain diversity in the specific results that were obtained. A similar outcome was observed for hESCs that were cultured on nanofibrous substrates (Smith et al., 2009). In contrast, ESCs grown on substrates built up by silica colloidal spheres with a diameter of 120–600 nm preserved a colony morphology and marker expression (*e.g.*, *Dpp5a*, *Nanog*, *Pou5f1*) similar to undifferentiated ESCs (compared to flat substrates) (Ji et al., 2012). Gold nanoparticle layers with a roughness R_q <392 nm also retained mESC pluripotency, whereas higher roughness >573 nm had the opposite effect inducing pluripotency exit and undirected differentiation (Lyu et al., 2014). Chen *et al.* observed instead an outcome somewhat contradictory to these latter studies. In their experiments, hESCs cultured on vitronectin-coated nanorough (R_q = 70 nm and 150 nm) areas (produced on silica-based glass wafers by reactive ion etching) showed lower Oct3/4 expression, loss of pluripotency and spontaneous differentiation, while on corresponding flat glass wafer areas stemness and Oct3/4 expression were maintained. These differences were accompanied by modulations of focal adhesion dimensions and distribution (Chen et al., 2012).

In a 3D setting, the pore sizes within a topography represent additional structural features with a potential influence on stem cell behaviour. These parameters define, *e.g.*, the distance between two adjacent anchoring points for the cell, but also the substrate curvature (Akhmanova et al., 2015; Reilly and Engler, 2010). If the pores are much larger than the cell, the effect will be similar to planar substrates. In the case of micrometric pores with a size in the same range of the cell, the cell will be able to attach in 3D which might lead to rounded cell morphology and lower tension (Reilly and Engler, 2010). However, this depends also on whether the surface curvature is concave or convex, the latter has, *e.g.*, been shown to induce osteogenic differentiation in hMSCs (Werner et al., 2016). At a certain point, pores of a few microns can also become constrictive for cell migration due to the difficulty to squeeze the nucleus through the pores (see **5.2.**). Interestingly, in hMSCs pores of 3 μm size led to nuclear damage, but also to increased osteogenesis, after constricted migration (at low cell density) (Smith et al., 2019). On the sub-micron pore size level, contrasting results have been reported for hMSCs, with works indicating either porosity and protein tethering as the decisive factor, versus stiffness, that influences differentiation (Trappmann et al., 2012), or rather stiffness as the determinant and little effect on differentiation for porosity (Wen et al., 2014).

In fact, these few selected works cited here highlight already the complexity and variety of the effects of topographical and structural stimuli on multi- and pluripotent stem cells. For further reading, we would like to direct the reader to more specialised reviews which provide also an extensive listing of the manifold observed impacts of stem cell interaction with a variety of nanotopographical biomaterials (Chen et al., 2014; Naqvi and McNamara, 2020; Ribeiro et al., 2024).

In general (as described in more detail in **3.1.2.**), how cells respond to topographical cues depends on the specific dimensionalities of the

topographical features, as well as their configuration in terms of (dis) order and (an)isotropy, and consequentially on their particular impact on mechanotransductive processes and structures, notably force loading in the molecular clutches (Dalby et al., 2014; Schulte, 2023). The underlying intricate (nano)topography-mediated mechanisms are, however, far less studied, and therefore less understood, compared to the substrate stiffness/(visco)elasticity-related effects, especially with respect to stem cells. This holds even more true regarding the impact that these (nano)topographical cues have on mechanotransduction and stem cell fate in 3D, particularly in the interplay with mechanical cues, also because it is really challenging to decouple the effects of the various parameters. However, compared to the 2D setting, it seems that in 3D the mechanotransductive response is determined less by the mechanical or structural substrate properties *per se*, but rather by how they influence the cell's capacity to remodel the substrate, or to be more precise, to spatially reorganise the adhesion sites (Saraswathibhatla et al., 2023; Zonderland and Moroni, 2021; Ribeiro et al., 2024). Overall, especially in 3D, the pivotal determinant appears to be how all these dimensional and biophysical parameters together in concert affect force loading within molecular clutches, channel mechanogating, Integrin clustering, and IAC maturation (Saraswathibhatla et al., 2023; Zonderland and Moroni, 2021).

4.1.3. Geometry

The geometry of the microenvironment has been determined as another crucial factor influencing stem cell fate. A study done on hMSCs has shown that the cells committed to adipocyte lineage if they were constrained to a round and small cell shape through micropatterned restrictive adhesion areas, whereas adhesion areas that allowed the cells to spread induced osteogenesis (McBeath et al., 2004). These effects are regulated by RhoA-mediated Acto-myosin contraction. A subsequent study (Kilian et al., 2010) specified that the influence of the cell shape on the cytoskeletal contractility is indeed the key factor.

Even if the provided adhesion/spreading area is the same, geometrical features that foster Acto-Myosin contraction (*i.e.*, star-shaped with concave edges and sharp points) promoted osteogenic differentiation, while cues that lowered cytoskeletal contractility (flower-like with convex curves) favoured adipocytogenesis (Kilian et al., 2010).

4.2. Integrin adhesion complexes and adhesion signalling of pluripotent stem cells

A study employing super-resolution microscopy observed scattered distribution of IACs in single mESCs (on laminin, gelatin or fibronectin) and a nanoscale organisation that was different from the canonical focal adhesion in stronger adhering, specialised cells (Xia et al., 2019b). The IACs were smaller in mESCs and the nanoarchitecture more compressed and compact, but the size was nevertheless dependent on Myosin II activity, and the IACs contain mature IAC components, such as Zyxin, FAK, and Paxillin (Fig. 3**A,C**).

Recent studies showed that Integrin adhesion-mediated structures in human induced pluripotent stem cells (hiPSC) colonies on Vitronectin-coated substrates are also different from adherent, specialised cells (Närvä et al., 2017; Stubb et al., 2019). However, while in the colony interior of hiPSCs larger IACs, similarly to the mESCs, were less prominent, they found a particular type of large adhesion sites at the colony edges and called them cornerstone focal adhesions. These structures were attached to thick ventral stress fibres, forming a contractile actin fence. Interestingly, Vinculin has a higher position (in the Actin regulatory layer) in mESCs and hiPSCs compared to classical focal adhesions of specialised, adherent cells (mainly force transduction layer) (Närvä et al., 2017; Stubb et al., 2019).

LIM domain-containing proteins are a pivotal fraction of the consensus Integrin adhesome (Horton et al., 2015) and they are employed in a Myosin II-dependent manner during IAC maturation (Schiller et al., 2011). Their expression level is reduced in mPSCs, compared to more adherent and specialised cells (Xia et al., 2019b). Zyxin, *e.g.*, which is a crucial protein for mechanosensing, cytoskeletal

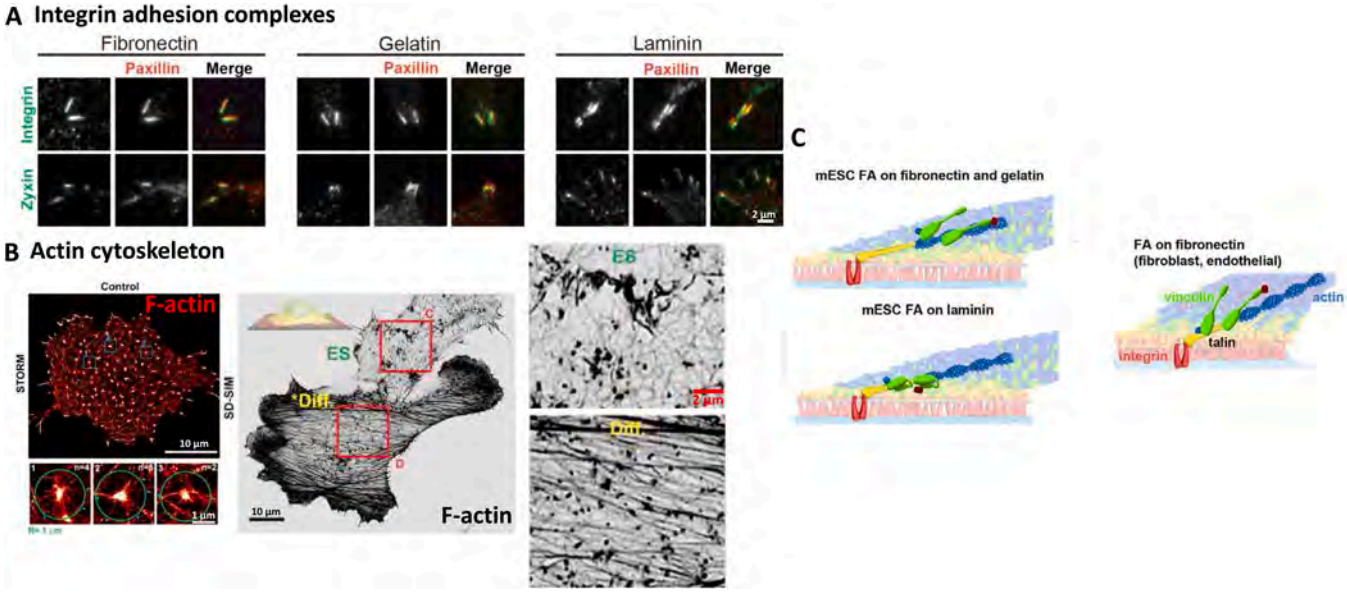

Fig. 3). Characterisation of the integrin-mediated ESC/microenvironment interface and cytoskeleton. (A) Immunfluorescence images of mESC focal adhesions showing the presence of classical focal adhesion markers, such as Integrin and Zyxin, on various substrates, such as Fibronectin, Gelatin, and Laminin. **(B)** Images on the left, STORM images of f-Actin in mESC (top) and magnifications of aster-like structures (bottom). Images in the middle and on the right, ventral z-slice of SD-SIM showing the Actin cytoskeletal organisation in mESCs (ES) and spontaneously differentiated cells (*Diff). **(C)** Schematic diagram of the focal adhesion nanoscale architecture in mESCs (left, on Fibronectin, Gelatin, and Laminin) and adherent, specialised cells (right, fibroblast on Fibronectin), highlighting the core Integrin–Talin–Actin connection (in red, yellow, blue, respectively). Vinculin is shown in green and marked by fluorescent protein probes at either the N- (blue) or C-termini (red). The different configuration of Vinculin in relation to the Integrin–Talin–Actin core is highlighted. Further details can be found in the main text. The figure and caption contains elements that are from two publications, reprinted (with adaptions) with permission from Xia et al., Copyright (2019) American Chemical Society (Xia et al., 2019b) and Cell Press (Xia et al., 2019a).

reinforcement and stress fibre formation (Yoshigi et al., 2005), might be essential for the regulation of the ESC status, as its downregulation results in enhanced expression of pluripotency marker genes (like *Nanog* and *Oct4*) in non-pluripotent cells (Parshina et al., 2020).

Interestingly, mESCs were found to be softer than differentiated cells deriving from them, and they were more susceptible to local apical stress applied through RGD-coated beads. The soft, undifferentiated mESCs responded to the mechanical stress with cell spreading, myosin light chain phospohorylation and increased traction forces at the cell edges which was dependent on Myosin II, Src and Cdc42 activity. The mechanical stress furthermore induced a downregulation of Oct3/4 expression (Chowdhury et al., 2010).

In line with this, weak Integrin-mediated adhesion was shown to promote mESC pluripotency, in contrast to strong adhesion induced by Mn^{2+} activation of Integrins, which triggers differentiation (Taleahmad et al., 2017). Consistently, omics-based studies indicated that mESCs which are kept in their pluripotency ground state (in 2i or R2i cultures) have a lower expression of IAC- and cell adhesion-related genes/proteins, compared to differentiation-inducing culture conditions (Marks et al., 2012; Taleahmad et al., 2017, 2015). In this context, it is interesting that the inhibition of Src (by CGP77675), an important Integrin signalling mediator, fosters the retainment of mESC pluripotency, overriding even substrate rigidity/culture condition-triggered differentiation (on 7.5 kPa substrates, + serum, -LIF) (Shimizu et al., 2012).

Another crucial Integrin downstream signalling intermediary is FAK, but its exact role in governing ESC behaviour remains unclear. The contribution of different Integrin subunits to FAK activity levels seems to be diverse. However, the related reported results are ambiguous and somehow conflicting between mPSCs and hPSCs.

For hESCs, inhibition or knockdown of FAK was shown to either lead to cell detachment and anoikis, or, if cells manage to stay adhered and escape anoikis, to a downregulation of pluripotency markers which was accompanied by changes in morphology and differentiation (Vitillo et al., 2016). Another study (Villa-Diaz et al., 2016) detected no FAK activity in undifferentiated hESCs and hiPSC. In those undifferentiated hESCs, the expression level of α6-Integrin, which forms a Laminin receptor together with β1-Integrin, is high and downregulated during hESC differentiation. α6β1-Integrin seems to be pro-pluripotent by preventing FAK Y397 phosphorylation and the reduction of pluripotency marker expression that would otherwise be induced by β1-Integrin. Indeed, global activation of Integrins (by Mn^{2+}) increased FAK Y397 phosphorylation, promoted the formation of focal adhesions, and reduced Oct4 expression. Congruently, α6-Integrin is upregulated during reprogramming of fibroblasts into hiPSCs and FAK inactivated. The capacity of hESCs to produce and deposit Laminin themselves contributes to this pathway, as Laminin α5 knockdown led to a reduction of α6-Integrin expression and triggered FAK Y397 phosphorylation and differentiation.

In mESCs, the situation seems different, although reports are somewhat contradictory. One study (Hayashi et al., 2007) showed that Fibronectin and Laminin activate FAK phosphorylation in mESCs, whereas inhibiting β1-Integrin by a neutralising antibody increased Nanog and reduced Fgf5 (a marker for primitive ectoderm) expression, maintaining mESC self-renewal. Another study (Toya et al., 2015), found that α6β1-Integrin in mESCs has the opposite effect compared to hESCs, *i.e.* Laminin-111/α6β1-Integrin interaction fosters (in cooperation with the Tetraspanin CD151) FAK phosphorylation and differentiation (at least in a differentiation scenario towards endothelial cell), whereas Laminin-111/α3β1-Integrin interaction antagonised the α6β1-Integrin effects. Contrary to this, RNAi-mediated downregulation of α6- and β1-Integrin in mESC was shown to counteract self-renewal and maintenance of pluripotency by another study (Cattavarayane et al., 2015).

There are furthermore studies (Cattavarayane et al., 2015; Domogatskaya et al., 2008; Rodin et al., 2010) reporting that Laminin-511 substrates promote long-term self-renewal and pluripotency in both, hESCs and mESCs, even in the absence of LIF; whereas Laminin-111, −332, −411 were not able to do that in mESCs (Domogatskaya et al., 2008). In hESCs, the effect of Laminin-511 was dependent on α6β1-Integrin (Rodin et al., 2010), in line with the abovementioned results by others (Villa-Diaz et al., 2016).

Altogether, albeit various downstream details remain to be clarified, it is apparent that a precise and spatiotemporally fine-tuned substrate-dependent Integrin signalling participates in the regulation of PSC survival, pluripotency maintenance or exit, and differentiation.

4.3. ESC actin cytoskeleton and mechanosensitive transcription factors

There are stark distinctions in the nanoscale actin cytoskeleton architecture, particularly the cortex region (see also **4.4**), between naïve pluripotent mESCs and spontaneously differentiating mESCs (Fig. 3**B,C**) (Chalut and Paluch, 2016; Xia et al., 2019a).

Naïve, pluripotent mESCs possess an isotropic, loose, and low-density cortical f-Actin meshwork with transient Arp2/3-dependent aster-like nodes. The meshwork is soft and shows only little higher organisation into stress fibres. In contrast to most other adherent and specialised cells, the mESC Actin cortex structure seems mostly independent of Myosin II, as Myosin II is physically excluded from it (Xia et al., 2019a). In fact, inhibiting Myosin II activity by blebbistatin neither affected the network organisation, nor the mechanics. The meshwork structure and homeostasis is instead controlled by Arp2/3, Formin and capping proteins (Xia et al., 2019a). In contrast, primed and spread mESCs, after spontaneous early differentiation, have a very different Actin cytoskeleton with an abundance of stress fibres (Chalut and Paluch, 2016; Xia et al., 2019a) (Fig. 3**B**). Congruently, a stiffening of mESCs during differentiation has been observed by AFM measurements (Bongiorno et al., 2018).

The Arp2/3 complex is critical in mESC for the realisation of morphological changes and actin architecture reorganisation during early differentiation, as well as regulating related gene expression (in particular, TBX3 target genes). The actions of the Arp2/3 complex are linked to MRTF and FHL2 (LIM domain-containing protein), both SRF co-transcriptional activators (see **3.2.**), which shuttle from their typical cytoplasmic localisation to the nuclear compartment (Aloisio and Barber, 2022). Congruently, the activity of MKL1/MRTF-A, which controls many Actin cytoskeletal genes, diminishes during mouse fibroblast reprogramming to iPSCs. Constitutively active MKL1/MRTF-A, instead, blocks pluripotency activation in a LINC-dependent manner (Hu et al., 2019). In line with this, the serine/threonine kinase LIM domain-containing LIMK2, which is essential for cytoskeletal remodelling by regulating ADF/Cofilin activity, is among the major barriers for somatic reprogramming of fibroblasts to hiPSCs (Sakurai et al., 2014).

The exact role of YAP in PSC maintenance/differentiation is still controversial and there are contrasting reports regarding the function of YAP/TAZ in early differentiation between mESCs and hESCs. Overexpression of YAP in hESCs and hiPSCs favours the naïve pluripotency state (Qin et al., 2016), whereas in mESCs early differentiation and lineage-specific gene expression are promoted (while YAP depletion causes maintenance of the undifferentiated state) (Chung et al., 2016). In hPSCs, it has been found that YAP-TEAD1 regulates expression of cytoskeleton-related genes, cytoskeletal dynamics and intracellular tension in response to substrate mechanics and directs mesoderm specification (Pagliari et al., 2021).

How the IAC-mediated force-dependent changes in nuclear shape and nuclear pore permeability for mechanosensitive transcription factors, such as MRTF and YAP (Andreu et al., 2022; Elosegui-Artola et al., 2017; Infante et al., 2019) (more details in **3.2.**), are involved in the regulation of stem cell fate is an important open question and requires investigation.

4.4. ESC membrane tension, actin cortex, cell/cell interaction mediated by E-Cadherin and epithelial-mesenchymal transition

The precise regulation of the cell membrane attachment to the Actin cortex and the consequential cortex mechanical properties are essential for many cell biological processes involving cell deformation and changes of the cell shape, such as cell migration and differentiation (Chugh and Paluch, 2018; Gauthier et al., 2012).

Two recent studies highlight the importance of membrane tension and modulation of membrane/cortex attachment in governing ESC fate transition (Bergert et al., 2021; De Belly et al., 2021). The observation of blebbing before the cell spreading, which accompanies mESC early differentiation, indicated weak membrane/cortex attachment and a decrease in membrane tension during this transition. This was confirmed by optical tweezer and force spectroscopy experiments. The membrane tension depends on β-Catenin/RhoA activity-mediated Ezrin-Radixin-Moesin (ERM) protein phosphorylation. In fact, expression of constitutively active Ezrin, which is one of the principal ERM mediators of membrane/cortex attachment, maintained a high cortex attachment and membrane tension, keeping the mESCs in the pluripotent state, even under culture conditions that induce differentiation. β-Catenin knockout instead decreased membrane tension and ERM protein phosphorylation in mESCs. RNA_{seq} data revealed furthermore an upregulation of pluripotency markers in the cells with constitutively active Ezrin, but also strong differential expression of genes belonging to the ECM-receptor interaction signalling pathway (De Belly et al., 2021). However, the effect was not limited to Ezrin *per se*, as also an engineered, purely mechanical membrane/Actin cortex linker had a comparable impact.

The same study showed furthermore that decreased membrane tension facilitates endocytosis. Rab5 overexpression-induced enhancement of endocytosis was able to even overcome the differentiation-blocking influence of the constitutively active Ezrin (De Belly et al., 2021).

It is long-known that dissociated hESCs undergo rapid apoptosis (Watanabe et al., 2007), due to a loss of E-Cadherin-mediated intercellular adhesion which triggers RhoA-induced Acto-Myosin hyperactivation. In fact, ROCK inhibition by Y27632 in dissociated hESCs mitigates this apoptosis and promotes their survival (Ohgushi et al., 2010; Watanabe et al., 2007). The pluripotency exit and early differentiation of hESCs is associated with epithelial-mesenchymal transition (EMT) events and a switch from E-Cadherin to N-Cadherin expression. Cell/cell contacts mediated by E-Cadherins, typical for the colonies of undifferentiated hESCs, diminish and the differentiating hESCs remodel their cortical Actin cytoskeleton and obtain a migratory, mesenchymal phenotype (Eastham et al., 2007; Soncin and Ward, 2011; Ullmann et al., 2007). Loss of Clathrin-mediated endocytosis in mESCs leads to reduced E-Cadherin levels which increases cell stiffness and stress fibre formation, accompanied by pluripotency exit and expression of differentiation marker (Mote et al., 2020; Narayana et al., 2019).

Interestingly, adhesive coupling between cells and cortical cellular tension are crucial regulators of mammalian blastocyst and early embryo development (Alvarez and Smutny, 2022; Firmin and Maître, 2021; Nelson, 2022). In 8-cell stage mouse embryos, pulsatile cortical contraction waves cause a compaction of the embryo, internalisation of cells and the formation of a sphere, in a process that is dependent on E-Cadherin (Maître et al., 2015). Asymmetries in cortical surface tension drive the cell sorting and lead to first inner cell allocation in mammalian blastocysts (Maître et al., 2016; Samarage et al., 2015; Firmin et al., 2024). Another study found that mESC colony architecture, pluripotency gene expression (Nanog and Oct4), and blastocyst development is regulated by E-cadherin and Rho/ROCK-dependent surface tension and compression, generated by a 3D supracellular Acto-Myosin cortex. High tension and compression forces at the colony edge maintain the pluripotency state, whereas a lowering of the tension and compression forces favours the downregulation of Nanog and Oct4 expression (Du et al., 2019).

In summary, these studies demonstrate the significance of cell surface mechanics and tension, in cooperation with endocytic processes, for the regulation of ESC fate (Fig. 4).

5. Nuclear mechanotransduction in (stem) cells

The force-based mechanotransductive dialogue between cells and the ECM eventually propagates into the nucleus modulating the nuclear shape, histone modification and 3D chromatin folding. This will eventually affect the cell identity by regulating the gene expression profile. However, the mechanisms defining this nuclear mechanotransduction and its impact on cell fate are among the least understood steps of the whole mechanotransductive sequence. That is even more true for the SCs context. Therefore, this overview of how biophysical cues, and the consequential cellular forces, influence the nucleus and (epi)genetic regulation includes relevant recent data obtained from other cell types and cell biological conditions (*e.g.*, cell migration in constrained environments).

5.1. Linker of Nucleoskeleton and Cytoskeleton complex and nuclear envelope

The cytoskeleton is structurally connected to the nucleus via the Linker of Nucleoskeleton and Cytoskeleton complex (or LINC complex) which, in turn, interacts with the nuclear envelope (Fig. 1). This structural aspect is gaining a lot of attention recently, as it has a lot of implications in terms of the impact on nuclear mechanotransduction and the subsequent cellular response (Hamouda et al., 2020; Uhler and Shivashankar, 2017b, 2017a).

The LINC complex includes Emerin and Nesprin proteins that reside within the outer nuclear membrane (ONM) and contain a C-terminal KASH (Klarsicht, ANC-1, Syne Homology) domain, which interacts with various isoforms of SUN (Sad1 Unc-84) domain proteins located on the inner nuclear membrane (INM) (Hamouda et al., 2020; Uhler and Shivashankar, 2017b, 2017a). The SUN proteins, in turn, bind to the nuclear lamina (Kirby and Lammerding, 2018). So the LINC complex provides the physical connection and mechanical interface through which forces applied by the cytoskeleton induce changes at the level of the nuclear shape and stiffness which are transmitted to the nuclear lamina (Bertillot et al., 2022; Kirby and Lammerding, 2018; Maurer and Lammerding, 2019; Tajik et al., 2016).

The nuclear lamina is a proteinaceous network underlying the inner nuclear membrane and is mainly composed by Lamins which are type V intermediate filament proteins. There are four different Lamin proteins. Lamin B1 and Lamin B2 are encoded by two different genes LMNB1 and LMNB2. Lamin A and Lamin C, instead, represent the long and the truncated transcript version produced by two alternative transcription starting sites of the LMNA gene (Uhler and Shivashankar, 2017a; van Steensel and Belmont, 2017). B-type Lamins are present in almost all cell types, but A-type Lamins are expressed predominantly in differentiated cells, suggesting a role in maintenance of the differentiated state. As structural components, Lamins provide shape and stability to the nucleus, defining thereby also its mechanical properties, but they represent also the anchoring point for heterochromatic portions of the genome, called Lamina Associated Domains (LADs) (Hamouda et al., 2020; Uhler and Shivashankar, 2017b, 2017a; van Steensel and Belmont, 2017).

The nuclear lamina is pivotal for the transfer of cytoskeletal forces into the nuclear compartment and is the primary element determining nuclear stiffness (Bertillot et al., 2022; Maurer and Lammerding, 2019; Tajik et al., 2016; Uhler and Shivashankar, 2017a). In fact, the expression levels of Lamins and the nuclear organisation adapt to the properties of the cellular environment (Crowder et al., 2016), *i.e.* stiffness (Swift et al., 2013) and nanotopography (Ankam et al., 2018). An important study on nuclear mechanobiology has shown that hMSCs grown in a stiff environment react to this mechanical microenvironmental stimulus by upregulating the Lamin A protein level (Swift et al.,

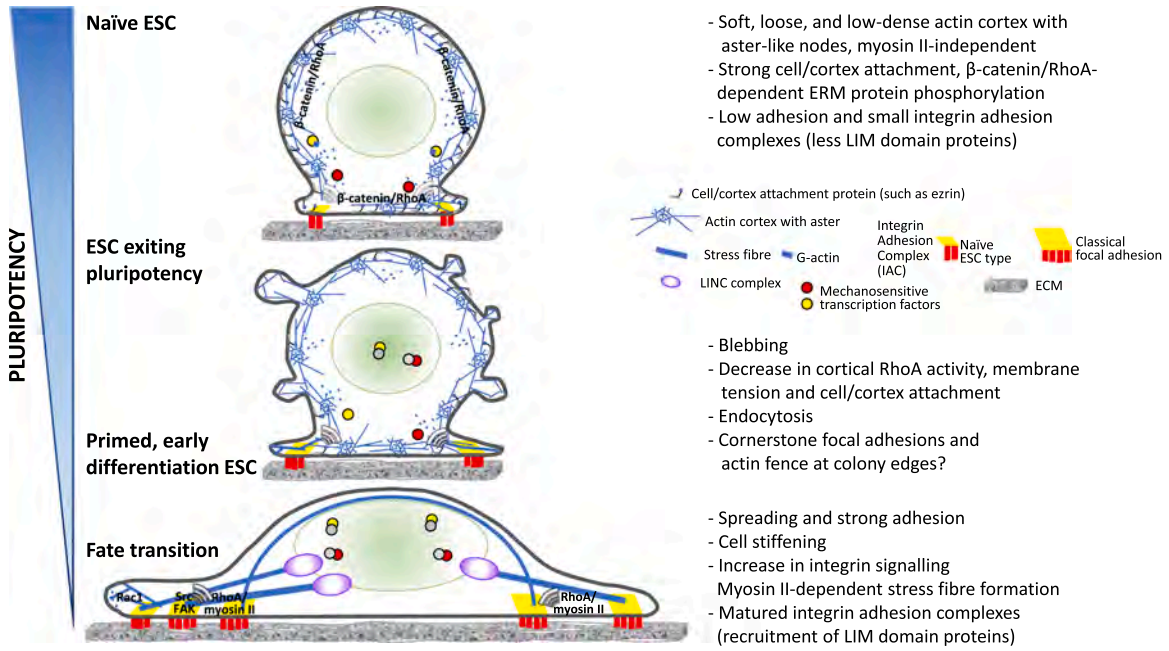

Fig. 4). Visual summary of major findings with respect to mechanobiological aspects and mechanotransductive processes in pluripotent stem cells that are presented in this review.

2013). Supposedly, this represents a mechanism by which the cell responds to mechanical stress, and which induces an increased nuclear stiffness. On the other hand, growing the cells in a softer environment leads to cell relaxation and a consequent decrease in the Lamin A protein level (Swift et al., 2013). hESCs have been shown to not express Lamin A/C and to have a high nuclear plasticity compared to differentiating cells where, together with the upregulation of the Lamin A/C, the nucleus stiffens (Pajerowski et al., 2007). Such phenomena may play an important role in the motility of SCs and, in particular, affect their ability to perform EMT and migrate through other cells and ECM scaffolds during *in vivo* embryogenesis (Ismagulov et al., 2021), or organ formation like in the case of the multipotent neural crest cells (Baggiolini et al., 2015). It is quite established that directional cell migration in dense 3D environments critically depends upon shape adaptation and is impeded or favoured depending on the size and rigidity of the nucleus (Bell et al., 2022; Lomakin et al., 2020). In this regard, the nucleus might work as a spatial sensor which determines the capability of the cell to migrate through a defined 3D microenvironment (Bell et al., 2022; Kirby and Lammerding, 2018; Lomakin et al., 2020).

Lamins contribute to the modulations of the chromatin structure which ultimately may affect gene expression (Tajik et al., 2016; Uhler and Shivashankar, 2017a; van Steensel and Belmont, 2017). Large portions of nonfacultative heterochromatin, marked by histone H3K9me3, are tethered to the nuclear lamina and associate with it in LADs (van Steensel and Belmont, 2017). This association provides a structural scaffold for 3D chromatin folding (Zheng et al., 2018). How mechanical cues translate into differences in the cellular phenotype via the modulation of epigenetic state is an exciting, and timely question in the field. However, the regulation and organisation of chromatin structure (and ensuing function) obtained through the integration of mechanotransductive signals, is the least understood part of the whole mechanotransductive process so far.

5.2. Chromatin remodelling and epigenetic regulation in response to mechanostimulation

Within the nucleus, the genome is physically organised into chromatin, which is further folded in a 3D fashion to fit the few μm space of this important organelle. The chromatin structure is dictated primarily by DNA methylation and post-translational modifications targeting the histone tails which change compaction of chromatin fibres. This eventually regulates the access of the general transcriptional machinery and, in turn, the gene expression. A further level of regulation is provided by the 3D chromatin organisation that defines long range interactions among different regulatory regions (like enhancers, promoters, or insulators) generating a physical frame in which the expressed and silenced genes are compartmentalized (Pombo and Dillon, 2015; Rada-Iglesias et al., 2018).

Several studies in the last years have demonstrated how mechanotransductive cues induce epigenetic regulation in the nucleus of the cells. For example, mechanical force derived from tensile loading in human epithelial progenitor cells can directly target gene expression and inhibit RNAPII elongation (Le et al., 2016). Notably, the same study has shown that this mechanical stimulation resulted in a switch from the constitutive heterochromatin markers H3K9me2 and H3K9me3 to H3K27me3, a facultative heterochromatin marker, mediated by the Polycomb repressive complex Ezh2. Similarly, cells subjected to hydrostatic pressure exhibited a switch from the constitutive heterochromatin markers H3K9me2 and H3K9me3 to H3K27me3 (Maki et al., 2021). Further mechanistic experiments on tensile and hydrostatic mechanical cues have shown that these epigenetic patterns are regulated by *JARID2*, *SUZ12,* Setdb1 and *Suv39H1* (Maki et al., 2021; Nava et al., 2020). The cell can also be induced to modulate chromatin regulation as a response to substrate stiffness. hMSCs seeded in tunable hydrogels show an upregulation of the acetyl transferase HAT1 and a consequent increase of the histone acetylation (active euchromatic chromatin marker) when grown on a stiff condition around 30 kPa (Killaars et al., 2019). Change of the stiff substrate to a softer one (5 kPA) induced a progressive reduction in the histone acetylation levels after one day, but the levels remained persistently higher than the control for at least five days (Killaars et al., 2019). Somehow in the same direction, super-resolution microscopy done on human mesenchymal stromal cells grown in methacrylated hyaluronic acid hydrogels with different stiffness, exhibited an increase of the active chromatin marker H3K4me3 and a decrease of the repressive marker H3K27me3 in the cells on the stiffer substrate (Heo et al., 2023). Interestingly, the increase signal of H3K27me3 seen in the softer substrate mainly accumulated at the nuclear periphery (Heo et al., 2023). Data coming from human breast

cancer cells partially confirmed this response. Cells that were grown on interpenetrating networks of reconstituted basement membrane matrix and alginate (which allows to have 3D cultures with soft or stiffer elastic moduli), have shown that stiffer conditions induce an increase on the euchromatic compartment with higher levels of H3ac and ATACseq increased accessibility (Stowers et al., 2019). Counterintuitively, HDAC class I inhibition decreased the number of accessible sites (Stowers et al., 2019). Electron microscopy results evidenced an altered nuclear morphology which appeared more wrinkled in cells grown in stiff matrices. However, the electron microscopy images have also shown a thickened electron dense signal at the nuclear periphery in the cells on stiff matrix which is compatible with an increase in the heterochromatic compartment which seems to point in the opposite direction of the abovementioned results (Stowers et al., 2019). Interestingly, results from gastric cancer cells demonstrate that soft substrates induce the methylation at the promoter of the YAP gene (Jang et al., 2021). Stiff substrates instead induce the expression of several DNA methylation inhibitors like TET2, KMT2A and GRHL which trigger the hypo-methylation of the YAP gene promoter, evidencing a link between stiffness and DNA methylation (Jang et al., 2021).

In a recent study from Vermeulen *et al.*, it has been shown that growing hMSCs on engineered substrates with different micro-topographies induced epigenetic reprogramming (Vermeulen et al., 2022). hMSCs responded to specific topographies by decreasing H3K27me3, H3K9me2 and H4K12ac (Vermeulen et al., 2022). Interestingly, they also observed a significant deformation in the nuclei of the cells. However, it is difficult to assess to which extent such morphology may be imposed to cells by the size of the elements of the used topography (μm range) (Vermeulen et al., 2022) which may expose them to a certain level of constriction. In the same study, they also reported that the used microtopographical conditions enhanced their quiescence, promoting the multipotency of the hMSCs (Vermeulen et al., 2022). These results are in line with two other studies where it was shown that transient nuclear deformation primes the epigenetic state and promotes cell reprogramming (Park et al., 2023; Song et al., 2022).

Confined migration is a process in which the cells squeeze through narrow interstitial spaces (1–20 μm in diameter) exposing the whole cell body, particularly the nuclear compartment, to intense mechano-transductive cues. During such a process, the nucleus can be subjected to moderate or extreme deformations. Two recent publications investigated the impact on the chromatin structure upon confined migration (Golloshi et al., 2022; Hsia et al., 2022). Human A375 melanoma cells were allowed to migrate through the filter of a transwell device with sized pores of 5 μm and they found that constricted cells showed a tendency to have more elongated nuclei compared with control cells (Golloshi et al., 2022). Cells showed no significant differences in the overall intensity of Lamin A/C. However, they noticed a consistent difference in the distribution of Lamin A/C at the nuclear periphery upon constricted migration, with areas that displayed higher/lower Lamin A/C signal which correlated with the wrinkling of the nuclear membrane (Golloshi et al., 2022). After investigating the intensities of the chromatin repressive marker H3K9me3, the results showed no difference between the migrating cells and their control. However, compared to normal cells showing the typical heterochromatic foci in the middle of the nucleoplasm, in the confined migrating cells, H3K9me3 was mainly distributed at the periphery of the nucleus (Golloshi et al., 2022). Complementary HiC dataset analysis revealed that cells which have passed through constrictions presented specific differences in their chromatin folding (Golloshi et al., 2022). In the other constrained migration study, they used a custom-made polydimethylsiloxane matrix which provide the great advantage of enabling a precise definition of the pore sizes that cells encounter during 3D migration, independent of the ECM concentration and stiffness (Hsia et al., 2022). Migration of the human HT1080 fibrosarcoma cells through the channels resulted in a decrease of the elongating form of the RNAPII (RNAPII-S2p) and a concomitant loss of transcription (Hsia et al., 2022). The effect on transcription was paralleled by a general increase on the constitutive and facultative heterochromatic markers represented respectively by H3K9me3 and H3K27me3, even though the Polycomb mediated H3K27me3 displayed a more pronounced increase compared to the H3K9me3 (Hsia et al., 2022). Interestingly, repeating the experiment using high- and low-concentration 3D collagen matrices, they did not observe the same general burst in the heterochromatic markers. Both H3K9me3 and H3K27me3 markers remained essentially unchanged in line with the results obtained by Golloshi *et al.* (Golloshi et al., 2022). The discrepancy in the results obtained in the study from Hsia *et al.* could be due to the viscoelastic properties of the matrix. Another interesting hypothesis might rely on the differences in the micro/-nanotopography of the collagen matrices.

Altogether, these data demonstrate that mechanotransductive cues are transmitted into the nucleus via LINC complex and Lamin meshwork which clearly affects the nuclear morphology and mechanics, as well as chromatin organisation and (epi)genetic regulation, in various cell biological frameworks.

6. Conclusions

In recent years there has been a fascinating expansion of our knowledge about the interplay between mechanotransductive signalling and chromatin regulation, and how this impacts on multi- and pluripotent stem cell dynamics, which we try to highlight in this review.

However, what shapes the life of a cell and its identity, from the outside (biophysical and structural cues of the cellular microenvironment) and from the inside (nuclear and chromatin organisation) and how it is connected (mechanotransduction and epigenetics) are highly relevant biological issues that are yet far from being understood in detail. Starting from the stem cell/microenvironment interface, a much more detailed understanding of the nanoscale events in this interface is necessary to unravel 1) what the SC perceives of the biophysical nature and the 3D adhesion site configuration and 2) how the immanent nanoscale information is interpreted and converted, through force-based actions, into a remodelling at the level of the IACs, cell/cell adhesions, cytoskeleton and nucleus.

Integrin-mediated mechanotransductive processes occur at the nanoscale with forces in the piconewton range and are potentially influenced by a plethora of microenvironmental physical factors, as we attempted to outline in this review. The dynamic interdependencies of these events and parameters have been hard to capture and dissect experimentally in its entirety until now. Integration of recent advances in computational modelling related to cell adhesion, Integrin dynamics and mechanotransduction into the stem cell and tissue engineering field could therefore contribute to a complementary and better understanding of the related mechanotransductive processes (Post et al., 2022; Sun et al., 2022).

Little variances in (extra)cellular biophysical parameters can strongly influence the cellular responses and there is still too little knowledge about how specific mechanotransductive cues affect the pluripotency/differentiation balance and dictate the differentiation trajectories. Many studies cited in this review exploited biomaterials of some kind (such as micro- or nanofabricated substrates produced, *e.g.*, by lithographic methods, or hydrogels made of various materials). Biomaterials have thus been very useful in understanding these processes by providing cell substrates in which certain biophysical parameters have been manipulated and by observing the cellular output behaviours in response to the substrates' cues. The insight obtained with these biomaterials leverages furthermore a potential of these substrates for the application in tissue engineering and regenerative medicine, *e.g.*, as platforms for the control of SC behaviour. By presenting appropriate mechanical and/or structural cues to the SCs, specific desired effects might be induced, such as maintenance of pluripotency, reprogramming somatic cells into iPSCs, or biasing the cell into particular differentiation directions. For further information on the potential tissue engineering

and regenerative medicine applications of these biomaterials that can influence stem cell fate, we refer the interested reader to more specific reviews on this topic (Chen et al., 2014; Crowder et al., 2016; Lenzini et al., 2019; Munoz-Robles et al., 2020; Naqvi and McNamara, 2020; Ribeiro et al., 2024; Li et al., 2017).

The mechanotransductive impact of biophysical cues might be achieved by simply activating some mechanosensitive downstream signalling targets, or possibly by establishing a mechano-induced memory in the cell by affecting the 3D chromatin folding and epigenetic profile, which eventually introduce differentiation biases influencing the cell fate. Despite the fact that in the nucleus of the cell there are no membrane-enclosed sub-compartments, most nuclear events are confined and spatially defined at specific nuclear landmarks (Ferrai et al., 2010). The DNA molecule which holds the genetic information of the entire organism is spatially organised in distinct higher-order chromatin domains, like the chromosome territories and condensed heterochromatin, but also by a number of protein sub-compartments condensates like transcription factories, nuclear lamina, speckles, the nucleolus and Cajal bodies (Ferrai et al., 2010). In this review, we report evidence pointing to the fact that mechanotransductive cues not only impact the nuclear shape, but that this is often associated with a significant regulation of chromatin marks, either in their moieties, or in their nuclear distribution. One speculation is that an altered sub-compartmentalisation of the nucleus could influence multiple parameters, including transcription factor accessibility and promoter enhancer strength of interaction, which cumulatively could affect cell fate ((Ferrai et al., 2017; Fraser et al., 2015).

Almost all the studies cited in this review were done in an *in vitro* framework, as mechanobiological aspects of pluripotent stem cells, *e.g.*, during embryogenesis, are hard to access *in vivo*. As aforementioned, it is known, e.g., that pre-implantation Acto-Myosin contractile force generation and cell surface tension control apicobasal polarity, positioning and fate specification of mouse and human blastomeres (Alvarez and Smutny, 2022; Firmin and Maître, 2021; Maître et al., 2016; Nelson, 2022; Firmin et al., 2024). However, the *in vivo* significance of mechanical forces and mechanotransduction in pluripotent stem cells, pre- and post-implantation, needs to be addressed more in detail in the future.

In vivo, stem cells are furthermore exposed to a whole variety of biophysical and biochemical stimuli at the same time. How such different signals are integrated spatially and temporally; regarding, *e.g.*, also the way biochemical factors (such as growth factors) crosstalk at the molecular level with mechanotransductive processes and *vice versa*, is basically unexplored in the SC framework and needs thus more research efforts.

Altogether, the complexity of the mechanotransductive sequence and the involved structures and processes still leaves the field with a lot to learn about the molecular mechanisms of mechanosensing at the stem cell/microenvironment interface and the translation of the information into differences in gene expression patterns by modulating the epigenetic state and 3D genome.

CRediT authorship contribution statement

Carsten Schulte: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Carmelo Ferrai:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

This is a review.

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