



Invited article

Trusting the forces of our cell lines

Marina Moro-López^a, Ramon Farré^{a,b,c}, Jorge Otero^{a,b,d}, Raimon Sunyer^{a,d,e,*}^a Unit of Biophysics and Bioengineering, School of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain^b Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBER-RES), Barcelona, Spain^c Institut Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Barcelona, Spain^d Institute of Nanoscience and Nanotechnology (IN2UB), University of Barcelona, Barcelona, Spain^e Centro de Investigación Biomédica en Red de Bioingeniería (CIBER-BBN), Barcelona, Spain

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ABSTRACT

Cells isolated from their native tissues and cultured *in vitro* face different selection pressures than those cultured *in vivo*. These pressures induce a profound transformation that reshapes the cell, alters its genome, and transforms the way it senses and generates forces. In this perspective, we focus on the evidence that cells cultured on conventional polystyrene substrates display a fundamentally different mechanobiology than their *in vivo* counterparts. We explore the role of adhesion reinforcement in this transformation and to what extent it is reversible. We argue that this mechanoadaptation is often understood as a mechanical memory. We propose some strategies to mitigate the effects of on-plastic culture on mechanobiology, such as organoid-inspired protocols or mechanical priming. While isolating cells from their native tissues and culturing them on artificial substrates has revolutionized biomedical research, it has also transformed cellular forces. Only by understanding and controlling them, we can improve their truthfulness and validity.

1. Introduction

In his book, *The Master Builder: How the New Science of the Cell Is Rewriting the Story of Life*, Martínez-Arias acknowledges that the creative tension between genes and cells lies at the heart of biology (Martínez-Arias, 2023). While Dawkins envisioned genes as selfish entities focused on maximizing their replication (Dawkins, 1976), Martínez-Arias presents a compelling counterpoint: genes may exhibit selfish tendencies, yet cells, in contrast, unveil remarkable selflessness. Cells within an organism cooperate with other cells to maintain homeostasis and function and even sacrifice themselves for the greater good. This selflessness is exemplified by apoptosis, or programmed cell death, which is essential for development, tissue remodeling, and elimination of damaged cells (D'Arcy, 2019). Another form of self-sacrifice is autophagy, where cells devour themselves, breaking down their complex molecules, and recycling them for new parts. This self-destruction process is crucial for maintaining cellular homeostasis and may even extend the organism's lifespan (D'Arcy, 2019). Some diseases can alter the altruistic behavior of cells. For example, cancer cells disregard the normal rules of cooperation and regulation that govern the behavior of tissues (Axelrod et al., 2006; Marongiu et al., 2021). They proliferate uncontrollably, invade neighboring tissues, and spread throughout the body, causing damage

and death (Hanahan and Weinberg, 2000; Brown et al., 2023). But cancer is not the only way to disrupt the altruistic nature of cells. The immortalization of cell lines and their culture on plastic labware disrupts the natural communication and interaction between cells and their environment, changing cell behavior fundamentally.

Cells isolated from their tissues and subsequently cultured *in vitro* face different selection pressures than those *in vivo*. These pressures favor cell survival and proliferation, often through the induction of mutations and epigenetic alterations. One of these alterations is the immortalization of cells, which allows them to divide indefinitely. Immortalization can result from random mutations or intentional genetic modifications by researchers (Stepanenko and Kavsan, 2012). In both cases, it often involves the loss of cell-cycle checkpoints (Maqsood et al., 2013), the upregulation of telomerase (Vaziri and Benchimol, 1998), and the overexpression of oncogenes (Garbe et al., 1999). The culture of HeLa cells for nearly seven decades illustrates the effects of long-term *in vitro* culture on cells (Silberman, 2010; Martínez-Arias, 2023). For decades, HeLa cells were considered a homogeneous and stable population (Lyapun et al., 2019). However, a recent study shows that HeLa cells are a family of cells with variations in genes, cell cycle duration, and chromosome number (Liu et al., 2019). In another experiment, the researchers cultured HeLa cells for three months to see

* Corresponding author at: Unit of Biophysics and Bioengineering, School of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain.
E-mail address: rsunyer@ub.edu (R. Sunyer).

how gene expression changed over time. They found a 6 % difference in gene expression between early and late cell generations. More generally, prolonged *in vitro* culture promotes changes in cell-cycle kinetics (Izadpanah et al., 2008; Gu et al., 2016), senescence (Izadpanah et al., 2008; Chen and Li, 2013), mitochondria function (Czernik et al., 2022), DNA methylation (Franzen et al., 2021), and gene expression (Fernández-Gonzalez et al., 2004). These changes occur in response to pressures imposed by factors such as nutrient availability, oxygen level, pH, and the mechanical properties of the *in vitro* environment. Among these factors, the last one stands out, suggesting that a significant part of this adaptation has a mechanical origin.

The role of mechanical constraints in the adaptation of cell cultures is partially imposed by the most widely used substrate for cell culture: plastic. Plastic labware provides a 2-dimensional, stiff, topology-free surface where cells must adhere and divide. These mechanical constraints impose selective pressures that change cellular forces. In this perspective, we will focus on the evidence that the mechanobiology of cell lines may differ from their *in vivo* counterparts in fundamental ways. We will review the mechanisms of mechanoadaptation and to what extent they are reversible. We will argue that this aberrant mechanobiology is often presented as a mechanical memory. We will suggest several approaches, such as the use of organoid-inspired procedures or mechanical priming, to reduce the negative impacts of *in vitro* cell culture on cellular mechanobiology. Because immortalized cell lines are easily accessible and can self-renew, they have been used extensively in scientific research for many years. We can enhance their efficacy and reliability in mechanobiology studies by comprehending and managing their altered mechanobiology.

2. From the animal tissue to plastic dishes: how polystyrene became the standard for tissue culture

By the end of the 19th century, biologists struggled to grow cells *in vitro*. There were two main challenges to overcome: the formulation of a cell culture medium that allowed cells to survive and proliferate, and the fabrication of a suitable substrate that enables cell adhesion (Yao and Asayama, 2017). A breakthrough occurred in 1907, when Ross Granville Harrison, an American biologist and anatomist, developed the first successful technique for *in vitro* culturing animal cells (Jedrzejczak-Silicka, 2017). He used a hanging drop method to grow frog neuroblasts in a lymph medium. His experiment demonstrated that cells could retain their normal functions and morphology outside the body and differentiate into specialized tissues under appropriate conditions. Harrison's success motivated Alexis Carrel, a French surgeon and biologist who won the Nobel Prize in Physiology and Medicine in 1912, to further advance tissue culture techniques. Collaborating with Harrison, Carrel adapted and expanded tissue culture methods, introducing innovations such as blood plasma instead of lymph, serial cultivation techniques, and the utilization of “embryo juice” to maintain cell size and prolong tissue growth (Selcer, 2008). In 1923, Carrel designed the Carrel flask, a round, flat vessel with a tilted neck that facilitated liquid cultures, aseptic conditions, and microscopic observation, which was widely utilized in research until the 1950s (Millet and Gillette, 2012). These reusable glass flasks, however, posed challenges for cell culture, such as the need for sterilization and cell adhesion problems.

Therefore, researchers experimented with disposable plastic vessels, mainly made of polystyrene (Lerman et al., 2018). Polystyrene had good optical clarity, moldability, and sterilizability by irradiation. However, it was also very hydrophobic and unsuitable for cell adhesion. The polystyrene surface had to be modified to be more hydrophilic to improve adhesion. This was achieved in 1966 by sulfonating the polystyrene surface (Rubin, 1966), creating the first tissue culture polystyrene (TCPS). TCPS enabled serum proteins like vitronectin and fibronectin to adhere and spread on the vessel bottom, providing an improved surface for cells. By the mid-1970s, TCPS was mass-produced and widely adopted by research laboratories worldwide. Today, TCPS

production methods have improved, and it is the standard for cell culture (Lerman et al., 2018).

3. Cells adapt to TCPS and change their mechanical phenotype, a process often understood as “mechanical memory”

The native support of cells within the organisms is the extracellular matrix (ECM). The ECM is a complex network of macromolecules that provides both mechanical and biochemical support to cells within tissues. The ECM contains a large number of components, mainly collagens, elastin, fibronectin, laminin, and proteoglycans (Frantz et al., 2010). These proteins form a fibrous structure with nonlinear viscoelastic properties and topology that adapt to the requirements of every tissue, from the soft brain to the hard cartilage (Levental et al., 2007). Cells attach to the ECM proteins *via* integrins, which are transmembrane receptors that, together with other proteins, cluster into focal adhesions, the structure that mediates cell-matrix adhesions (Kechagia et al., 2019). In contrast, cell attachment on TCPS flasks is mediated by nonspecific adhesions or by ECM proteins present in the serum of the cellular medium and absorbed into the hydrophilic surface. In these conditions, focal adhesions have different morphology and dynamics than *in vivo* (Yamaguchi and Knaut, 2022). This difference sparked debates in past decades about whether focal adhesions were merely artifacts of cell culture or if they genuinely occur within living organisms (Wozniak et al., 2004). These historical discussions underscore how the rigid, flat surface of TCPS flasks introduce new constraints for cells, forcing them to adapt and grow in an environment far from the soft viscoelastic environment of their native tissue.

TCPS imposes physical constraints that alter the phenotype of cells. These alterations persist even when the cells are transferred to a different scaffold or reimplanted into living animal tissue. This phenomenon is often termed *mechanical memory* because the cell “remembers” past physical signals (Dudaryeva et al., 2023; Kanoldt et al., 2019; Balestrini et al., 2012). This memory induces alterations in the cytoskeleton, focal adhesions and the activation of transcription factors that ultimately lead to gene expression. Unlike differentiation, mechanical memory does not necessarily involve a change in the cell's identity or function, but rather influences how the cell behaves in response to its physical environment. The term mechanical memory might lead to a misinterpretation of the underlying mechanisms. When cultured in TCPS, cells acquire adaptations that allow them to attach to stiffer environments and proliferate. These adaptations change cellular metabolism, trigger gene expression, and impose epigenetic changes on the genome (Dudaryeva et al., 2023). When cells are subsequently transferred to a softer environment, these signatures remain, giving the impression that cells remember their mechanical history. More than a memory, it is an adaptive response. The shift in phenotype is not merely a retention of past signals but rather an ongoing adaptation to the prevailing mechanical conditions.

The term mechanical memory was coined in 2012 when Balestrini et al. showed that primary fibroblasts seeded in stiff elastomers for a few weeks led to sustained myofibroblast activation even after returning them to soft cultures (Balestrini et al., 2012). Conversely, seeding fibroblasts on soft elastomers offered protection against myofibroblast activation when shifted to stiff conditions. This initial exposure to either stiff or soft conditions is often termed “mechanical priming” or “mechanical preconditioning”. Subsequent studies experimented with different priming conditions, ranging from brief periods of a few hours (Heo et al., 2015) to more extended periods spanning days (Yang et al., 2014; Caliri et al., 2016; Li et al., 2017) and weeks (Balestrini et al., 2012). While most studies have concentrated on elucidating the impact of substrate stiffness, a subset has explored the influence of other mechanical factors, including viscosity (Dingal et al., 2015) and various forms of mechanical stimulation (Heo et al., 2015; Park et al., 2023) and confinement (Roy et al., 2018, 2020). These findings indicate that cells adapt to priming conditions at different timescales, in a process called

mechanoadaptation.

4. Stiff priming conditions promote the adhesion reinforcement of cells

To understand how stiff priming conditions, such as TCPS, may alter cell adhesion, we need to use the conceptual framework proposed by the molecular clutch model. This model, initially proposed by Abercrombie in 1978 and later named by Mitchison and Kirschner in 1988, compares the interaction between the cytoskeleton and the ECM with a clutch engaging and disengaging its power transfer between an engine and a shaft (Abercrombie, 1980; Mitchison and Kirschner, 1988). In 2008, Chan and D.J. Odde developed and experimentally tested a mathematical version of the molecular clutch (Chan and Odde, 2008). The model gained further refinement as Elosegui and Roca-Cusachs, and others extended it by integrating adhesion reinforcement (Elosegui-Artola et al., 2014). In the molecular clutch model, cellular contractile forces are exerted by myosin motors and transmitted to the ECM through integrins and adaptor proteins. The key parameter controlling this force transmission is the loading rate, which depends on the stiffness of the substrate, the number of myosin motors, and the molecular properties of the clutch elements. These properties include the integrin-ECM binding affinities, the spring constant of the clutch and its rupture force (Bangasser et al., 2013). As myosin contracts, it generates an actin flow that goes from the edge of the cell (where adhesions with the ECM form) to the center of the cell. This flow opposes to actin polymerization (or cell protrusion) and extends the cell membrane outward (see Fig. 1a).

The molecular clutch model predicts how traction forces depend on substrate rigidity (Fig. 1b). When cells adhere to soft substrates, they experience a “frictional slippage” regime, leading to low force generation (Elosegui-Artola et al., 2018; Fortunato and Sunyer, 2022) (Fig. 1b, inset i). When cells adhere to rigid substrates, they undergo a “load and

fail” or “stick-slip” regime, which causes periodic force buildup and release, also resulting in low force generation (Fig. 1b, inset ii). Cells maximize their force output only at intermediate substrate rigidities (Elosegui-Artola et al., 2016; Bangasser et al., 2017) (Fig. 1b, inset iii). This rigidity range is called “optimal stiffness”, and it suggests a significant physiological implication: cells would exert maximal force at the physiologic stiffness of their native tissue. Different tissues in the body have different rigidities, ranging from brain (260–490 Pa) and liver (640 Pa) to kidney (2.5 kPa), skeletal muscle (12–100 kPa) and cartilage (950 kPa) (Reviewed in Ref. (Levental et al., 2007)). The concept of optimal stiffness suggests that cells in these tissues are likely to be adapted to exert maximal force in the specific rigidity of their native tissue. While this concept would make sense physiologically, this maximal force is rarely observed in experiments because most cell types respond to a certain force threshold by reinforcing their adhesions (Fig. 1b, inset iv). This involves the unfolding of talin, a protein that connects integrins to the cytoskeleton and exposes vinculin binding sites (Elosegui-Artola et al., 2018, 2016; del Rio et al., 2009). As a result, the density and size of focal adhesions increase, and the cell produces more force by having more integrins. Adhesion reinforcement is the key mechanoadaptation mechanism that enables cells to adhere to very stiff substrates.

5. Downstream effects after adhesion reinforcement: from cytoskeleton alterations to nuclear deformation and transcription factor activity

To adhere to stiff conditions such as TCPS, cells initiate adhesion reinforcement, a process mediated by integrin adhesion complexes such as zyxin, paxillin, alpha actinin, VASP, FAK and talin. Among them, the role of talin is the most extensively studied (Chastney et al., 2021). Talin connects the integrins with the actin cytoskeleton, transmitting

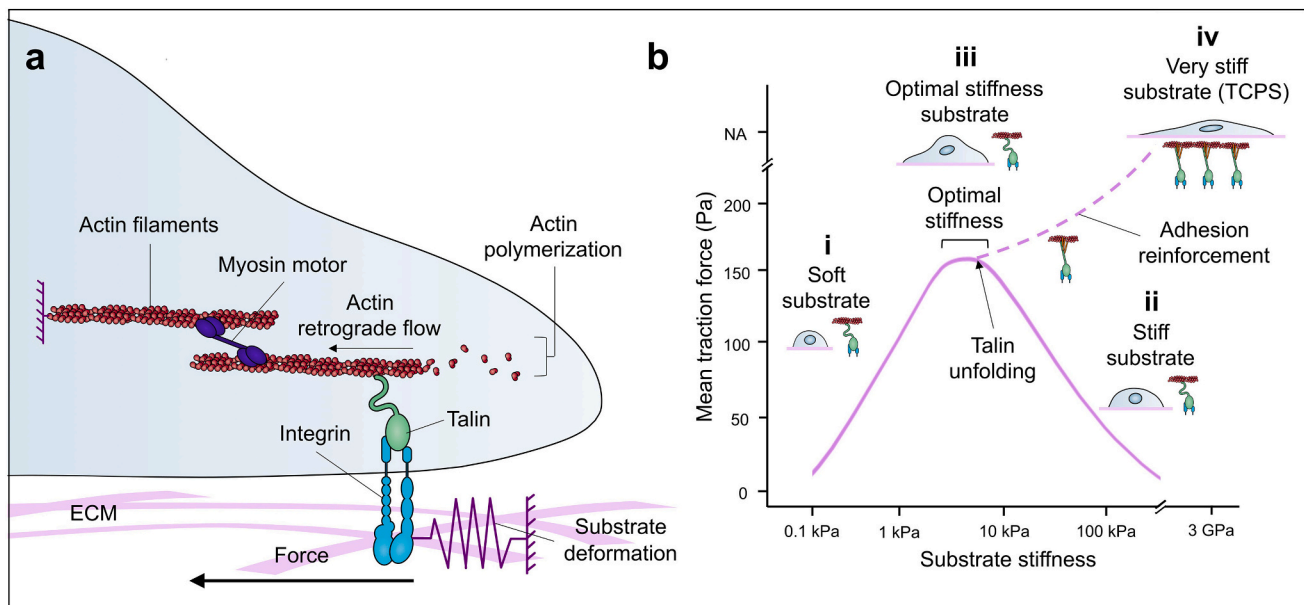


Fig. 1. The molecular clutch model offers insights into how cells adapt to rigid substrates such as tissue culture polystyrene (TCPS). (a) Simplified diagram of the clutch model. Cells exert forces on their extracellular matrix (ECM) by contracting the actin cytoskeleton with myosin molecular motors. Substrate deformation is represented as a spring. Integrins and accessory proteins at the cell edges act as clutches with specific binding and unbinding rates. Myosin-driven contraction generates a retrograde flow from the cell periphery toward the center, opposing actin polymerization and extending the cell membrane outward. (b) The molecular clutch model predicts how traction forces depend on the rigidity of the substrate (solid line). On soft stiffness substrates, cells experience a “frictional slippage” regime, resulting in low force generation (i). On stiff stiffness substrates, cells are in a “load and fail” or “stick-slip” regime that causes periodic build-up and release, also resulting in low force generation (ii). The molecular clutch predicts that cells maximize their force output only at intermediate substrate rigidities (iii). This rigidity range is called “optimal stiffness”, and its value depends on the particularities of each cell type such as the number of myosin motors, the ECM-integrin affinity, etc. This optimal stiffness is often overshadowed by talin/vinculin-mediated adhesion reinforcement (dashed line). Above a particular stiffness, cells that display adhesion reinforcement increase the density and size of their focal adhesions, producing more force by having more integrins. Adhesion reinforcement serves as a critical mechanoadaptation mechanism that facilitates cell adhesion to rigid environments, such as TCPS (iv).

contractile forces from the myosin motors to the ECM (Fig. 2a). But talin is not just a passive link. It responds to mechanical force by unfolding and refolding, efficiently dissipating energy. This dissipation is so effective that new shock-absorbing materials made of talin can withstand the impact of ultrasonic projectiles (Doolan et al., 2023). Above a specific stiffness threshold, talin unfolds and exposes cryptic vinculin sites, which recruit vinculin (Hytönen and Vogel, 2008) (Fig. 2a–b). Vinculin stabilizes, enlarges, and increases the density of focal adhesions, providing more contact points between the cell and the substrate (Iskratsch et al., 2014; Critchley et al., 1999). Vinculin also interacts with paxillin and other cytoskeletal proteins and regulates the formation and alignment of actin stress fibers (Gupta et al., 2015) (Fig. 2c). The behavior of cells without adhesion reinforcement was studied by Elosegui et al. using mouse embryonic fibroblasts that lacked talin-1 and had reduced talin-2 expression (Elosegui-Artola et al., 2016). They found that below a certain stiffness threshold, there was no difference

between talin-depleted cells and control cells. However, above that threshold, talin-depleted cells had smaller cell areas, smaller focal adhesions, and lower traction forces on the substrate. These results contrast with the typical behavior observed in most immortalized cell lines, which show increased cell area and force with stiffness, suggesting a strong adhesion reinforcement (Janmey et al., 2020; Discher, 2005).

Adhesion reinforcement alters not only cell adhesion and cytoskeleton architecture but also nuclear mechanics and structure (Elosegui-Artola et al., 2017). When the cell contracts on a stiff substrate, it transmits mechanical forces to the nucleus through the cytoskeleton (Fig. 2d–e). These forces deform the nuclear envelope and activate signaling pathways that increase the expression of lamin A/C, a protein that stiffens the nucleus and counteracts deformation (Dingal et al., 2015) (Fig. 2e). However, the most significant effect of applying force to the nucleus is that it changes the nuclear permeability for transcription factors. Transcription factors are proteins that bind to specific DNA

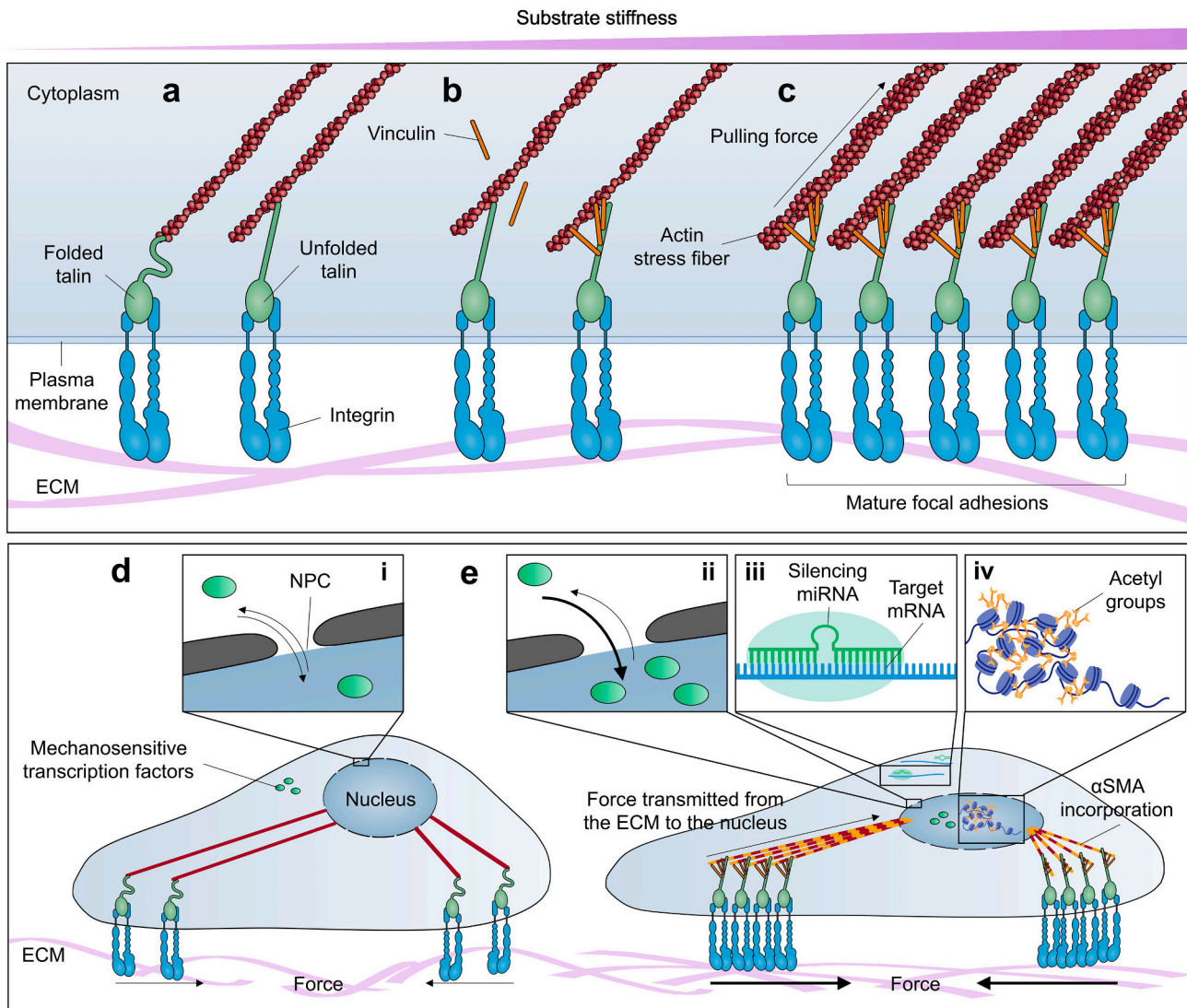


Fig. 2. Culturing cells on stiff substrates alters their mechanical phenotype. Stiff substrates, such as tissue culture polystyrene (TCPS), trigger adhesion reinforcement. (a) Adhesion reinforcement starts with talin unfolding. (b) Unfolding of talin reveals cryptic vinculin sites. (c) Vinculin recruitment to these sites stabilizes and enlarges focal adhesions, augmenting their density. (d) On soft substrates, adhesions are not reinforced, talin is mostly unfolded and forces fail to transmit to the nucleus. Transcription factors import and export to the nucleus through nuclear pore complexes (NPCs) remain balanced (inset i). (e) On stiff substrates, forces are transmitted to the nucleus altering its morphology. Nuclear deformation affects NPC permeability, influencing the import and export dynamics of transcription factors (inset ii). These transcription factors regulate the expression of specific genes, including α SMA, which confer resistance to mechanical stress, thereby facilitating cellular adaptation to a stiff microenvironment. Notably, certain transcription factors like YAP and RUNX2 preferentially localize to the nucleus on stiff substrates, while others like NKX2.5 exhibit the opposite trend. Importantly, when cells transition to a softer environment, some adaptations persist. Epigenetic modifications such as miRNA regulation (inset iii) or histone acetylation (inset iv) contribute to maintaining this altered phenotype.

sequences and regulate gene expression. When mechanical forces deform the nuclear envelope, the nuclear pore complexes (NPCs) open, allowing cargoes with a certain molecular weight and affinity for nuclear transport receptors to move in or out of the nucleus (Andreu et al., 2022) (Fig. 2d–e, insets i–ii). These transcription factors regulate the expression of specific genes that confer resistance to mechanical stress, enabling the cell to adapt to the stiff environment.

Some transcription factors that are sensitive to nuclear pore opening include YAP/TAZ, MRTF-A, NKX2.5, and RUNX2. These factors have different roles in the regulation of gene expression and function. The most studied mechanosensitive transcription factor is YAP/TAZ, a co-activator of the Hippo pathway, that translocates to the nucleus on stiff substrates (Dupont et al., 2011). Among many other functions, YAP/TAZ activates genes involved in actin polymerization (Yang et al., 2023), cell adhesion (Nardone et al., 2017), cell proliferation (Camargo et al., 2007) and fibrosis (Talele et al., 2015). But YAP/TAZ translocates to the nucleus not only in response to substrate stiffness. Mechanical stretch (Andreu et al., 2021) and externally applied forces to the cell also trigger YAP/TAZ translocation. Atomic Force Microscopy (AFM) experiments showed that this translocation occurs rapidly, in seconds (Elosegui-Artola et al., 2017). Another mechanosensitive transcription factor is myocardin-related transcription factor A (MRTF-A, also known as MKL1/MAL). MRTF-A is sequestered in the cytoplasm by G-actin, but when the actin cytoskeleton is disturbed by mechanical stimuli, MRTF-A translocates to the nucleus and interacts with serum response factor (SRF) (Gau and Roy, 2018), regulating actin cytoskeletal organization, focal adhesion assembly and α -smooth muscle actin (α SMA) (Parreno et al., 2017). α SMA is a common marker for myofibroblasts, which are present in healing wounds, scars, and fibrocontractive lesions and contribute to fibrosis (Hinz et al., 2001). α SMA has also been used as a marker of mechanical memory (Balestrini et al., 2012; Walker et al., 2021). However, not all transcription factors translocate to the nucleus in response to stiffness. NKX2.5 does the opposite. NKX2.5 is a homeobox transcription factor that is essential for cardiac development and maintenance, but it is likely to modulate other functions in other tissues. NKX2.5 accumulates in the nucleus of cells on soft substrates, repressing the expression of α SMA (Dingal et al., 2015). On stiff substrates, NKX2.5 localizes in the cytosol, and it no longer represses α SMA. Therefore, α SMA incorporates into the stress fibers, increasing their stability and contractility (Cao et al., 2023).

Taken together, the combined effects of mechanosensitive transcription factors change the native phenotype of cells to a new one more adapted to stiff conditions. This phenotype features abundant stress fibers, large focal adhesions, and lamin A/C-enriched nuclear envelopes. These features allow cells to generate higher forces on stiff substrates, remodel their cytoskeleton more dynamically, and migrate and invade more effectively (Nasrollahi et al., 2017; Watson et al., 2021; Almeida et al., 2023).

6. Reversibility of the mechanical adaptation

The effects of culturing cells on stiff substrates do not always disappear when cells are transferred to a softer environment. The persistence and reversibility of this phenotype depend on several factors, such as how long cells remain on the stiff substrate, which is often referred to as the priming time (Balestrini et al., 2012; Yang et al., 2014). For example, when human mesenchymal stem cells (hMSCs) were cultured on a stiff substrate for one day, they exhibited a reversal in the elevated levels of RUNX2 and YAP two days after being transferred to soft substrates. However, after being cultured on the stiff substrate for five days, the changes in nuclear levels became irreversible (Yang et al., 2014). Similarly, the expression of α SMA in some fibroblasts also depends on the priming time. Hence, cells that were primed on stiff conditions for 5 days or less could lower their α SMA levels after being transferred to soft conditions, but longer priming times led to irreversible changes (Walker et al., 2021). These results indicate that when the

cell adapts to stiff conditions, it stores its mechanical phenotype somewhere. The molecular mechanisms behind this process are still unknown, but some early identified mechanisms include changes in small non-coding RNA (miRNAs) molecules and epigenetic chromatin alterations.

miRNAs are short RNA molecules that do not code for proteins, but they can control gene expression by binding to mRNA molecules that have complementary sequences (Fig. 2d, inset iii). This can cause mRNA to be degraded, inhibited, or destabilized (Chuang and Jones, 2007). Following the evidence that miRNAs regulate fibrotic cell programs, Xi Li et al. showed that miRNA-21 was a key regulator of the mechanical phenotype (Li et al., 2017). During stiff priming, the miRNA-21 levels in MSC increased gradually due to MRTF-A regulation and stayed high for 2 weeks even after the stiff environment was removed. Knocking down miRNA-21 levels at the end of the stiff exposure was enough to eliminate the stiff mechanical phenotype. Interestingly, reducing miRNA-21 levels improved the therapeutic potential in animal models.

The reversibility of cellular mechanoadaptation may be influenced by other epigenetic factors, such as histone acetylation (Fig. 2d, inset iv). Histone acetylation is a process that involves the addition or removal of acetyl groups to the amino-terminal tails of histone proteins, which are the main components of chromatin (Sharda et al., 2019). This modification affects the accessibility of transcription factors and other regulatory molecules to the DNA. Histone acetylation is controlled by two types of enzymes: histone acetyltransferases (HATs), which add acetyl groups, and histone deacetylases (HDACs), which remove them. The balance between HAT and HDAC activity determines the level of histone acetylation and the expression of genes involved in cellular mechanoadaptation. Nuclear deformation alters the activity of HAT and HDAC, but the effect seems to depend on the type of stimulus and cell type. For example, hMSCs grown on stiff surfaces have less HDAC activity, which leads to more open chromatin and osteoblast differentiation (Killaars et al., 2020). When the mechanical link between the cytoskeleton and the nucleus is disrupted, the chromatin becomes more condensed, and the cells do not differentiate into osteoblasts. This can be reversed by artificially inhibiting HDAC activity with Trichostatin A (TSA). Conversely, mechanical tension increases HDAC activity in fibroblasts, resulting in more condensed chromatin and myofibroblast transformation. Myofibroblasts can return to their normal state by inhibiting HDAC with the TSA and culturing them on soft substrates (Walker et al., 2021). The distinct roles that chromatin condensation plays between hMSCs and fibroblasts highlight the cell-type-specific nature of mechanoadaptation and underscore the intricate role played by histone acetylation in this process.

Besides histone acetylation and miRNAs, other epigenetic mechanisms likely maintain the mechanical phenotype of cells. One such mechanism is DNA methylation, which could provide a more stable phenotype than histone alterations. Although no direct evidence links DNA methylation to the maintenance of the mechanical phenotype, an increasing body of research suggests its involvement. Indeed, several studies have reported aberrant methylation patterns in cells cultured on tissue culture polystyrene (TCPS) (Franzen et al., 2021; Sturm et al., 2019). Similarly, diseases characterized by increased tissue stiffness, such as fibrosis or cancer, have been associated with changes in DNA methylation (Das and Singal, 2004; Lyu et al., 2023). Another possible mechanism is the mechanical force itself. The nucleoskeleton-cytoskeleton (LINC) linker connects the cytoskeleton to the nuclear lamina, allowing bidirectional force transmission (Bouazid et al., 2019). The LINC complex plays a significant role in chromatin condensation. Recent studies showed that exogenous local forces applied for 2 to 10 min to the cell, decondensed chromatin, and increased chromatin and nucleoplasm proteins within the nucleus. This leads to elevated diffusivity of single protein molecules in the nucleoplasm, tens of minutes after cessation of the exogenous force (Rashid et al., 2023). This effect was so powerful that it counteracted the action of demethylase inhibitors. Moreover, newly identified mechanosensitive mechanisms,

such as force-induced mitochondrial changes in structure and dynamics, could also contribute to the irreversibility of mechanical adaptation (Bartolák-Suki et al., 2017). Collectively, results imply that multiple factors, such as histone acetylation, miRNAs, the LINC complex, and possibly, DNA methylation and mitochondria mechanosensitivity, regulate the mechanical phenotype of cells. However, ascertaining the exact mechanisms and interactions of these factors need further investigation.

7. Besides mechanoadaptation, are we (involuntarily) selecting cells?

We have seen that cells mechanoadapt to stiff mechanical environments by modifying their adhesion patterns, cytoskeleton architecture, nuclear shape, and epigenetic landscape. Some of these changes persist even after transferring cells to a more physiological soft environment (Price et al., 2021). Yet, mechanoadaptation is not the only process that can adapt cells to specific mechanical conditions. Another mechanism, cell selection, could favor specific phenotypes over others depending on the mechanical environment. Primary cells are isolated directly from donor animals or humans, usually by dissecting a tissue of interest and placing it in a TCPS dish, sometimes with enzymatic solutions that digest ECM (Richter et al., 2021). This is called explant culture. Over time, primary cells migrate out of the explant to the surface of the dish (Fig. 3a). Cells unable to adhere to the stiff surface of the dish remain poorly adhered, and they are usually eliminated when the cell media is replaced (Fig. 3b). After several passages, only the cells with strong adhesion reinforcement remain on the TCPS dish (Fig. 3c). Therefore, current protocols for isolating primary cells could select cell populations with a strong adhesion reinforcement, which are most likely to adhere to TCPS dishes.

For this involuntary selection process to happen, cells must display mechanical heterogeneity, a phenomenon demonstrated by many experiments. For instance, epithelial tissues under uniform pressure accommodate deformations with a very heterogeneous response, with some cells reaching strains up to five times greater than the average cell strain (Latorre et al., 2018). In another study, the application of a microfluidic flow was able to separate two cell populations from genotypically similar cells (Yeoman et al., 2021). These two populations

displayed different adhesion patterns, migratory speed, and contractility, and had a different ability to respond to stiffness gradients. Moreover, tumors exhibit a large degree of heterogeneity that can affect the response to therapy and the progression of the disease (Fisher et al., 2013). Despite evidence of mechanical heterogeneity in tissues, it is unclear how our tissue culture techniques select specific cell populations, favoring those cells more adapted to a particular mechanical environment.

A possible way to alter the selection pressure is to avoid plastic when isolating primary cells, a procedure carried out in few studies. In one of them, the authors observed that primary mouse mammary gland stromal fibroblasts were able to spread efficiently, generate high forces, and display nuclear YAP on soft collagen-coated substrates, resembling the soft mammary gland tissue (Lerche et al., 2020). This contrasts with immortalized cell lines in which cell spreading increases with cell area, and YAP translocates to the nucleus at specific stiffness. In another study, the authors isolated primary human colon cells from healthy and cancerous surgical human samples avoiding TCPS (Ali et al., 2015). They observed that at 10 kPa stiffness substrate, cancer cells were much more spread than healthy cells and they were able to exert much larger forces. This difference aligns with the concept that the stiffness of the tumor microenvironment mechanically primes cancer cells (Panciera et al., 2020; Cambria et al., 2024). Finally, a study comparing the hanging drop technique to conventional substrate-supported culture revealed that tissues exhibited distinct patterns of actomyosin contraction in response to chemical treatments (Gullekson et al., 2017). These studies indicate that plastic-free protocols provide primary cells with a different mechanobiology than standard culture techniques, but the role of cell selection is still unclear. A potential approach to address this question is to compare the mechanobiology of cells that adhere to substrates with varying stiffness and ECM composition with cells that remain in suspension. While this study could be technically challenging, it would reveal whether substrate adhesion *per se* is selecting specific cell phenotypes.

8. Strategies to mitigate stiff mechanoadaptation in cell culture

Using intact organisms as models may be the most effective strategy to preserve the mechanical niche of cells. Over the last decade, various

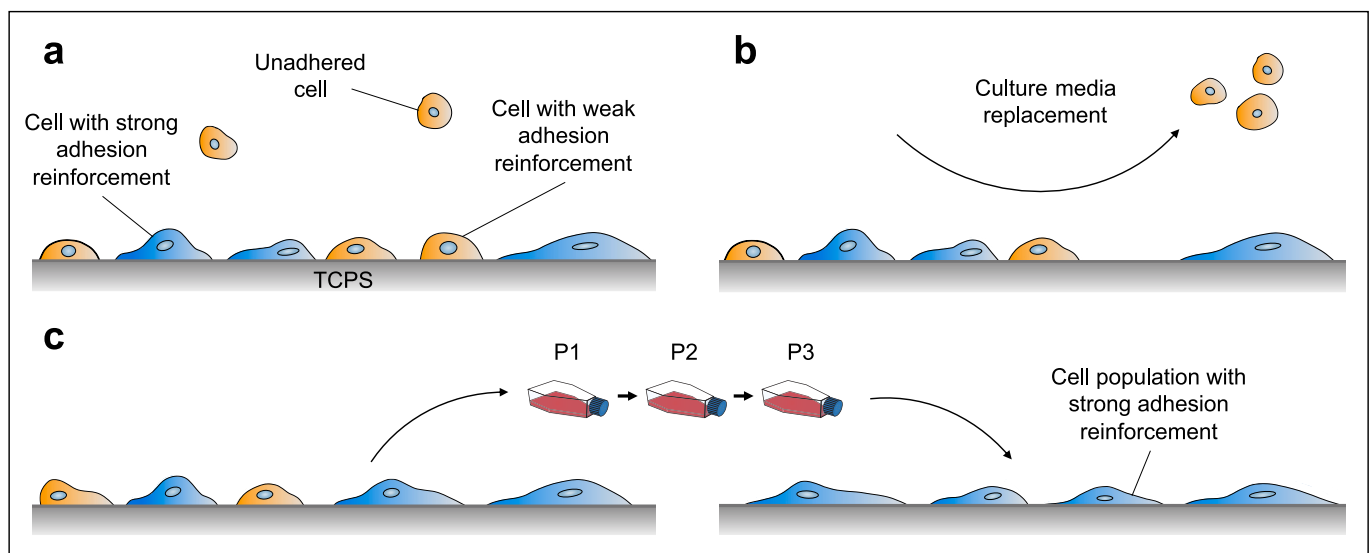


Fig. 3. Cell selection and mechanoadaptation when isolating primary cells. This figure illustrates how primary cells isolated from a tissue explant are affected by the stiffness of the culture surface. (a) A heterogeneous population of primary cells is cultured on a tissue culture polystyrene (TCPS) dish. Cells with strong adhesion reinforcement (blue cells) attach firmly to the stiff surface, while cells with weaker adhesion reinforcement (orange cells) have a lower attachment strength. (b) When the media is replaced, some of the cells with weaker adhesion strength are washed away. (c) After several passages, only the cells with strong adhesion reinforcement remain on the TCPS dish. In parallel, cells adhered to TCPS mechanoadapt to the stiff environment as shown in Fig. 2.

methods have been developed to investigate how cells sense and respond to mechanical forces in living organisms, including *Xenopus*, *C. elegans*, Zebrafish, and *Drosophila*, providing valuable insights into cellular forces in their native environment (Gómez-González et al., 2020). Some of these techniques, such as optical tweezers (Favre-Bulle et al., 2019; Català-Castro et al., 2022), Brillouin microscopy (Prevedel et al., 2019) and atomic force microscopy (AFM) (Krieg et al., 2019), have been adapted to measure the mechanical properties of tissues inside organisms. For example, AFM was used to demonstrate that the neural crest, an embryonic population, self-generates a stiffness gradient in the adjacent placodal tissue, and follows this gradient by a process called durotaxis (Shellard and Mayor, 2021). Besides the measurement of the mechanical properties of living tissues, other techniques, such as droplet inclusions, can measure stresses in the tissue. This approach consists of introducing force transducers into the tissue and measuring their deformations with a microscope (Campàs et al., 2014). These techniques not only allow a passive measurement of the stresses in the tissue. The use of liquids such as ferromagnetic fluids allows the application of forces. With this technique, Mongera and co-workers found that cells in zebrafish presomitic mesoderm differentiate by probing the stiffness of the supracellular tissue architecture, with stress relaxation leading to a perceived microenvironment stiffness that decreases over time (Mongera et al., 2018). Despite measuring cells in their native mechanical state, these techniques and models are not yet widely adopted in the scientific community. Moreover, their utility is limited when it comes to studying specific diseases that affect particular tissues such as lung fibrosis, or that simply do not lend themselves well to study in model organisms, like certain genetic disorders or rare diseases (Marian, 2011). For this reason, *in vitro* culture remains indispensable. Alongside this, strategies to minimize the stiff mechanoadaptation of cells are needed.

One solution to minimize cell transformation in cultures would be to maintain them in plastic-free environments resembling their natural niches. This, however, is technically challenging for several practical reasons. First, the fabrication of soft substrates for cells is time-consuming and expensive for regular cell culture. Second, cells on soft substrates tend to proliferate slower than those on plastic, which can delay experiments (Hadjipanayi et al., 2009). Third, besides the stiffness of the environment, other factors such as the ECM architecture and the soluble signals are difficult to replicate. Fourth, cells might remodel their environment over time, depositing ECM and altering the mechanical conditions of the culture (d'Alessandro et al., 2021). Finally, cell retrieval on 3D cultures can be challenging, limiting the downstream experiments (Abuwatfa et al., 2024). Although these limitations have not been fully addressed, some strategies have been proposed to mitigate mechanoadaptation to stiff culture conditions.

The first of these strategies is to mechanically prime cells under soft conditions before experiments (Fig. 4a). This method, widely applied in studies of mechanical memory, has shown to improve the therapeutic potential of MSCs (Li et al., 2017; Killaars et al., 2018; Scott et al., 2023; Nonaka et al., 2020), decrease the invasiveness of cancer cells (Watson et al., 2021), and reduce myofibroblast activation (Balestrini et al., 2012; Walker et al., 2021). However, as discussed above, soft mechanical priming cannot fully revert prolonged culture on TCPS. Consequently, a second strategy targets the epigenetic factors that maintain the stiff mechanical phenotype, for example, by lowering miRNA-21 levels or inhibiting HDACs with TSA (Fig. 4b). These methods have been shown to restore the therapeutic potential of MSCs (Li et al., 2017) and prevent myofibroblast activation (Killaars et al., 2018). This approach is promising for cell therapy but has not been explored beyond mechanical memory studies. Finally, a third strategy exploits the fact that the acquisition of a stiff phenotype is mediated by contractility (Fig. 4c). Thus, culturing Cal27 cells on stiff substrates with contractility inhibitors produced a similar invasive phenotype to those cells primed on soft substrates (Moon et al., 2023). This simple approach does not need soft hydrogels that mimic the physiological ECM environment.

However, its long-term effects are unknown and need further investigation.

To see how these strategies are being applied, we can look at the emerging field of organoid culture, where maintaining the mechanical phenotype is essential for the development of functional organoids. Organoids are often cultured in ECM variants that mimic the mechanical properties of the native tissue environment. The most common one is Matrigel, a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Matrigel offers a microenvironment rich in ECM proteins such as laminin, collagen IV, and entactin, as well as various growth factors (Passaniti et al., 2022). Serving as a scaffold, Matrigel facilitates the three-dimensional architecture of organoids, including porosity and stiffness, which are key for cell signaling and organoid integrity (Fig. 4d). Besides Matrigel, hanging drop techniques (Kyriakopoulou et al., 2023) (Fig. 4e) and PEG-based scaffolds are being increasingly used to overcome the batch variability of Matrigel (Kratovichil et al., 2019; Kozłowski et al., 2021). Besides these scaffolds, studies have shown that applying mechanical forces, such as cyclic stretch, can enhance the expansion and regeneration of organoids (Meng et al., 2022) and improve the therapeutic potential of MSCs (Nonaka et al., 2020) (Fig. 4f). This approach is particularly relevant for organoids derived from tissues that are naturally subjected to mechanical forces, such as heart, lung, muscle, tendon or intestine.

Besides the mechanical niche, in living organisms, cells receive signals from the environment through the ECM or other cells. Organoid culture mimics these signals by either growing different types of cells together or adding specific factors to the culture medium (Zhao et al., 2022) (Fig. 4g). One example is the culture of alveolar organoids. Alveoli are spherical structures in the lungs that facilitate gas exchange between the pulmonary capillaries and the alveolar air. Initially, alveolar organoids were grown by co-culturing alveolar type II cells, which are progenitor cells in the lungs, with fibroblasts expressing PDGFR α , a protein that marks the stem cell niche (McQualter et al., 2010; Demchenko et al., 2022). By analyzing the interactions between alveolar type II cells and fibroblasts, Katsura et al. identified several ligands that were expressed by fibroblasts and recognized by alveolar type II cells, as well as some antagonists that inhibited their communication (Katsura et al., 2020). Based on this information, they developed a culture medium that could support a non-differentiated population of alveolar type II cells and the formation of alveospheres without the need for fibroblast co-culture. In the future, organoid techniques could help preserve the native mechanical phenotype of immortalized cells.

9. Conclusions and perspective

The evolution of cell culture techniques from their early days a century ago to the widespread adoption of TCPS has shaped our understanding of cellular behavior in health and disease. However, the transition from native tissues to artificial substrates triggers significant alterations in the cellular phenotype. This issue particularly affects immortalized cell lines, which are usually cultured in conventional plastic substrates and are the most accessible cells of modern biomedical research.

Current culture methods necessarily induce adhesion reinforcement, a process that enables cells to attach to rigid substrates by increasing their adhesion points, strengthening their cytoskeleton with actin fibers, translocating transcription factors in the nucleus and modifying its epigenetic landscape. These changes persist even when cells are transferred to softer environments, creating so-called “mechanical memory”. These changes, however, are not merely a reflection of previous mechanical signals, but rather a continuous adaptation to the stiff culture conditions. Intriguingly, this mechanoadaptation may be accelerated by another factor: cell selection. Current cell isolation techniques, especially those based in explant culture on TCPS, may unintentionally select for cell subpopulations that exhibit strong adhesion reinforcement, further altering the forces displayed by cell lines, potentially resulting in

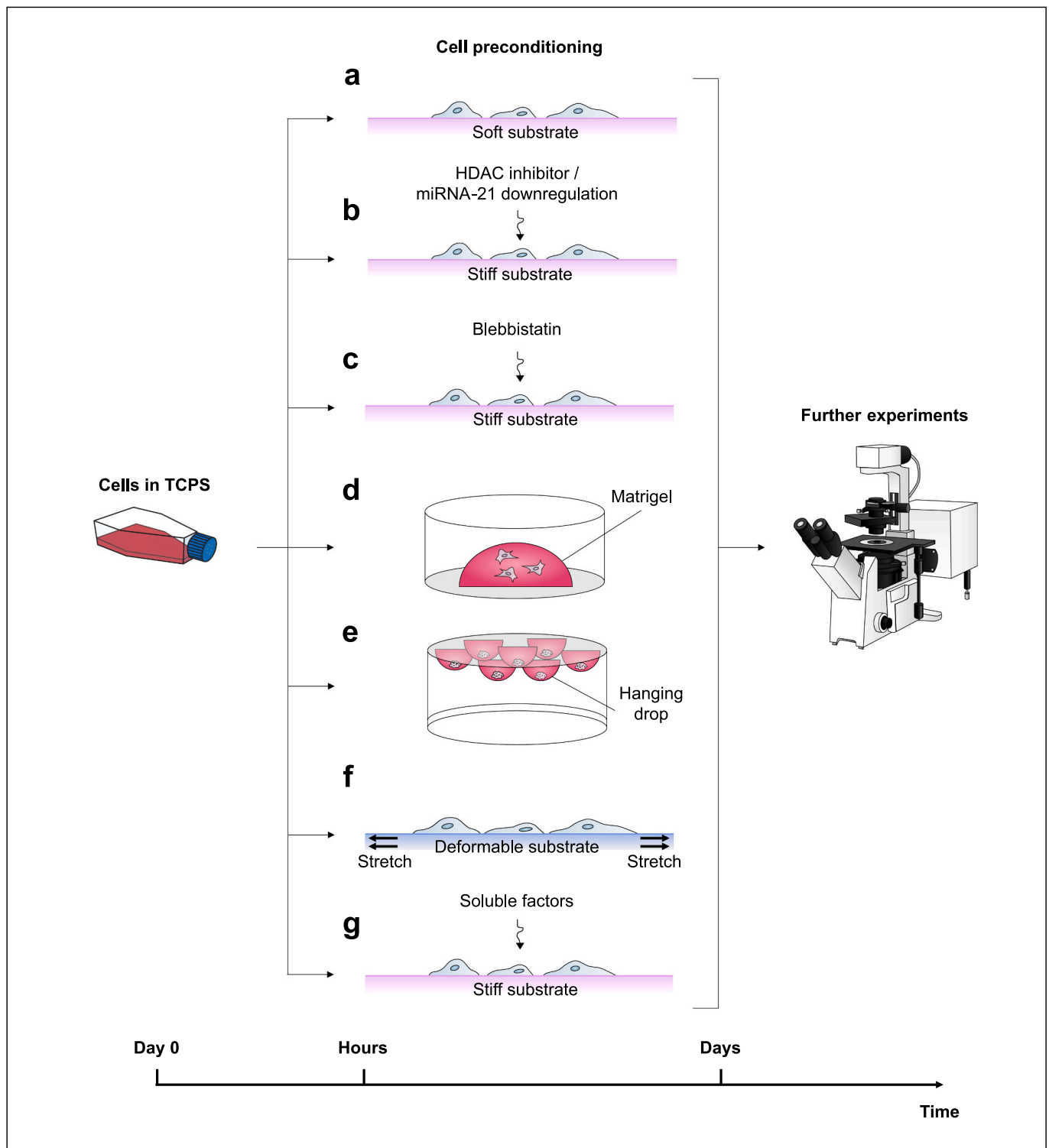


Fig. 4. Strategies to mitigate stiff mechanoadaptation in cell culture. (a) Mechanical priming on soft substrates: Cells are primed on soft conditions before experiments for a period that can range from a few hours to several weeks. This method can enhance the therapeutic potential of mesenchymal stem cells (MSCs) and lower the invasiveness of cancer cells, but it cannot revert the effects of long-term culture on tissue culture polystyrene (TCPS). (b) Targeting epigenetic factors: Modulating epigenetic factors such as reducing miRNA-21 levels or inhibiting histone deacetylases (HDACs) can restore the soft mechanical phenotype of cells. The latter method exhibits varying effects depending on cell type. (c) Inhibiting contractility: Culturing cells on stiff substrates with contractility inhibitors such as blebbistatin can mimic soft substrate effects, simplifying the culture process without needing soft environments. Long-term effects require further investigation. (d) Matrigel and extracellular matrix (ECM) mimic: Matrigel is commonly used in organoid culture to offer a 3D environment with mechanical properties that resemble physiological conditions. Recent efforts have aimed to create a synthetic ECM that replicates Matrigel. (e) Hanging drop culture: the hanging drop is a matrix-free technique that permits cells to self-aggregate and form spheroids, a structure that mimics the cell niche *in vivo*. (f) Application of mechanical forces: cells from organs that experience stretch such as the lungs and heart may need the application of mechanical forces to reproduce their physiological niche. (g) Adding soluble factors: the addition of specific factors may be needed for preserving the mechanical phenotype. These factors can be provided either by neighboring cells in a co-culture or by simple addition of these factors to the cell medium.

methodology-induced bias when interpreting cell mechanobiology at the physiological level.

Various strategies have been proposed to mitigate the effects of stiff mechanoadaptation in cell culture, including mechanical priming on soft substrates, targeting epigenetic factors, and modulating cell contractility. Moreover, the emerging field of organoid culture offers exciting prospects for preserving the natural mechanical phenotype of cells *in vitro*. These approaches show promise in restoring cellular function and phenotype closer to physiological conditions, offering new avenues for improving cell-based therapies and experimental models. Overall, we can improve the truthfulness of the forces derived from our cell models by gaining a deeper understanding of how our cell culturing techniques alter the mechanobiology of cells and how these alterations can be mitigated.

CRedit authorship contribution statement

Marina Moro-López: Writing – review & editing, Visualization. **Ramon Farré:** Writing – review & editing. **Jorge Otero:** Writing – review & editing, Supervision, Conceptualization. **Raimon Sunyer:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Writeful and MS Copilot (free versions) to improve the clarity of certain sentences. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication. The use of AI tools was limited to improve writing and all ideas presented in this work remain original.

Data availability

No data was used for the research described in the article.

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