Metabolic and Mechanical Cues Regulating Pluripotent Stem Cell Fate

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The ability to shift between metabolic states and to tightly regulate cellular mechanical properties have been described as crucial events in the achievement of correct embryonic development. Indeed, metabolic and mechanical manipulations in vitro have led to the discovery of new methods to control cell fate. As these two modulators are usually studied separately, in this review article, we describe how cellular mechanics and metabolic characteristics regulate embryonic development in vivo and describe the role of these cues in the regulation of pluripotency and differentiation in vitro. We also pinpoint possible connections between metabolism and mechanotransduction, highlighting recent findings in the Yes-associated protein, phosphoinositide 3-kinase, and AMP-activated protein kinase signaling pathways, and how they may be relevant in modulating cell fate in other contexts.

Embryonic Development

Embryogenesis is a highly complex and dynamic, yet precise process. It begins with fertilization of the oocyte with a sperm cell, which gives rise to a totipotent cell, the zygote. This cell has the potential to produce all embryonic and extraembryonic lineages [1]. Importantly, embryogenesis is a continuous process. As development takes place cells face important fate decisions, which culminate in a new individual organism (Figure 1).

The need for a better understanding of early mammalian development has led scientists to look for in vitro models that can recapitulate cell pluripotency, commitment, and differentiation. Thus, different cell populations have been successfully isolated in vitro including mouse embryonic stem cells (mESCs) [4,5], human ESCs (hESCs) [6], induced pluripotent stem cells (iPSCs) reprogrammed from somatic mouse and human cells [7,8], and epiblast (EPI)-derived stem cells (EpiSCs) [9,10] (Figure 1). While ESCs are derived from preimplantation blastocysts, EpiSCs are obtained from the post-implantation EPI and iPSCs are reprogrammed from somatic cells (Figure 1). These cell models have led to the recognition that maintenance of each developmental stage is achieved through a balance of transcription factor networks (Figure 2). How these different transcription factors are maintained or modulated is still not completely understood. Biochemical modulators, such as leukemia inhibitory factor (LIF) and inhibitors of glycogen synthase kinase-3 (Gsk3) and nitrogen-activated protein kinase kinase (MEK), maintain mESCs in a pluripotent state, while their withdrawal leads to differentiation [11]. Moreover, the successful isolation of EpiSCs and iPSCs in culture led to the ability to compare different cell states and to the discovery that metabolism (see Glossary) is a keystone in stem cell fate, responsible not only for the production of energy but also for the synthesis of precursors, which are crucial for sensing the energetic and redox status of the cell in the developmental process, chromatin remodeling, and gene transcription regulation. Finally, the discovery that the physical characteristics of culture conditions, notably that substrate

Highlights

Metabolic and mechanical cues control stem cell fate in vivo and in vitro.

There is a synergistic intertwining of metabolism, stem cell fate, differentiation, and reprogramming.

The mechanical properties of embryonic cells are crucial for their precise development. Recent knowledge of the mechanical properties at different cell stages of embryonic development has brought to light the role of mechanosensing in cell fate decision.

In vitro, biophysical cues are key modulators in ESC fate and cellular reprogramming.

The connection between metabolic and mechanotransduction signaling pathways is emerging as key in determining cell fate but remains largely unexplored.

Evidence suggests that YAP, MAPK, and PI3K signaling pathways act as molecular crosstalk between metabolism and mechanotransduction.

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**stiffness**, can regulate the fate of mesenchymal stem cells (MSCs) [12] opened a new research window that led to the discovery that physical modulators, such as stiffness and topography, are able to control ESC fate [13,14]. In this review article, we highlight the importance of metabolism and mechanical cues in cell fate regulation.

**Metabolism: An Important Player in Stem Cell Fate**

Cells rely on two processes to produce energy in the form of ATP: glycolysis and oxidative phosphorylation (OXPHOS; Box 1). In addition to its role in energy production, glycolysis and the Krebs cycle provide intermediates for other molecular pathways. Cells have the plasticity to shift between metabolic pathways to match their requirements for ATP and cell precursors. Nonetheless, most differentiating cells rely on OXPHOS to achieve their energetic demands.

**Glycolysis and OXPHOS in Embryonic Development**

During embryonic development, metabolic pathways are tightly regulated to satisfy demands for both energy and precursor molecules, which are essential for cellular replication and specialization (Figure 1). It all starts before fertilization, during oogenesis, where a boost in mitochondria biogenesis results in a two- to threefold increase in mitochondrial content [16]. Mitofusin-2 regulates oocyte development as its downregulation reduces mitochondrial membrane potential as well as fertilization and cleavage rate [16]. Since mitochondria do not replicate between fertilization and the blastocyst stage, the increase in mitochondrial content before fertilization is crucial to ensure sufficient mitochondrial number and to meet energetic and precursor demands in all embryonic cells [17].

After fertilization, the embryo relies on OXPHOS energy metabolism (Figure 1) [18,19]. In the early embryonic stages, mitochondria are small, globular shaped, and contain poorly developed cristae; nevertheless, they are still active. After several embryonic divisions, the mitochondrial content in each cell declines [20], and at the morula state, there is an increase in glucose transporters in each cell, which gradually shifts the embryo to a glycolytic phenotype with the preimplantation EPI combining an OXPHOS and glycolytic metabolism (Figure 1) [19–21]. After implantation, glycolysis is the major metabolic pathway in the EPI, where glucose is metabolized to lactate, presumably due to the hypoxic uterine wall environment. OXPHOS is reestablished and mitochondria biogenesis and maturation occur again after blood flow begins in the embryo [22] (Figure 1). Importantly, when comparing the different in vivo embryonic states with in vitro-derived ESCs and EpiSCs, equivalent mitochondrial morphologies and metabolism requirements are seen: ESCs seem to retain similar characteristics to the preimplantation inner cell mass (ICM), while EpiSCs are similar to the post-implantation EPI [23] (Figure 1).

**Glycolysis and OXPHOS in ESCs and EpiSCs**

Naïve mESCs have small and spherical mitochondria, with immature cristae and reduced mitochondrial DNA (mtDNA) copy number, equivalent to what is found in the preimplantation ICM cells in the blastocyst. Nevertheless, these cells have a bivalent metabolism and can switch between glycolysis and OXPHOS [23]. Importantly, mitochondrial function is dependent not only on the expression of mtDNA genes but also on the expression of nuclear genes. For instance, using mESCs as a model system, signal transducer and activator of transcription 3 (Stat3), a downstream effector of the LIF pathway, bound mtDNA and increased the expression of mitochondrial transcripts, enhancing OXPHOS metabolism [24]. These results suggest a synergetic role between the mitochondria respiratory chain and the nuclear transcription of pluripotent genes mediated by Stat3.

Similar to the post-implantation EPI, mEpiSCs have a more mature mitochondrial network, but still less mature than those found in differentiated cells [23]. mEpiSCs are more glycolytic;
consequently, their basal and reserve levels of respiration are lower than that of naïve mESCs [23]. These features have been related to the low expression of multiple nuclear-encoded proteins of the respiratory chain complexes in EpiSCs, namely, cytochrome c oxidase (from complex IV), and nicotinamide adenine dinucleotide (NADH) dehydrogenase (from complex I), compared to mESCs [23]. In addition, hypoxia-inducible factor 1α (Hif1α) levels are increased in mEpiSCs compared to mESCs [23]. Hif1α is a transcription factor that acts as an oxygen sensor inside the cell. Low levels of oxygen promote the expression of Hif1α, which controls the expression of essential genes that promote glycolysis, such as lactate dehydrogenase A, responsible for the conversion of pyruvate to lactate; and pyruvate dehydrogenase kinase 1 (PDK1), responsible for the phosphorylation of pyruvate dehydrogenase (PDH) and, consequently, its inactivation [25]. Interestingly, ectopic expression of Hif1α or reduced oxygen concentrations promote the transition of mESCs from the naïve state to the primed state, which is the first step towards pluripotent cell differentiation [23,26]. In addition, dichloroacetate, an inhibitor of PDK1 and therefore an activator of the PDH complex, promotes differentiation of mESCs, suggesting PDK as a metabolic gatekeeper of pluripotency [27]. Also, 3-bromopyruvate, an inhibitor of hexokinase I and glyceraldehyde-3-phosphate dehydrogenase, which are crucial enzymes in the glycolytic pathway, led to a metabolic switch and loss of pluripotency, even in the presence of pluripotent cell culture conditions [28]. Stressing the notion that reduction of OXPHOS in the primed state is essential for maintenance of pluripotency, antinycin A (AA), an inhibitor of complex III of the electron transport chain (ETC), is able to maintain pluripotency in hESCs and inhibit differentiation of mESCs [29–32].

Epigenetic Modulators and Reprogramming

Besides its role in energy production, metabolism is also implicated in the generation of cellular building blocks involved in epigenetic regulation. Glycolysis and the Krebs cycle provide precursors for the synthesis of nucleotides, non-essential amino acids and lipids, used to produce macromolecules essential for cell division, as well as intermediates used in enzymatic reactions, such as acetylation and methylation (Figure 3, Key Figure) [33,34]. For instance, acetyl-coenzyme A (CoA) contributes not only to the Krebs cycle but also to the synthesis of lipids and acetylation of amino acid residues, such as lysine, on histones and other proteins [35,36], while α-ketoglutarate, a Krebs cycle intermediate, contributes to the demethylation of DNA and histones [37].

The acetylation/deacetylation of histones regulates global chromatin architecture and, consequently, the control of gene transcription and stem cell fate. There are two main protein families involved in the control of this process: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of histones is associated with open chromatin structures and plays a critical role in the maintenance of stem cell pluripotency [38]. Inhibition of glycolytic enzymes decreases histone acetylation, connecting glycolytic production of acetyl-CoA with histone acetylation and the maintenance of the pluripotent state [36]. By contrast, HDAC promotes histone deacetylation, which is one of the primary features upon ESC differentiation [36,39]. HDACs are divided in two main groups: ‘classical’ HDACs, which are Zn²⁺ dependent, and sirtuins, which are dependent of the NAD⁺ pool. Shifts from anaerobic (glycolysis) to aerobic (OXPHOS) metabolism and vice versa are linked to changes in the NAD⁺/NADH ratio, which make sirtuins important cellular metabolic sensors [40].

Methylation is another epigenetic mark that plays a crucial role in the maintenance of stem cell pluripotency. Methylation/demethylation promotes a modulation of chromatin structure, and this process is tightly interconnected with the metabolic status of the cell [41]. DNA and histone methylation are regulated by DNA methyltransferases (DNMTs) and histone

Glossary

Anabolic pathways: pathways responsible for the production of macromolecules, such as nucleotides, amino acids, and lipids.

Anabolic pathways require energy from ATP or nicotinamide adenine dinucleotide (NADH).

Blastoid: an in vitro 3D combination of ESCs and trophoblast stem cells that resembles the transcription profile and the structure of the blastocyst.

Blastomere compaction: process in which blastomeres flatten against each other, their cell-cell contact is maximized, and they can no longer be distinguished morphologically. This takes place before the morula stage of embryo development.

Catabolic pathways: pathways that break down molecules into smaller units to produce energy or building blocks.

Gastruloid: an in vitro multicellular model of gastrulation.

Glycolytic flux: the rate at which molecules progress through glycolysis.

Metabolism: combination of anabolic and catabolic mechanisms that occur inside a cell.

Naïve state: ESCs capable of unlimited self-renewal capacity and able to differentiate into all three germ layers in vitro and to form teratomas and chimeras in vivo. The earliest stage of pluripotency.

Organoid: an in vitro multicellular structure that contains many cell types resembling a specific adult organ.

Pluripotent cells: cells that can differentiate in vitro as well as in vivo, giving rise to all three embryonic lineages and germ cells, as well as being capable of self-renewal.

Primed state: ESCs that are not capable of forming chimeras in vivo, although they are still able to form teratomas and differentiate into all three germ layers in vitro. A later stage of pluripotency.

Self-renewal: cells that can proliferate indefinitely, while maintaining pluripotency.

Stiffness: reported in Pascal (Pa) units, it is the ability of a material to resist to a force. It is formally defined as Young’s elastic modulus (E) and results from the relationship between the force applied to a defined area.
methyltransferases, respectively. In ESCs, the lack of DNMTs, for example, promotes maintenance of pluripotency and self-renewal, while these cells fail to differentiate [42,43]. Histone demethylases can be lysine-specific demethylases, which target histone 3-lysine 4 residues and histone 3-lysine 9 residues and require flavin adenine dinucleotide to catalyze this process, or lysine and arginine demethylases, which require α-ketoglutarate to promote demethylation [41]. α-Ketoglutarate is a cofactor of α-ketoglutarate/Fe²⁺ dioxygenases (enzymes responsible for histone and DNA demethylation), whereas succinate is a competitive inhibitor of these enzymes. Thus, in ESCs, an elevated α-ketoglutarate-to-succinate ratio, maintained by glucose or glutamine metabolism, promotes pluripotency through histone and DNA demethylation [37]. Furthermore, this demethylation pattern matches the epigenetic markers characteristic of pluripotency in ESCs that are responsible for the suppression of differentiation and expression of pluripotent genes.

Epigenetic regulation is also crucial during somatic cell reprogramming to iPSCs. Upon induction of pluripotency, cells must remodel their chromatin to reactivate pluripotency-associated genes, while silencing differentiation-related genes. Metabolically, the increase of PDK and hexokinase II or the inhibition of the ETC through AA or rotenone increases reprogramming efficiency [44,45]. Moreover, reprogramming factors, with the exception of Oct4, can be replaced by stimulating glycolytic gene expression through the activation of key metabolic enzymes [46], suggesting that metabolism and the cell state are also interconnected during cellular reprogramming [47].

In addition to metabolism, biophysical cues influence somatic cellular reprogramming to iPSCs (for a detailed review, see [48]). For example, 2D soft matrices as well as 3D matrices of defined stiffness increase the reprogramming efficacy of fibroblasts through the facilitation of mesenchymal-to-epithelial transition (a crucial step for the success of reprogramming) [14,49]. Accordingly, substrate topography such as microgrooves (10 μm in width) or aligned nanofibers significantly improve reprogramming efficiency by promoting chromatin structure rearrangement [13]. These conditions induce the reorganization of the cytoskeleton and actin-myosin contractility, which results in the alteration of key epigenetic regulators of expression, including a decrease in HDAC activity and an increase in methyltransferase activity (WD repeat domain 5) and consequently increases in histone H3 acetylation and di- and trimethylation of lysine 4, respectively [13]. iPSCs formed by the combination of gene transfer and soft substrates or specific substrate topography are able to generate teratomas in vivo and embryoid bodies in vitro, both of which are hallmarks of pluripotent cells [13,14]. The inhibition of actomyosin contractility with blebbistatin results in the reduction of epigenetic changes and reprogramming efficiency, which suggests that the cell epigenetic state is also regulated by mechano-transduction processes [13].

Interestingly, studies focused on metabolism and iPSC formation suggest that the glycolytic shift occurs before expression of pluripotency genes, reinforcing metabolism as a key player in cellular fate [50,51]. In addition, studies focused on the role of mechanotransduction and iPSC formation propose that gene transfer and biophysical cues synergistically stimulate pluripotent gene expression, and the increase of reprogramming efficacy. It will be interesting to address whether these two aspects are related, for example, if biophysical cues play a role in the glycolytic shift, and whether there is any synergy between metabolism and the mechanotransduction signal that could be tapped to increase cellular reprogramming efficiency.

Mechanical Cues: A Forgotten Piece in Regulating Stem Cell Fate
Mechanical cues have recently been recognized as important regulators of cell fate. Changes in extracellular matrix (ECM) mechanics, substrate stiffness, topography, and physical forces such as shear stress, tension, and compression are examples of external mechanical cues that

(stress) and the resultant deformation of the material (strain). A stiff matrix is more resistant to deformation than a soft matrix.

Teratoma: a multicellular type of rare tumor used as an in vivo pluripotency assay, the only one that can be performed with human cells. The teratoma assay assesses the ability of cells to spontaneously differentiate into tissues from the three germ layers when injected into immunocompromised mice. If they are pluripotent cells should always form teratomas.
Figure 1. Embryonic Development in Mammals and In Vitro-Derived Cells: From Preimplantation to Gastrulation. (A) Schematic of cell fate decisions that lead to the formation of the three embryonic germ layers and the extraembryonic lineages. After fertilization, the zygote is formed. The zygote gives rise to two cells, four cells, and then eight cells that are morphologically distinct from each other. These cells rely mostly on oxidative phosphorylation (OXPHOS) for ATP. At the eight-cell stage, the cells undergo compaction, polarization, and asymmetric cell division, giving rise to a compact morula, and the first cell-fate decision occurs: trophectoderm (TE) (green) and inner cell mass (ICM) (purple) are formed. At the 32-cell stage, the blastocoele cavity starts to form inside the embryo, giving rise to the blastocyst, where the second cell-fate decision occurs: ICM gives rise to the epiblast (EPI) (orange) and the primitive endoderm (PrE) (blue) [2]. PrE is positioned facing the blastocoele cavity, while EPI is positioned between the PrE and the TE. At this stage, cells rely both on glycolysis and OXPHOS. After Epi, PrE, and TE specification, the blastocyst is ready to be activated and to implant. During implantation, glycolysis is the main source of ATP. After implantation, PrE gives rise to parietal endoderm (PE) and visceral endoderm (VE), and EPI will give rise to mesoderm, definitive endoderm, and ectoderm [3]. These cells have a shift in their metabolic requirements and rely mostly on OXPHOS. (B) Different pluripotent cell populations have been isolated and propagated under in vitro conditions. Embryonic stem cells (ESCs) are derived from the ICM of the preimplanted blastocyst, whereas epiblast-derived stem cells (EpiSCs) are derived from the post-implantation EPI. Similar to the ICM, ESCs rely both on glycolysis and OXPHOS, while EpiSCs shift their metabolism to glycolysis. Induced pluripotent stem cells (iPSCs) are a result of the molecular reprogramming of somatic cells in vitro by the forced exogenous expression of a combination of key pluripotent transcription factors such as Oct4, Klf4, Sox2, and c-myc. These cells shut down OXPHOS and rely on glycolysis as their main source of ATP.

Influence cell fate through a mechanotransduction process (Box 2). It is now accepted that mechanical cues work together with biochemical cues in the regulation of embryogenesis and organogenesis, while cell mechanical properties are associated with a readout of physiological functions or pathological features.

Mechanical Cues in Embryonic Development
Recently, mechanical characteristics have been taken into consideration and different methods have been developed to analyze oocyte and zygote quality. During fertilization, the sperm cell first penetrates the zona pellucida, a specialized extracellular matrix made of a network of glycoproteins that surrounds and protects the oocyte [52,53]. After gamete fusion, there is a triggering of biochemical signals that induces a 3D architectural change of the zona pellucida known as zona reaction or ‘hardening’ [53]. Mechanically, different studies have been conducted to analyze zona pellucida elasticity before and after fertilization [54–59]. Although different techniques were used, all studies show that the zona pellucida becomes stiffer after fertilization [54–59]. Moreover, the viscoelasticity properties of the oocyte after fertilization were recently associated with embryo viability [58,59]. These results suggest that the mechanical properties of the zona pellucida can function, in an early stage, as a predictive factor of embryonic development.
Figure 2. Role of Transcription Factors and Stiffness in Naive Pluripotency, Primed Pluripotency, and Somatic Lineage Differentiation. The main transcription factors involved in naive and primed mouse embryonic stem cell (mESC) pluripotency, as well as in lineage specification (mesoderm and ectoderm), are represented. The core pluripotency transcription factors Oct4, Sox2, and Nanog are represented in yellow. When together, Oct4, Sox2, and Nanog maintain ESCs in a pluripotent state. In blue are the transcription factors specific for the naive state of pluripotency, with black arrows representing some of the pathways that activate these genes. Specific primed transcription factors responsible for maintaining cells in a primed pluripotency state are represented in green. Upon differentiation, specific transcription genes are maintained in each lineage: (i) mesendoderm [Oct4, Sox17, Lefty2, Foxa2, T (T-brachury)] and (ii) ectoderm [Sox2, Fgf5, Nestin, and Zeb2]. While soft substrates (represented in light red) have been shown to induce naive pluripotency, the increase of stiffness (represented in dark red) has been correlated with somatic lineage differentiation.

The mechanical properties of embryonic cells are also crucial for their precise development and recent knowledge of the changes in mechanical properties during different stages of embryo development have brought to light the role of mechanosensing in cell fate decisions. For example, the evaluation of surface tension in the eight-cell stage blastomere, using a micropipette aspiration technique, demonstrated that the increase of surface tension, promoted by

Box 1. Summary of Glycolysis and OXPHOS

Glycolysis is the pathway responsible for the breakdown of one molecule of glucose into two molecules of pyruvate, and the production of two net molecules of ATP and two net molecules of NADH [21]. Pyruvate, the end product of glycolysis, can usually then go one of two ways: under anaerobic conditions, pyruvate is reduced to lactate that is excreted to the extracellular space; whereas under aerobic conditions, pyruvate is oxidatively decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex (PDH), in the mitochondrial matrix, with production of one NADH molecule. Acetyl-CoA is then the fuel of the Krebs cycle, located inside the mitochondria. In the Krebs cycle, three molecules of NADH, one moiety of flavin adenine dinucleotide (FADH2) of the succinate dehydrogenase enzyme, and one molecule of guanosine triphosphate (GTP) are produced from each acetyl-CoA that gets into the Krebs cycle [21]. At the end, from each glucose molecule, four molecules of ATP, ten molecules of NADH, and two reduced FADH2 (part of the enzyme succinate dehydrogenase) are generated. The NADH and succinate (FADH2) produced thus far are oxidized, and, on average, 34 additional ATP molecules per molecule of glucose are generated [21]. Therefore, mitochondria are known as the powerhouse of the cell. It should however be noted that many cells, including some types of stem cells, can carry out aerobic glycolysis, using the Krebs cycle mostly for precursor production, not ATP generation.
Figure 3. (1) The transmission of mechanical forces between adjacent cells. Cadherins are responsible for the force transmission between adjacent cells. Inside the cell, cadherins recruit catenins, which have associated cytoskeleton-binding proteins responsible for signaling transmission. The energy sensor AMP-activated protein kinase (AMPK) is also associated to the cadhesome (a pool of structural and regulatory protein components of the adherens junctions), and its activation promotes glucose uptake and consequently an increase in glycolysis and ATP production, which are important for cytoskeleton rearrangement. (2) The transmission of...
Box 2. Mechanotransduction

Inside the cell, mechanical stimuli are converted to biochemical signals, in a process called mechanotransduction. This process enables cell adaptation to the surrounding environment and can be divided into three stages: mechanotransmission, mechanosensing, and mechanoresponse [123]. Mechanotransmission is the transmission of mechanical forces, through protein–protein interactions, between the ECM and the cell or between neighboring cells. Transmembrane integrins are the bridge between the cell and the ECM and work as both force transmitters and mechanosensors. The mechanical linkage between the ECM and the actin cytoskeleton is mediated by focal adhesion complexes [124].

Inside the cell, structural scaffolds bind to the cytoplasmic domain of integrins and continue the transduction pathway mechanically, with the transduction of force to the actin cytoskeleton, with consequent regulation of actomyosin structure, and transmission of force to the nucleus through the LINC (linker of nucleoskeleton and cytoskeleton) complex [123,125]. These forces can be transmitted for long distances and are faster than biochemical signals since the propagation of the mechanical force is dependent on the resistance of the protein–protein interactions.

Cells can also receive mechanical signals through their neighboring cells via cell-cell adhesions [126]. Cadherins are the transmembrane proteins responsible for the transmission of the force between adjacent cells, and similar to integrins, they act as both force transmitters and mechanosensors [126]. The cytoplasmic domain of cadherins recruits catenins, which have associated cytoskeleton-binding proteins, whereas the cadherin extracellular domain forms intercellular bonds with cadherins of neighboring cells.

Mechanosensing is the ability to convert the mechanical force into a biochemical output through molecular changes of focal adhesions and conformational changes of their individual components. Inside the cell, there are also signaling scaffolds that bind to the cytoplasmic domain of integrins and continue the transduction pathway biochemically [75,123]. Finally, the mechanoresponse, as implied by its name, is the downstream effect of the applied force that in the short term promotes the modulation of cell-ECM binding of focal adhesions stability and of cytoskeleton strength. In the long term, it induces the activation of signaling pathways and changes of gene expression [75,123].

Actomyosin contractility, is essential to induce blastomere compaction [60]. Blastomere compaction has long been known to be related to an increase in adhesion molecules such as E-cadherin [61], but the molecular pathways that regulate this event are poorly understood. Recently, E-cadherin was shown to be present in filopodia-like protrusions that occur at the eight-cell stage, which have been proposed to be responsible for maintaining tension to control cell shape and promote compaction [62]. Although this study proposes that E-cadherin-dependent filopodia generate the mechanical forces to promote compaction, an independent study proposes that compaction is generated by actomyosin cortex tension, where E-cadherin prevents an increase in contractility at cell–cell contacts [60]. One possible explanation for these opposing results is that the actomyosin cytoskeleton can also be disrupted upon filopodia laser ablation, causing blastomeres to decompact [63]. In addition, the authors show that tension at cell–cell contacts is smaller than at the embryo surface, suggesting that adhesive forces, such as filopodia, cannot be the main driver of compaction, as they operate by pulling on neighboring cells [63].

During blastomere compaction, an apico-basal cell polarity in each blastomere is established, and blastomeres undergo two rounds of symmetric or asymmetric division [64]. In the first case, both daughter cells inherit the apical domain resulting in two polar cells, both maintaining their mechanical forces between the extracellular matrix (ECM) and a cell. Broken arrows indicate a presumable interaction between mechanotransduction and metabolism upon ECM–cell interaction. Cells sense the substrate through ECM-integrin interactions. Inside the cell, interactions between focal adhesions lead to the signaling cascade initiated by focal adhesion kinase (FAK), which activates the phosphoinositide 3-kinase (PIK3) pathway. The force activates Rac, resulting in actin cytoskeleton turnover and release of aldolase to the cytoplasm, which increases glycolysis flux. PIP3, phosphatidylinositol (3,4,5)-trisphosphate. (2) Metabolism as a source of intermediates used in enzymatic reactions, such as acetylation and methyltransfer. Citrate and α-ketoglutarate (α-KG) produced in the mitochondria through the Krebs cycle contribute to DNA and histone acetylation and methylation, respectively. (3) Mechanotransduction and metabolism as regulators of Yes-associated protein (YAP)/transcriptional co-activator with postnuclear density 95/disc-large/zone occludens (PDZ)-binding motif (TAZ) location. stiff substrates induce F-actin polymerization and consequently YAP and transcriptional co-activator with PDZ-binding motif (TAZ) translocation to the nucleus, whereas cell-cell adhesions are responsible for activating the Hippo signaling pathway and induce YAP/TAZ degradation signaled by large tumor suppressor kinase (LATS). By contrast, cholesterol biosynthesis and glycolysis are responsible for the translocation of YAP/TAZ to the nucleus and their binding to the TEAD family of transcription factors.
outer position. In the second case, only one daughter inherits the apical domain, resulting in one polar (outside cell) and one apolar cell (inside cell). This eventually leads to the outside layer of cells forming the trophoblast or trophectoderm (TE) that will participate in placental development and an interior cluster of ICM cells that contributes to the fetal lineage. However, how a blastomere adopts an internal or external position within the embryo has long been a topic of debate, with cell polarity, cell position, and contractility models described as independent regulators of TE/ICM fate [65,66]. Recently, mechanosensing was shown to be crucial for the correct positioning of cells during TE/ICM specification, acting as the bridge between polarity, position, and cell fate [67]. This model suggests that upon asymmetric division, the two daughter cells acquire different levels of contractility; apolar blastomeres have low levels of atypical protein kinase C (aPKC) and increased levels of cortical myosin, which increases cell contractility and regulates Yes-associated protein (YAP) localization in the cytoplasm, in opposition to polar blastomeres [67].

In addition to the mechanical properties of the embryo, the uterus has been suggested as an external mechanical force involved in embryonic anterior–posterior axis formation, which is critical for post-implantation development [68,69]. With the use of microfabricated cavities to recapitulate the external constraints promoted by the uterus during implantation, one group reported that mouse embryo egg-cylinder shape acquisition and distal visceral endoderm (DVE) specification are dependent on the external spatial restriction, with the width and stiffness of the cavity as vital players in the correct DVE development and anterior-posterior axis acquisition [68]. However, doubts about this model have emerged due to the ability to induce the DVE in embryos cultured in a hanging drop method or in a dish without a physical constraint, raising the importance of the self-organization properties of these cells [70,71]. Nevertheless, mechanical forces derived from cell–cell contacts or ECM-cell adhesion are increasingly recognized as key regulators in gastrulation and organ formation, where cells undergo changes in their motility and shape, which are either affected by mechanical forces, or generate mechanical forces that are crucial for correct embryo development [72–75].

Indeed, ESCs cultured in different confinement experiments in 2D patterns or within 3D suspension or matrices have led to the development of blastoids, gastruloids, and organoids, cells on a spatial organized shape and signaling environments that try to recapitulate the different states of in vivo development [76]. Many different organoid models have been generated to study the chemical and physical requirements for stem cells to differentiate into different tissue types [77,78]. Specific mechanical and ECM environments were recently shown to be crucial to intestinal stem cell maintenance and differentiation [79]. Fibronectin-based adhesion and high matrix stiffness were described as being responsible for stem cell survival and proliferation, through activation of YAP; whereas laminin-based adhesion and soft substrates were required to promote intestinal stem cell differentiation [79].

Considering the importance of cells sensing the correct force, geometric confinement of ESCs was shown to promote a self-organized pattern and induce trophectoderm, ectoderm, and mesendoderm primitive-streak markers in embryoid bodies with a similar cell type distribution as the gastrula during embryogenesis [80]. By contrast, blastoids were just recently established using ESCs and trophoblast stem cells cultured in 3D [81,82]. This model recapitulates the transcription and morphological patterns seen in the blastocyst stage 3.5 of the embryo and opens the door for new approaches to study genetic and mechanical principles in blastocyst formation and implantation. All things considered, there is a clear pattern showing a closer in vitro recapitulation when the native microenvironment of cells in the different tissues and development stages is considered.
Stiffness in ESC Fate

ESCs fate in vitro has also been shown to be regulated through biophysical cues in addition to biochemical cues. Generally, ESCs are cultured in tissue culture polystyrene, and their fate is regulated only using biochemical factors (i.e., liquid media manipulation of growth factors or inhibitors). However, reports have shown that ESCs sense mechanical properties of the culture dishes. When mESCs and hESCs are cultured on dishes of different stiffness ranges, their fate changes accordingly (Figure 2). This effect was first reported in MSCs, where the commitment to a cell lineage was shown to be dependent on the similarity of the substrate elasticity to tissues in vivo [12]. Soft substrates (~1 kPa) mimic the preimplantation embryo, the uterine epithelium, and the brain; substrates of intermediate stiffness (~10 kPa) mimic the muscle; and stiffer substrates (~100 kPa) mimic the bone [12,56,83]. mESCs cultured on very soft substrates (0.2–0.6, and 2-kPa polyacrylamide gels) maintain pluripotency for many passages, even in the absence of the pluripotency growth factor LIF [84,85]. Under these conditions, mESC cultures are homogeneous, with round and compact colonies. These cells can develop teratomas, thus showing pluripotent properties [84]. An increase in substrate stiffness promotes an increase in basal traction forces in mESC colonies and affects cell spreading, but not cell attachment [84,86].

Upon induction of spontaneous mESC differentiation, substrate stiffness also plays an important role. mESCs cultured in the absence of LIF on 7.5-kPa substrates tend to differentiate into mesendoderm [85]. Embryoid bodies derived from mESCs show an increase in cardiomyogenic differentiation when cultured on substrates with an elasticity of approximately 6 kPa [87]. Nevertheless, the use of substrates of high stiffness (40–2700 kPa) to culture mESCs in the absence of LIF leads to a positive correlation between genes expressed in the primitive streak during gastrulation and substrate stiffness [86]. The differentiation-related genes T-brachyury, Eomes, Foxa2, and N-cadherin increase their expression as stiffness increases, an effect that is independent of cell density, suggesting that substrate stiffness has a direct rather than an indirect effect on cell differentiation [86]. In the presence of osteogenic supplements, stiffer substrates promote an increase in osteogenic differentiation, compared to mESCs cultured on soft substrates [86]. Similar results have been reported in hESCs triggered to differentiate, where cells were cultured in 3D scaffolds of different stiffness to model germ layer specification upon gastrulation. The culture of hESCs in scaffolds of high elasticity (1.5–6 MPa) induces a transition from primitive streak expression to a mesoderm lineage, scaffolds of intermediate elasticity (0.1–1 MPa) induce endoderm-specific gene expression, and softer scaffolds (<0.1 MPa) increase the expression of ectoderm-specific genes [88]. In addition, hESCs cultured on soft flat (2D) substrates (0.1 and 0.7 kPa) form neural ectoderm more efficiently that hESCs cultured on stiffer substrates (75 kPa) but, contrary to mESCs, hESCs do not maintain their pluripotency on soft substrates in the absence of biochemical pluripotency cues [89]. Interestingly, mESCs cultured on 2-kPa polyacrylamide gels in the presence of the pluripotency-inducing 2i medium (combination of an MEK inhibitor, PD0325901, and a GSK3 inhibitor, CHIR99021), but in the absence of LIF, have higher expression of ectoderm lineage markers, indicating that these cells tend to differentiate even in the presence of specific pluripotency-maintaining inhibitors [85]. However, the presence of a Src inhibitor (GP77675), in combination with GSK3 in serum-free medium, designated as ‘alternative 2i medium’, was able to inhibit mESC differentiation and sustain mESC self-renewal and pluripotency, with these cells able to form teratomas and produce chimeric mice [85]. Therefore, Src is proposed to be an early mechanotransduction signal that induces differentiation when mESCs are cultured on substrates with specific elasticities. This mechanism was shown to involve the Src-Src homology/collagen A mitogen-activated protein kinase (MAPK) pathway [85]. In addition, cell stiffness also dictates cell sensitivity in response to mechanical stimuli. Upon the induction of a
local small cyclic stress, soft cells such as mESCs are more prone to spread and to lose Oct4 expression than stiffer cells, with Src, myosin II contractility, and cdc42 involved in the induction of this focal adhesion cell spreading [90]. These results suggest that small forces might have an important role during embryonic development, where cells are known to have soft properties. In addition, the initiation of force-induced spreading response and consequently differentiation are induced by activation of Src through the integrin cytoskeleton pathway.

The Connection between Mechanotransduction and Metabolism: What Do We Know?
The simultaneous deregulation of both mechanotransduction and metabolic pathways has been described as a cause (or consequence) of diseases, such as osteoarthritis, cardiovascular diseases, and cancer [91–93]. These two pathways seem to be responsible for the cell energy imbalance and the deregulation of post-translational modifications in proteins responsible for the mechanosensing and the mechanoresponse in these diseases. Thus, in a simplistic way, mechanotransduction induces a rearrangement of the cytoskeleton, and this rearrangement requires energy. Furthermore, mitochondria need to interact with the cytoskeleton to maintain their normal function and proper localization, which is crucial for cell metabolism. However, the molecular crosstalk between these two signaling pathways is only now starting to be revealed, and the role of this crosstalk in pluripotency remains to be established. Nevertheless, many interesting aspects are being discovered, notably the differentiation of cardiac tissue and fate commitment in cancer biology, which may also be relevant given the metabolic similarities between pluripotent stem cells and many cancer cell types [21].

For example, the number and morphology of mitochondria are dependent on fission and fusion processes, which are carefully balanced in the cell as they are required to maintain a functional mitochondrial population. The cytoskeleton has been shown to be crucial in mitochondrial dynamics, and new research has found that mitochondria fission is triggered by mechanical forces [94]. Moreover, stretch, which mimics cell contractility, was shown to induce a structural organization of actin, microtubules, and mitochondria, promoting an increase of mitochondrial membrane potential in cardiac differentiation [95]. Nevertheless, while microtubules and vimentin were shown to have a crucial role in mechanotransduction and consequently mitochondrial membrane potential, non-muscle myosin II, the microtubule motor kinesin, and the mitochondrial fission protein dynamin-related protein 1 (Drp1) were crucial to the maintenance of the mitochondrial membrane potential during stretch fluctuations [95]. Taken together, mitochondria dynamics and activity seem to be clearly dependent on the mechanical context of the cell, with the cytoskeleton playing a major role.

YAP is a very promising candidate in bridging metabolism and mechanotransduction. YAP and transcriptional co-activator with postsynaptic density 95/disc-large/zona occludens (PDZ)-binding motif (TAZ) are localized in the nucleus when transcriptionally active and are known to interact with the TEAD family of transcription factors. YAP/TAZ have a role in cellular polarization, migration, organ growth, and induction of stem cell self-renewal and inhibition of differentiation and apoptosis [96]. YAP/TAZ are also well known cell mechanosensors and mechanoresponders and have been shown to be regulated by the Hippo signaling pathway, Wnt signaling pathway, and actin cytoskeleton integrity [96,97]. In addition, metabolism has recently been suggested to also regulate YAP/TAZ activity [98]. The mevalonate pathway, involved in cholesterol biosynthesis, was shown to regulate YAP/TAZ activity through geranylgeranylation of Rho GTPase [99,100]. Through a different mechanism, glycolysis was also shown to regulate YAP/TAZ activity, with active phosphofructokinase, an enzyme that mediates the first committed step of glycolysis, shown to interact with TEAD and stabilize its interaction with YAP/
TAZ [101]. Moreover, the AMP-activated protein kinase (AMPK), which is activated upon glucose starvation or energy stress, was shown to induce the phosphorylation of YAP and consequently promote its cytoplasmic retention and inactivation [102–104]. It is also well known that YAP/TAZ activation is related to tumor malignancy, and these results suggest that metabolism is directly linked to cellular proliferation through YAP/TAZ regulation. Indeed, in breast cancer cells both glucose metabolism and the mevalonate pathway are strongly associated with YAP/TAZ activity and with the progression of these tumors towards more advanced and malignant stages, with phosphofructokinase acting downstream of the mevalonate pathway [99–101]. YAP was also shown to promote transcription of the GLUT3 glucose transport receptor and to control the transcription of enzymes involved in nucleotide biosynthesis [102,105,106]. Taking into account that YAP has also been described to be activated upon tumor ECM stiffening [107,108], YAP might perhaps contribute to a positive feedback mechanism between cellular stiffening, DNA replication, and the increase of energy requirements characteristic of tumor cells. As noted previously, this is particularly relevant given that the metabolic characteristics of proliferating tumor cells are similar to pluripotent stem cells [25].

Although multiple studies have recently shown that metabolic alterations are correlated with mechanotransduction changes in disease, the opposite is also true. Sarcomere mutations (known to induce cardiomyopathies) have been described to change the energetic cost of cardiac contraction and therefore induce metabolic changes in these cells [109]. In addition, proteome studies of a mouse model of collagen VI deficiency, which is associated with a myopathic phenotype, demonstrated that ECM architectural alterations induce mechanotransduction changes through cytoskeleton tension [specifically focal adhesion kinase (FAK) and Rho-associated, coiled-coil-containing protein kinase 1 increase] and consequently cause changes in muscle metabolism, such as a glycolysis decrease [110]. Indeed, cardiomyocytes cultured on soft substrates have an extended lifetime and retain a greater respiratory capacity compared to cardiomyocytes cultured on stiff substrates [111]. Furthermore, the same group recently showed that ECM elasticity is directly correlated with mitochondria basal respiration, ATP production, and maximum respiration capability [112]. Interestingly, the authors coupled ECM elasticity with substrate alignment and showed that there is a pronounced trend in basal respiration, ATP production, and maximum respiration capability compared to isotropic substrates, suggesting that both ECM and tissue architecture better regulate the adaptability of mitochondria to stress and the demand to higher cellular ATP levels [112]. In addition to mitochondrial activity, substrate stiffness was also shown to regulate the mitochondrial network, the nature of which is also linked to function [113]. However, the molecular crosstalk between ECM-cell adhesion, cell-cell adhesion, cytoskeleton, and mitochondria metabolism has just started to be unveiled.

The phosphoinositide 3-kinase (PI3K) pathway is activated in response to ECM-cell adhesion (Figure 3) [114]. The PI3K signaling pathway regulates cell metabolism through Akt and induces cytoskeleton rearrangements through the Rac pathway [115]. Recently, PI3K was described to regulate glycolysis via the direct regulation of cytoskeleton dynamics, independently of Akt. One group showed that PI3K induces the disruption of the actin cytoskeleton via Rac, and this cytoskeleton disruption induces the release of aldolase (a glycolysis pathway enzyme) from F-actin to the cytoplasm, increasing its activity and consequently the glycolytic flux [116]. Interestingly, the decrease of mechanistic target of rapamycin kinase (mTOR) signaling, a downstream effector of the PI3K/Akt pathway, was shown to induce the endocytosis of β-integrin and its ECM substrate in epithelial cells [117,118]. This ECM internalization is used as a source of energy and building blocks to produce amino acids, which enhances mTOR
activity and prevents the death of starved cells \[118\]. These results suggest that the PI3K pathway is responsible for the coordination between cell energy and cell mechanotransduction.

By contrast, the already mentioned cell energy sensor AMPK is activated by a low ATP/AMP ratio, and it induces the increase of **catabolic pathways** while switching off **anabolic pathways** \[119\]. Recently, AMPK and one of its activators, liver kinase B1 (LKB1), have been shown to colocalize with the cadherin adhesion complex in a force-dependent manner in epithelial cells \[Figure 3\] \[120\]. This colocalization is crucial for the initiation of mechanotransduction signals. Indeed, one study elegantly showed that AMPK is upstream of Rho-A-mediated contractility, acting as a mechanoresponsive protein \[120\]. In addition, AMPK was shown to increase glucose uptake and oxidation upon induction of force on E-cadherin. Thus, AMPK appears to connect mechanotransduction and energy homeostasis in cell–cell adhesion.

**Concluding Remarks**

During embryonic development, cells are in contact with extracellular matrices of varying stiffness, which trigger different cell fates. However, to progress to different fates, cells need to tightly regulate their metabolism to achieve the correct balance of energy and production of precursors for distinct biosynthetic purposes. Recent in vivo studies have highlighted the importance of mitochondrial dynamics, metabolic switches, and mechanical cues on embryo development. The currently accepted models suggest that metabolism and mechanotransduction are important contributors to cell fate specifications from fertilization to organogenesis; however, their interaction has not yet been investigated in detail. It is known that cells that give rise to the fetus rely first on OXPHOS and then switch to a glycolysis-based metabolism upon implantation, and finally rely again predominantly on OXPHOS upon tissue differentiation. By contrast, cell tension and contraction have emerged as important regulators of the first cell fate decisions during embryonic development. Moreover, the recent studies involving blastoids, gastruloids, and organoids bring to light the importance of cells sensing the appropriate microenvironment to recapitulate in vitro the structural and biochemical development that occurs in vivo. Thus, these models open the door to the study of possible interactions between metabolic and mechanical cues, which remains a largely unexplored research area.

Deciphering the signaling pathways in vitro that mechanical or biochemical cues induce in ESCs, EpiSCs, and iPSCs highlights the importance of these cues on chromatin remodeling and consequently gene transcription regulation. In addition, the findings that independent modulation of metabolism, substrate stiffness, and substrate patterning are crucial to maintain pluripotency, to promote differentiation, or to induce somatic cell reprogramming stress the importance of these two cues working in synergy to induce different cell fates. It will be interesting to decouple the effect of each modulator on stem cell fate and evaluate the impact of biophysical modulators on metabolism and vice versa.

Indeed, the latest research is now focusing on the link between mechanotransduction and metabolism in differentiated and cancer cells, which are metabolically similar to pluripotent cells. Key signaling pathways such as YAP, PI3K, and MAPK seem to connect both energy and precursor requirements for cell proliferation and maintenance of state. YAP/TAZ expression was recently shown to reprogram and induce tissue-specific stem cells from differentiated cells, with an independent report showing that YAP is able to bind to promoters of stem cell genes and stimulate their expression \[121,122\]. Considering that the ectopic expression of YAP prevents ESC differentiation and that during embryonic stem cell differentiation cellular mechanics is altered and cells go through a metabolic shift, it will be interesting in the future
to analyze whether YAP connects the effects of metabolism and mechanotransduction during embryonic stem cell differentiation. Moreover, the recent results suggest AMPK and PI3K as common signaling transducers of metabolism and of cell-cell and of ECM-cell adhesion signaling, respectively. These new results emphasize the importance of understanding the robust integration of these different stimuli in determining cell fate and open the door to a panoply of questions (see Outstanding Questions) that can lead to different cellular reprogramming models and novel solutions for tissue engineering.

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