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Cell fate decisions: emerging roles for metabolic signals and cell morphology

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Abstract

Understanding how cell fate decisions are regulated is a fundamental goal of developmental and stem cell biology. Most studies on the control of cell fate decisions address the contributions of changes in transcriptional programming, epigenetic modifications, and biochemical differentiation cues. However, recent studies have found that other aspects of cell biology also make important contributions to regulating cell fate decisions. These cues can have a permissive or instructive role and are integrated into the larger network of signaling, functioning both upstream and downstream of developmental signaling pathways. Here, we summarize recent insights into how cell fate decisions are influenced by four aspects of cell biology: metabolism, reactive oxygen species (ROS), intracellular pH (pHi), and cell morphology. For each topic, we discuss how these cell biological cues interact with each other and with protein-based mechanisms for changing gene transcription. In addition, we highlight several questions that remain unanswered in these exciting and relatively new areas of the field.

Keywords cell fate; cell morphology; intracellular pH; metabolism; reactive oxygen species

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See the Glossary for abbreviations used in this article.

Introduction

Cell fate decisions are tightly regulated by many layers of control. A change in cell fate is ultimately defined by the acquisition of new characteristics that come about largely through changes in transcription. Protein-based signal transduction cascades leading to changes in transcription factor activity are the most direct causes of transcriptional changes and are among the most well-studied aspects of the cell fate decision process. In contrast, much less is known about how other aspects of cell biology such as changes in metabolite concentration or mechanical forces contribute to cell fate decisions. This is due in part to the difficulty of studying cues that are not directly encoded in the genome. However, technological advances, including the generation of new biosensors that can be used for live cell imaging, improvements in quantitative fluorescence microscopy, and the development of more sensitive biochemical methods for detecting small molecules are making it easier to identify previously unrecognized control mechanisms. In this review, we discuss recent advances in understanding the role of metabolism, reactive oxygen species (ROS), intracellular pH (pHi), and cell morphology and adhesions to cell fate decisions, particularly during differentiation in adult, embryonic, and induced pluripotent stem cell lineages.

Metabolism

The metabolic state of a cell is the result of a complex array of inputs, including cell signaling, availability of nutrients and oxygen, energy needs, and biomass demands. These inputs and demands combine to influence the rate of ATP production from glycolysis versus oxidative phosphorylation, as well as the rate of side reactions that produce anabolic intermediates. As cells differentiate, the change in these inputs causes the metabolic state to shift. However, the metabolic state of the cell is not merely a consequence of differentiation. Instead, shifts in metabolism can have permissive and, in some cases, even instructive roles in promoting differentiation [1]. This perspective positions metabolism as a key node in the regulation of cell fate transitions. In this section, we summarize the metabolic programs of cells at different stages of differentiation, briefly review some of the major cell signaling regulators of metabolic state, and discuss how changes in metabolic state contribute to cellular differentiation (Fig 1).

Changes in metabolism, often collectively referred to as "metabolic reprogramming", can shift the amount of energy and biomass produced by glycolysis versus oxidative phosphorylation to regulate changes in cell fate. In adult stem cell lineages, less active long-term progenitors, such as quiescent hematopoietic stem cells (HSCs) or satellite cells (stem cells of skeletal muscle) utilize glycolysis over oxidative phosphorylation, whereas more actively growing and proliferating cells are bivalent and utilize both glycolysis and oxidative phosphorylation [2–5]. Embryonic stem cells (ESCs) transition through several metabolic states during differentiation. ESCs in the most undifferentiated, or "naive" state, have relatively high levels of oxidative phosphorylation [6–8], although these cells still consume high amounts of glucose and glutamine [6,9]. As ESCs differentiate

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Glossary

ECM	extracellular matrix
ESCs	embryonic stem cells
HSCs	hematopoietic stem cells
iPSCs	induced pluripotent stem cells
Metabolic reprogramming	Changes in metabolism that accompany and can sometimes be necessary or instructive for changes in cell fate
MSCs	mesenchymal stem cells
Niche	A specialized microenvironment in the tissue that maintains cells in the stem cell state
NSCs	neural stem cells
pH sensor	Selective proteins with post-translational modification by protonation/ deprotonation regulating activity or ligand binding.
ROS	reactive oxygen species
SAM	S-adenosyl methionine
Satellite cells	Stem cells of the skeletal muscle

toward the "primed" state, ATP production becomes decoupled from oxidative phosphorylation, and the metabolic program is shifted toward the use of glycolysis for energy and biomass production [6,10] through a process that is regulated by the conserved RNA-binding protein, LIN28 [7]. Energy production from oxidative phosphorylation then increases again as differentiation proceeds beyond the primed state. Likewise, the reprogramming of differentiated somatic cells into induced pluripotent stem cells (iPSCs) requires a shift from a bivalent metabolic program of glycolysis and oxidative phosphorylation toward a primarily glycolytic state that resembles the metabolism of primed ESCs [11,12]. Recent evidence indicates that this metabolic shift occurs prior to changes in gene expression, suggesting that it is a prerequisite for reprogramming rather than a consequence of the cell fate change [13].

Nonetheless, in most cases, metabolic changes are initiated by cell signaling molecules, including AMPK, HIF1α, AKT, and Myc. AMPK, which is activated by high [AMP]/[ATP] ratios that indicate low nutrient availability and metabolic stress, increases glycolytic energy production, activates FOXO proteins to promote the expression of antioxidants and autophagy genes, and restricts growth by inhibiting mTor [14,15]. This stress response program is important for maintaining cellular homeostasis in general, and thus functions during both self-renewal and differentiation. HIF1α is an oxygen sensor that is stabilized by low oxygen levels and promotes a steady state level of energy production during periods of relatively low activity in quiescent and slowly dividing adult stem cells, such as HSCs [2], mesenchymal stem cells (MSCs) [16], and satellite cells [17]. HIF1α shifts the metabolic program toward glycolysis over oxidative phosphorylation, which is conducive to the hypoxic environments of stem cell niches that maintain quiescent stem cells, and also minimizes the damage caused by ROS produced from mitochondrial respiration. In contrast, Akt and Myc promote an increase in energy production from oxidative phosphorylation and a switch in the utilization of glycolysis from a source of energy production to a source of anabolic intermediates. Akt activates mTor by inhibiting the Tsc complex, and several studies have found that this pathway promotes differentiation of adult stem cells including HSCs, NSCs, and ISCs [18–20]. Akt signaling also increases ROS levels by inhibiting FOXO proteins, which has the effect of further promoting differentiation in some types of stem cells (see next section). Myc is also required for differentiation in the HSC and epidermal stem cell lineages [21,22]. In addition, Myc is an important factor for reprogramming into iPSCs, and inhibition of mTor or induced expression of metabolic enzymes can substitute for Myc in iPSC reprogramming [23,24]. Thus, shifts in metabolic state are a prerequisite for differentiation in cases where the shift is needed in order to meet the energetic and anabolic demands of the new cell state.

Metabolic state can also influence cell fate decisions by affecting the availability of metabolites that are important for the epigenetic regulation of gene expression [24]. Epigenetic regulation occurs primarily through the modification of histones and DNA, and histone acetylation and deacetylation as well as histone and DNA methylation and demethylation all can be regulated by metabolites. Histone acetyltransferases (HATs) use acetyl-CoA, which is a key metabolic intermediate between glycolysis and the TCA cycle, as a substrate for histone acetylation. In the absence of sufficient acetyl-CoA, global histone acetylation is reduced, and thus, the regulation of gene expression is impaired. This connection was clearly demonstrated in a study of in mouse adipocytes [25]. The authors found that knockdown of ATP-citrate lyase, which generates acetyl-CoA from citrate, caused a decrease in histone acetylation and prevented the upregulation of genes such as glucose transporters that are required for differentiation. Likewise, deacetylation is also sensitive to acetyl-CoA concentrations in the cell. For example, the addition of acetate (which increases acetyl-CoA levels) to the culture media of human or mouse ESCs blocked histone deacetylation and delayed differentiation, whereas inhibition of glycolysis (which decreases acetyl-CoA levels) accelerated differentiation [26]. The effect of glycolysis inhibition could be reversed with the addition of acetate to the media, and pharmacological inhibition of the enzyme that produces acetyl-CoA for histone acetylation produced a similar phenotype, but the effect on histone deacetylation was not tested directly. Deacetylation by sirtuins is also responsive to metabolic inputs [27]. Sirtuins are deacetylases with a broad range of targets including histones and transcription factors. These enzymes are considered metabolic sensors because they use NAD^+ as a cofactor and thus become more active when $[NAD^+]$/[NADH] ratios are high. In addition, sirtuins both regulate and are regulated by AMPK [28]. Sirtuin 1 (SIRT1) has been well studied during mammalian cell differentiation and may function through different mechanisms to both repress differentiation in some contexts and promote differentiation in others. For example, SIRT1 is highly expressed in ESCs, iPSCs, and early morula stage embryos, where it promotes pluripotency and is downregulated upon differentiation [29,30]. In contrast, genetic and pharmacological studies indicate that SIRT1 promotes differentiation in hematopoietic and neural lineages [31,32].

The epigenetic regulators that catalyze the addition of methyl groups, DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs), use S-adenosyl methionine (SAM) as a substrate. The rates of histone methylation are different at active versus inactive promoters, and the concentration of intracellular SAM can directly influence these rates. SAM concentrations are relatively high in human and mouse ESCs and iPSCs, and SAM is required for histone methylation to maintain the pluripotent state in these cell types [33–35]. In adult tissues, there is a well-established role for SAM in the regulation of DNA and histone methylation during

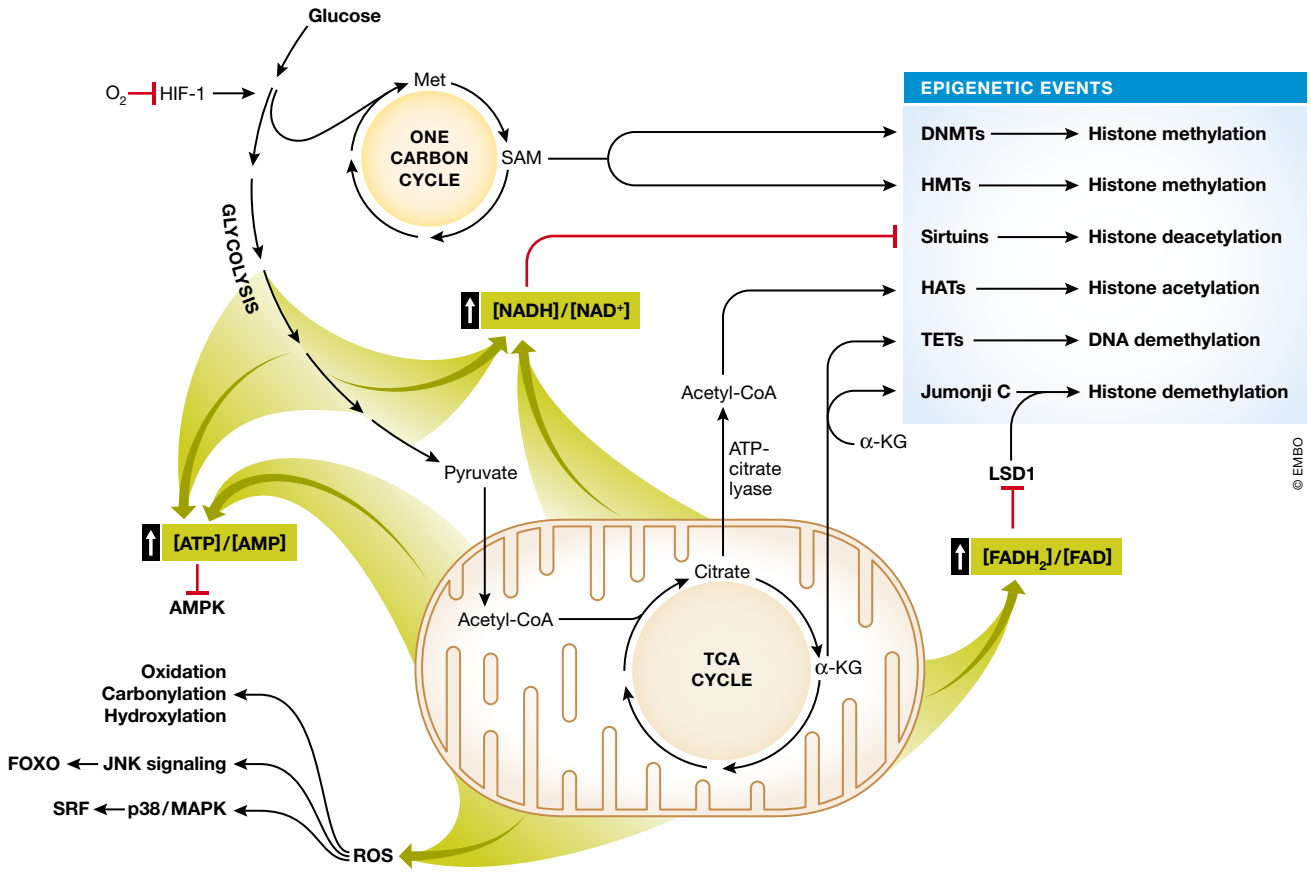

Figure 1. The connections between metabolism and cell fate decisions.
Metabolic inputs regulate epigenetics and cell signaling to promote changes in cell fate. Glycolysis produces metabolic intermediates that feed into the folate and one carbon metabolism cycle to produce S-adenosylmethionine (SAM), which is a cofactor for DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs). The energy released from glycolysis and oxidative phosphorylation also converts AMP to ATP and NAD^+ to NADH. AMP stimulates AMPK activity, and NAD^+ is a cofactor for sirtuins, so increased energy production decreases the activity of these enzymes. Glucose-derived acetyl-CoA enters the tricarboxylic acid (TCA) cycle to form citrate, which can be converted back to acetyl-CoA by ATP-citrate lyase. This source of acetyl-CoA (but not acetyl-CoA derived from fatty acid oxidation) contributes to the pool of nuclear acetyl-CoA that is essential for histone acetylation by histone acetyltransferases (HATs). α-ketoglutarate (α-KG), which is produced in the TCA cycle and in the cytoplasm, is an essential cofactor for TET and Jumonji C enzymes, which demethylate DNA and histones, respectively. The energy released from oxidative phosphorylation converts FAD to $FADH_2$, and FAD is a cofactor for lysine-specific demethylase 1 (LSD1), so a reduction in FAD levels inhibits LSD1 activity. Increased oxidative phosphorylation also generates reactive oxygen species (ROS), which promote oxidation, carbonylation, and hydroxylation as well as increase the levels of JNK and p38/MAPK pathway activity. Low levels of oxygen (O_2), for example in the HSC and satellite cell niches, increase the activity of the hypoxia inducible factor-1 (HIF-1), which promotes glycolysis.

oncogenesis [36], and though less is known about the role of SAM in adult stem cell differentiation, many adult progenitors, including HSCs [37], ISCs [38], and epidermal progenitors [39] require DNMTs and HMTs [40]. Thus, changes in the concentration of SAM influence cell fate transitions in many different cell types.

Likewise, enzymes that catalyze the removal of methyl groups from histones and DNA are sensitive to the availability of specific metabolites. For example, the Jumanji C family of histone demethylases and the TET-family enzymes, which catalyze the first step of DNA demethylation, require both the TCA cycle intermediate α-ketoglutarate (α-KG) and the reduced (Fe^{2+}) form of iron [41,42]. Iron is more commonly in the Fe^{3+} form but can be reduced to Fe^{2+} by vitamin C, and several recent studies revealed the importance of vitamin C for promoting the activity of Jumonji C or TET-family enzymes in ESCs [43–46], adult stem cells [47,48], and during iPSC reprogramming [43,49]. Another important histone demethylase, lysine-specific demethylase 1 (Lsd1), is also sensitive to metabolic changes as it relies on FAD as a cofactor [50]. LSD1 is required in mouse ESCs (mESCs) to silence self-renewal genes during differentiation [51], and the homologous gene, *Su(var)3-3*, is also required in the somatic cells of the *Drosophila* ovary to promote germ cell differentiation [52,53]. Collectively, these findings demonstrate that metabolic processes can influence epigenetic regulation of gene expression at multiple levels.

In addition to the permissive roles for metabolism in cellular differentiation described above, metabolic cues can also be instructive, causing changes in cell signaling and gene expression sufficient to drive the change in cell fate. For example, in satellite cells, increased glycolysis during exit from quiescence causes a decrease in NAD^+, which reduces SIRT activity and thus increases H4K16 acetylation, ultimately leading to the expression of key differentiation genes, such as MyoD [54]. Another interesting example comes from a recent study that found that intestinal stem cells (ISCs) utilize lactate provided by the neighboring Paneth cells to sustain a high

level of oxidative phosphorylation [55]. Increased oxidative phosphorylation in ISCs causes an increase in reactive oxygen species (ROS), which activates the p38-MAPK pathway (as discussed in the following section). Paneth cells are part of the ISC niche, so this suggests that metabolic cues can function as niche signals. Additional examples in which metabolic changes feed into signaling networks to instruct cell fate decisions involve mTOR, which is a master regulator of cell growth and proliferation. Several studies have demonstrated that mTOR is essential for the maintenance of pluripotency and the repression of differentiation genes in ESCs grown under standard conditions [56]. In addition, a more recent study found that partial inhibition of mTOR in mESCs induces the cells to adopt a "paused" state resembling embryonic diapause [57]. The mechanism of this effect is not fully understood, but the authors speculate that the paused state is induced by the combined effects of mTOR inhibition on transcription, translation, and metabolism. Lastly, in quiescent HSCs, activation of mTOR induces mitochondrial biogenesis, which activates proliferation and induces differentiation [58].

Two recent studies demonstrated that changes in pyruvate metabolism can contribute to the regulation of proliferation and differentiation in epidermal and intestinal cell lineages [59,60]. Pyruvate is the end product of glycolysis and can either enter be converted to lactate in the cytoplasm, or be transported into the mitochondria, where it is converted to acetyl-CoA and oxidized in the TCA cycle. These studies provide evidence that hair follicle and intestinal stem cells are more glycolytic than their non-stem cell progeny, and suggest that increased conversion of pyruvate to lactate drives stem cell proliferation whereas increased mitochondrial oxidation of pyruvate promotes differentiation. The downstream mechanism was not investigated, but both studies provide evidence suggesting that high levels of Myc in the stem cells may promote the shift toward lactate production. Interestingly, a separate study of intestinal differentiation in zebrafish found that Wnt signaling also regulates pyruvate metabolism [61]. Wnt signaling is generally high in epithelial stem cells [62] and promotes Myc expression [63,64], suggesting a model in which Wnt signaling, Myc, and pyruvate metabolism function together to promote epithelial stem cell identity.

Taken together, these studies demonstrate that changes in metabolism influence cell fate decisions in a variety of ways. In many cases, the link between the metabolic cue and the cell fate decision is reactive oxygen species as described in the next section.

Reactive oxygen species

Metabolic pathways can influence stem cell fate decisions through the activity of ROS (Fig 1). ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are formed by the reduction of molecular oxygen (O_2). The toxic effects of these ROS have been studied extensively in the context of cell proliferation, DNA damage, and apoptosis. Additionally, ROS play a crucial role in regulating cellular processes like oxidative stress responses, aging, and stem cell fate decisions. In this section, we review recent advances in the understanding of the role of ROS in cell differentiation. ROS are commonly generated as by-products of metabolic reactions occurring in the mitochondria, mainly in the electron transport chain. ROS levels are controlled by several proteins, such as NADPH oxidases, which have activity that results in formation of superoxides, superoxide dismutases (SOD), which reduce O_2^- to H_2O_2, and other enzymes, including thioredoxins, glutathione peroxidases, and peroxiredoxins [65,66].

Recent studies identified examples in which specific ROS regulators are necessary for stem cell differentiation. For example, Kim *et al* [67] found that peroxiredoxins, PrxI and Prx II, promote mouse embryonic stem cell differentiation into neurons by regulating ROS levels. In addition, Hochmuth *et al* [68] found that Nrf2, which controls transcription of antioxidant enzymes like thioredoxins and peroxidases, and Keap1, a negative regulator of Nrf2, regulate *Drosophila* intestinal stem cell proliferation by altering intracellular ROS. Other studies have focused on the downstream effects of changes to ROS levels, and in general, these studies find that increased ROS levels are associated with differentiation. During *Drosophila* testes germline stem cell (GSC) differentiation, GSCs maintain reduced levels of ROS, regulated by Keap1 and Nrf2 [69]. An increase in ROS in GSCs caused a decrease in the number of GSCs and promoted differentiation. In mammalian HSCs, an elevation in ROS levels occurs during differentiation into common myeloid progenitors [70]. Likewise, quiescent multipotent hematopoietic progenitor cells in the *Drosophila* lymph gland have elevated levels of ROS, which promotes differentiation [71]. In these studies, scavenging ROS by expressing antioxidant proteins like catalase *in vivo* or by the addition of N-acetylcysteine, delayed differentiation, whereas increasing ROS by adding paraquat or mutating mitochondrial complex I proteins like ND75, promoted differentiation [69,71,72]. During vascular smooth muscle cell differentiation, inhibition of ROS activity decreased the cellular expression of differentiation proteins, whereas an elevation in ROS activity increased expression of these differentiation markers [73]. In contrast to this trend, two studies show that elevated ROS levels promote self-renewing, proliferative neural and mouse spermatogonial stem cell fate [74,75]. Additionally, elevated ROS levels promote *Drosophila* intestinal stem cell proliferation [68].

Reactive oxygen species instruct stem cell fate decisions by regulating key signal transduction pathways. A mechanism by which ROS control signaling pathways that affect stem cell differentiation is by affecting post-translational modifications of regulatory proteins, such as phosphatases. For example, ROS have been shown to mediate cysteine and methionine oxidation, protein carbonylation, and hydroxylation (reviewed by [66]). Another mechanism by which ROS influences differentiation decisions is by directly affecting the activity of transcription factors and essential signaling pathway proteins responsible for activating genetic differentiation programs. The most commonly studied signaling pathways in this regard are the JNK and p38 MAPK pathways and an increase in ROS typically activates these pathways to promote differentiation [68,71–73]. For example, in *Drosophila* hematopoietic progenitor cells, elevated ROS levels stimulate the JNK pathway to promote differentiation by activating transcription factor FoxO and the derepression of polycomb activity [71]. However, FoxO also increases antioxidant activity, which reduces ROS levels and thus creates a negative feedback loop that eventually brings FoxO activity back down. Likewise, in mammalian hematopoietic stem cells (HSCs), low levels of ROS are necessary for HSC self-renewal whereas elevated levels of ROS promote differentiation by stimulating the activity of p38 and mTOR signaling pathways [76]. During vascular smooth muscle

differentiation and mouse spermatogonial stem cell self-renewal, increased ROS levels activate p38 MAPK signaling pathway, which promotes the transcription of serum response factor (SRF), and ultimately increases the activity of differentiation proteins, such as α-actin and calponin [73]. Additionally, high levels of ROS in *Drosophila* GSCs promote differentiation by increasing the transcription of the epidermal growth factor receptor ligand, Spitz, thereby activating the MAPK signaling pathway [69]. Collectively, these studies demonstrate that ROS concentrations are tightly controlled during cellular differentiation and that changes in ROS concentrations play important roles in the cell fate decision process.

Intracellular pH

A long-held view is that pHi is constitutively maintained between 7.2 and 7.4 in normal mammalian cells and only dysregulated from this narrow range in diseases, including being constitutively increased in cancer [77,78] and decreased in neurodegenerative disorders [79,80]. However, emerging evidence indicates there are transient increases in pHi in normal mammalian cells during cell cycle progression [81], directional migration [82,83], and differentiation [84–87]. Although the role of pHi dynamics in regulating cell fate decisions remains understudied, we highlight recent findings on this topic and emphasize questions that remain to be addressed (Fig 2).

Increasing evidence suggests that changes in pHi are necessary for embryonic stem cell differentiation. We recently showed a transient increase in pHi during differentiation of clonal naïve mESCs to primed epiblast-like cells (EpiSC), which when prevented, blocks differentiation as indicated by attenuated expression of epiblast cell markers, including Pax6, Brachyury, and Fgf5, as well as the miRNA cluster *mir-302* [84]. The increased pHi from ~7.40 to ~7.65 occurs during the first 3 days of spontaneous differentiation and then returns to pHi values seen in naïve cells, which suggests that the higher pHi is necessary for the differentiation process but not for maintaining a differentiated state. Consistent with this prediction, an earlier study by Edwards *et al* [88] found that pHi increases from zygote to the morula stage. In a different embryonic cell model, Li *et al* [87] showed that inhibiting activity of the plasma membrane

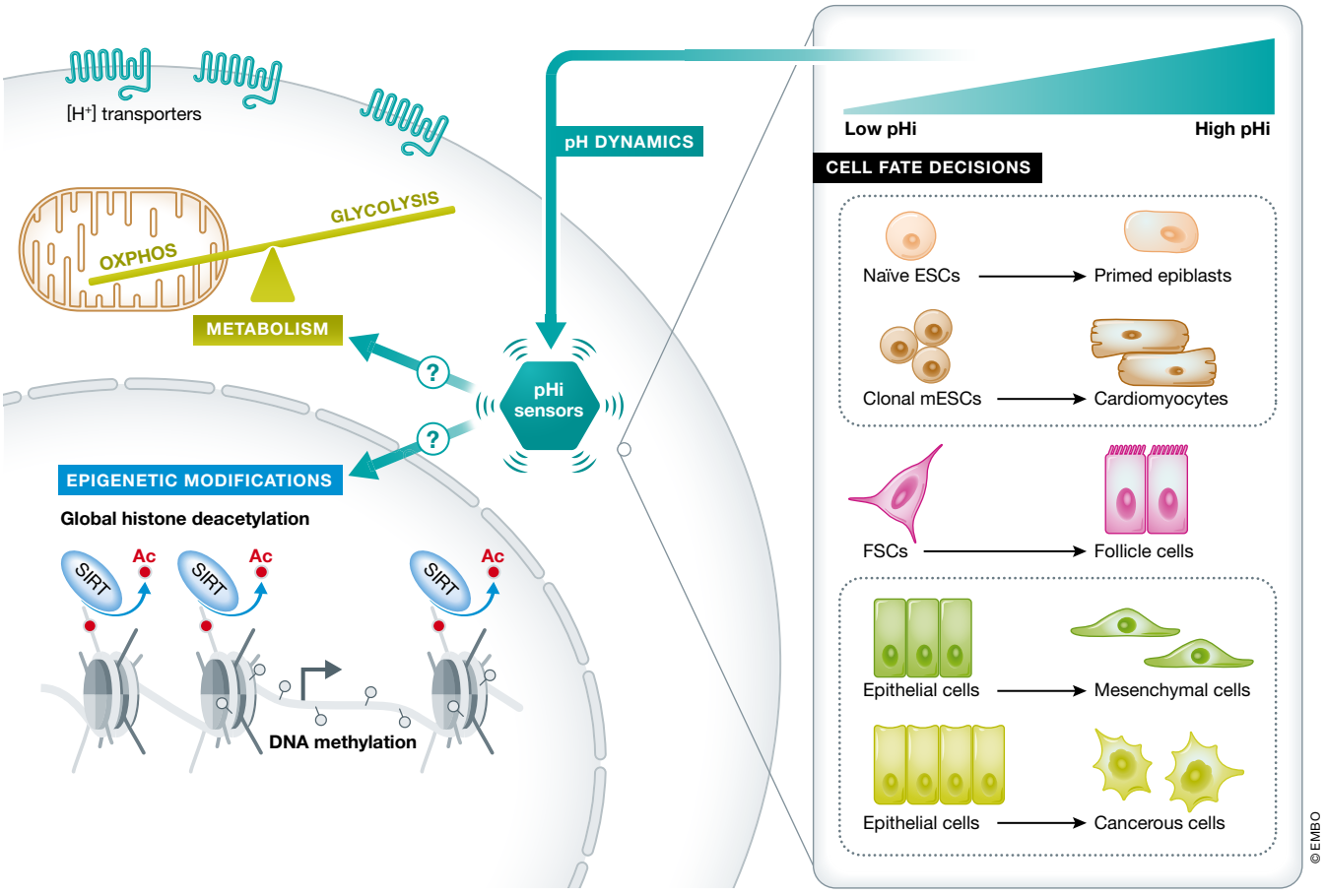

Figure 2. Mechanisms by which pHi could regulate cell fate decisions.
pHi increases during embryonic and adult stem cell differentiation, epithelial-to-mesenchymal transitions, and carcinoma transformations. Theoretically, pH-sensitive proteins ("pH sensors") that undergo protonation or deprotonation upon changes in pHi could regulate cell fate decisions by affecting proton transporter activity, cellular metabolism, and epigenetic modifications like histone deacetylation and DNA methylation. However, in most cases, the specific mechanisms by which pHi could regulate cell fate decisions are unknown.

Na-H exchanger-1 (NHE1), markedly attenuates differentiation of CGR8 clonal mESCs into cardiomyocytes resulting in a decreased expression of the transcription factors Nkx2-5 and Tbx5 and decreased abundance of α-myosin heavy chain. Although changes in pHi during differentiation were not determined, inhibiting NHE1, which is an acid extruder, is predicted to lower pHi. This group also found that NHE1 activity potentiates differentiation of P19 embryonal carcinoma cells into neurons [89]. In contrast, umbilical cord-derived human mesenchymal stem cells (MSCs) have a higher pHi than differentiated cells. Lowering pHi of these cells by pharmacological inhibition of NHE1 promotes differentiation to an osteogenic lineage but has no effect on differentiation to an adipogenic lineage [90].

Recent studies, including studies from our laboratories, suggest that differentiation occurring during *Drosophila* adult epithelial follicle stem cell lineages requires changes in pHi. Using the genetically encoded pHi biosensor pHluorin, we showed a lower pHi in follicle stem cells of the adult *Drosophila* ovary compared with differentiated daughter cells. Preventing the increased pHi by loss of *Dnhe2*, the *Drosophila* ortholog of mammalian NHE1, inhibits differentiation, impairs germarium morphology, and results in infertility [84]. Krüger and Bohrmann [91] also found an anteroposterior pHi-gradient in follicle and nurse cells of the *Drosophila* ovary, although significance in oogenesis was not determined.

It remains to be determined whether the lower pHi in self-renewing cells or the higher pHi in differentiating cells is an active process. In endometrial epithelial cells, LeftyA inhibits NHE1 to actively maintain a lower pHi [92]. Likewise, in mESCs, our findings that the increased pHi with differentiation is transient and seen only during the first 72 h are consistent with an active regulation of pHi [84]. Because increased pHi promotes proliferation, the decrease after 72 h may function to limit proliferation. As described below, a constitutively higher pHi is seen in most cancers and can induce hyperproliferation and dysplasia even in the absence of activated oncogenes [93].

Considered more broadly, a role for pHi dynamics in differentiation, epithelial plasticity, and morphogenesis remains understudied. Increased pHi is reported to enable or be necessary for the differentiation of $CD4^+$ T helper 9 (Th9) cells [85], epithelial-to-mesenchymal transition (EMT) [86], and neural fates from ectoderm during Xenopus development [94]. In contrast, expression of the Cl-HCO3 exchanger AE2, which as an acid loader facilitating HCO3 efflux should lower pHi, is necessary for clonal mouse macrophages to differentiate into osteoclasts [95], although a role for pHi dynamics was not determined. Recent work shows that a glycolysis gradient in mouse and chick embryo tail bud generates a more acidic extracellular pH (pHe) in the tail bud, which when experimentally manipulated to be more alkaline, results in slower axis elongation [96]. Additionally, decreased pHe from extracellular lactic acid generated by lactate dehydrogenase enables myofibroblast differentiation in an EMT-like fibrosis by increasing acid-induced activation of latent TGF-β in the extracellular matrix [97], and an acidic pHe enables differentiation of MSCs into cancer-associated fibroblasts through a mechanism involving a pH-sensitive GPCR that regulates Yes-associated protein (YAP) signaling [98]. In normal adult tissues, pHe is ~7.4 and higher than pHi of ~7.2. In most cancers, this gradient is reversed, with pHe being ~7.0 or lower while pHi is ~7.6 and higher [77,78]. An intriguing prediction that remains to be verified is whether cancer initiating cells, analogous to stem cells, might have a lower pHe and higher pHi than differentiated cancer cells. In support of this prediction, an acidic pHe promotes self-renewal of glioma stem cells by increasing stability of hypoxia inducible factor 2a [99].

The mechanisms by which pH dynamics regulates differentiation remain largely unknown. However, based on our previous findings on pHi-dependent cell behaviors such as proliferation and migration, we speculate important roles for pH sensors, defined as selective proteins with post-translational modification by protonation/deprotonation regulating activity or ligand binding [100]. Our findings with pHi-regulated *Drosophila* follicle stem cell differentiation suggest pH sensing by the hedgehog signaling pathway [84]. Previous findings in *Drosophila* eye epithelium indicate pHi-dependent Wnt signaling with a higher pHi enabling binding of disheveled to the plasma membrane and being necessary for planar cell polarity [101]. With regard to pH-dependent post-translational modification, a decreased pHi is associated with global histone deacetylation [102]. Epigenetic modifications such as histone modification and DNA methylation have established roles in cell differentiation by changing chromatin structure to activate or inhibit gene expression. Although unique for each stem cell lineage, in general, DNA silencing by methylation of CpG islands suppresses the expression of genes involved in cell cycle exit and terminal differentiation and hence preserves the progenitor self-renewing state [103].

Metabolic reprogramming is another potential mechanism for pHi-regulated epigenetic modifications [104]. Recent findings show that a more acidic pHi promotes promiscuous enzymatic activity of lactate dehydrogenase to convert α-ketoglutarate to the L enantiomer of 2-hydroxyglutarate, compared with conventional lactate dehydrogenase conversion of pyruvate to lactate [105,106]. L- and D-2-hydroxyglutarate antagonize α-ketoglutarate-regulated chromatin modifications associated with differentiation and also stabilize expression of HIF-1α [85,86]. Additionally, stabilized HIF-1α promotes reprogramming to a glycolytic metabolism during the ESC to EpiSC transition [6]. Hence, increased pHi during stem cell differentiation could enable reprogramming to a more glycolytic phenotype, which could be mediated by glycolytic enzymes that are pH sensors with increased activity at higher pH, such as phosphofructokinase-1 [77,78]. While studies have begun to uncover the integral role of pH dynamics in regulating cell fate changes, an important future direction is to identify the mechanisms mediating this effect.

Cell morphology and adhesion dynamics

Although differentiation often includes changes in cell shape and cell adhesion, including both cell–cell and cell-matrix adhesion, we have an incomplete understanding of how these changes are regulated during differentiation and contribute to the differentiation process. Understanding the underlying cell biology of differentiation, especially during *in vivo* development, requires knowledge of how the cell interprets its niche through cell shape and adhesion-derived mechanical forces. In this section, we review recent progress in how cell morphology and mechanical cues instruct cell fate decisions (Fig 3).

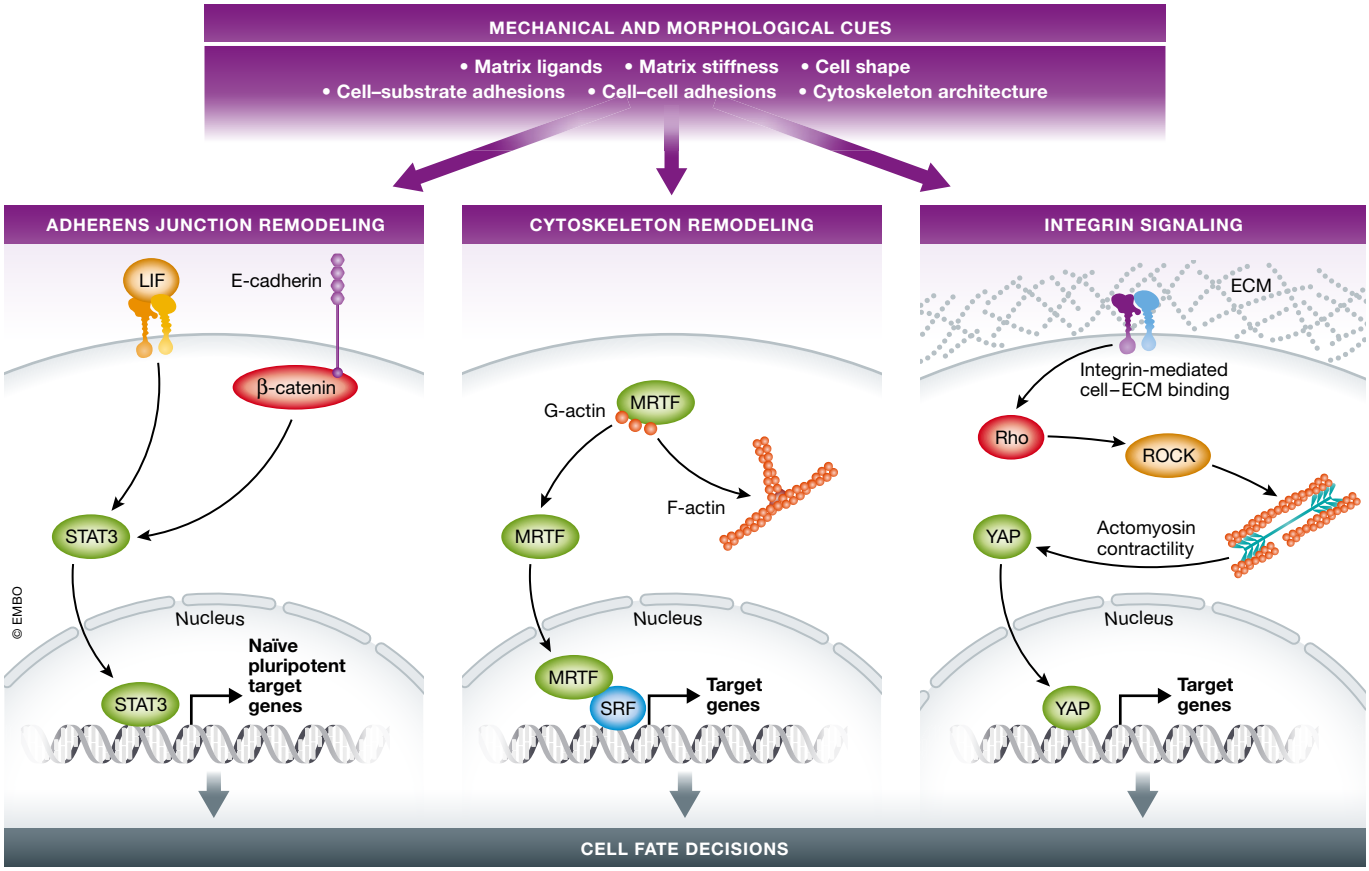

Figure 3. Mechanical and morphological cues regulate cell fate decisions through distinct signaling mechanisms.
Cues provided by extracellular matrix (ECM) ligands, ECM stiffness, cell shape, cell-substrate adhesion, cell–cell adhesion, and cytoskeleton architectures inform the cell of its surrounding niche (right panel). The naïve state of clonal embryonic stem cells is routinely maintained in medium supplemented with leukemia inhibitory factor (LIF), which activates STAT3 to induce expression of naïve pluripotent target genes. However, expression of E-cadherin in pluripotent stem cells is sufficient to promote LIF-independent self-renewal by activating STAT3 to induce expression of naïve pluripotent target genes. This later effect requires the β-catenin-binding region of E-cadherin (left panel). With increased actin polymerization, myocardin-related transcription factor (MRTF), which is retained in the cytoplasm by binding to G-actin, translocates to the nucleus where it binds the transcription factor serum response factor (SRF) to activate genes regulating differentiation programs (middle panel). In response to integrin-mediated cell-substrate adhesion, the low molecular weight GTPase Rho activates Rho-associated protein kinase (ROCK) to generate actomyosin contractility, which results in nuclear translocation of yes-associated protein (YAP) (right panel).

Cell-substrate adhesion

Extracellular matrix (ECM) interactions with integrins and the changes in cell shape and tensional forces they generate provide instructive cues in stem cell fate decisions for both embryonic and adult stem cells, although downstream pathways result in divergent outcomes depending on the cellular context. Variable matrix elasticity directs MSC lineage specification with a greater selectivity than through biochemical cues and generates cellular fate memory that persists after cells are removed from a given matrix [107]. Furthermore, pre-committing naive MSCs on a matrix stiffness that most closely recapitulates *in vivo* niche stiffness improves microenvironment adaptation upon implantation [107]. The effect of cell shape on MSC fate decisions has also been shown by plating cells on small fibronectin islands, which reveals that cells with a rounded morphology differentiate to adipogenic lineages, while cells with a flattened cell shape differentiate to osteogenic lineages [108]. This morphology-driven differentiation is dependent on activity of the low molecular weight GTPase RhoA, indicating that the mechanical cues of cell shape and contractility contribute to lineage commitment in MSCs. In support of this finding, McBeath and colleagues [108] suggest that changes in cell shape sensed through integrin binding of ECM ligands, which provide tensional forces, can drive signaling cascades that result in altered gene expression in MSCs.

In contrast to these MSCs that respond to integrin signaling with self-renewal, ECM–integrin interactions facilitate differentiation in mouse ESCs [109]. Teasing apart the roles of mechanical forces resulting from integrin-mediated cell–ECM adhesion versus E-cadherin-mediated cell–cell adhesion, Uda *et al* [110] found that force via integrins but not E-cadherins decreases Oct3/4 expression in mouse ESCs, suggesting mechanical forces from distinct force transduction pathways can play divergent roles in embryonic stem cell biology.

Divergent roles for cadherin-mediated and integrin-mediated force transduction pathways may occur in the stem cell niches present in *Drosophila melanogaster* gonad development. DE-cadherin, the *Drosophila melanogaster* homolog of E-cadherin, mediates cell–cell adhesion between germline stem cells and other

cells within the *Drosophila* ovary niche for both proper recruitment and anchoring [111]. Somatic stem cells within the *Drosophila* ovary generate follicle progenitor cells and several differentiated cells within the chamber. These epithelial stem cells are similarly anchored to the surrounding niche by DE-cadherin in order to prevent differentiation [112]. In addition to this cadherin-mediated cell–cell adhesion, integrins also enable follicle stem cells in the *Drosophila* ovary to adhere to surrounding basal lamina in the niche, anchoring them in position to respond to cues regulating their differentiation [113]. During gonad morphogenesis in the *Drosophila* testis, germline stem cells contact hub cells in the niche. Integrin-dependent adhesion but not DE-cadherin-dependent adhesion positions the hub cells such that ECM surrounding the gonads anchors the niche and the germline stem cells [114,115]. Somatic stem cells within the *Drosophila* testis must also contact hub cells to maintain self-renewal and proliferation, but these contacts are DE-cadherin-mediated [115]. Though dependent on distinct anchoring mechanisms, positioning of both germline stem cells and somatic stem cells along hub cells within the *Drosophila* testis allows cooperation during gametogenesis as both cell types respond in different ways to local JAK-STAT signaling within the niche [116]. A recent study suggests that DE-cadherin affects signaling in the *Drosophila* ISCs through a feedback loop that couples enterocyte cell death to ISC divisions [117]. In this tissue, β-catenin is typically sequestered at the adherens junctions in enterocytes, but enterocyte cell death disrupts these junctions and thus causes the release of β-catenin. β-catenin then translocates to the nucleus where it activates the expression of *rhomboid*, which promotes the secretion of EGF ligands and ultimately leads to increased ISC proliferation.

We highlight here merely some advances in our understanding of how cell–ECM interactions and cell shape contribute to stem cell fate decisions. For more comprehensive discussions, we refer readers to reviews on ECM, integrins, and growth factors directing stem cell fate [118], nanoscale features of integrin–matrix interactions, matrix stiffness and 2D versus 3D cultures [119], and integrin- and cadherin-mediated adhesion in maintaining a supportive niche for stem cell anchoring, self-renewal, and differentiation [120–122].

Cell–cell adhesion

The role of cadherin-mediated cell–cell adhesion in pluripotent cells is currently an area of active investigation. In mouse embryos, the adherens junction protein E-cadherin is highly expressed until gastrulation, when E-cadherin is downregulated as epithelial epiblasts undergo an epithelial-to-mesenchymal transition (EMT) and germ layers are specified. Animals null for E-cadherin are unable to complete embryogenesis beyond this point [123,124], which may be due in part to the lack of mechanical forces at adherens junctions [125]. However, heterozygous loss of E-cadherin combined with N-cadherin knock-in results in normal embryonic development [126]. Whether the *in vivo* role for E-cadherin is similar for differentiation of embryonic stem cells *in vitro* remains controversial. Spencer *et al* [127] found that mouse ESC differentiation involves traditional markers of EMT such as an E-cadherin to N-cadherin switching, increased expression of the E-cadherin repressors Snail and Slug, and increased cell motility. Also in support of a pluripotent self-renewal promoting role for cell–cell adhesion, E-cadherin-mediated cell–cell contacts promote mouse ESC self-renewal and induced pluripotent stem cell (iPSC) generation [128,129]. In agreement with this proposed role, mouse ESCs null for E-cadherin have a transcriptional profile that more closely resembles differentiated epiblast stem cells than self-renewing naïve ESCs [130]. Interestingly, genes most differentially expressed in self-renewing ESCs from E-cadherin$^{-/-}$ compared with WT mice are not limited to cell adhesion and motility but also includes transcripts related to metabolic processes, catabolism, and apoptosis [130]. A comprehensive evaluation of the roles for E-cadherin in embryonic stem cells, pluripotency, and self-renewal is beyond the scope of our discussion of lesser-studied regulators of stem cell biology, but we refer the reader to several excellent reviews on this topic [131–133].

Like E-cadherin, the role of β-catenin in stem cell self-renewal and differentiation is currently controversial, despite consensus on the importance of repressive transcriptional activity Tcf3 downstream of canonical Wnt signaling, as described more completely in recent reviews on embryonic [134] and adult [135] stem cells. For embryonic stem cells, conflicting findings may result from distinct β-catenin functions as an adherens junction protein and a signaling molecule in the Wnt pathway, with perhaps a cell–cell adhesion function being more critical. In brief, for embryonic stem cells, one view is that β-catenin is not necessary for the self-renewal and expansion of naïve mESCs, but its absence eliminates the self-renewal response to Gsk3 inhibition [136]. Another non-contradictory view is that a complex of β-catenin, E-cadherin and Oct 4 but not β-catenin transcriptional activity is necessary for pluripotency [137]. Additionally, β-catenin may be necessary for subsequent differentiation stages because mesendodermal germ layer formation and neuronal differentiation are defective in β-catenin-null mESCs [138]. Redundancy between catenins may also explain conflicting findings because in β-catenin-null mESCs, loss of γ-catenin promotes exit from pluripotency [139], which further suggests the importance of the adherens junctions but not signaling function of β-catenin in embryonic stem cell self-renewal and differentiation.

Actin filaments

Although actin filament dynamics regulate cell-substrate adhesion, cell–cell adhesion, and cell morphology, we have limited understanding of its direct role in stem cell differentiation and lineage specification. Moreover, how actin cytoskeleton dynamics might regulate transcriptional programs in cell differentiation is incompletely understood, although current evidence implicates roles for YAP, transcriptional activator with PDZ-binding motif (TAZ), and myocardin-related transcription factor (MRTF), which are transcriptional regulators responding to mechanical force or actin remodeling. YAP and TAZ, transcriptional cofactors in the Hippo signaling pathway, are both required for early mouse embryo development [140]. In response to mechanical cues, YAP and TAZ translocate from the cytoplasm to the nucleus where they bind the transcription factor TEAD and other promoter-specific transcription factors (reviewed in [141]). Higher stiffness of the surrounding extracellular matrix results in nuclear YAP/TAZ localization by an unclear mechanism that senses cell tension [142]. Multiple types of mouse stem and progenitor cells, including ESCs, are characterized by upregulated YAP expression, suggesting that Hippo signaling promotes pluripotency-related pathways [143]. Additionally, Yorkie, the *Drosophila* homolog of Yap, causes increased ISC proliferation in

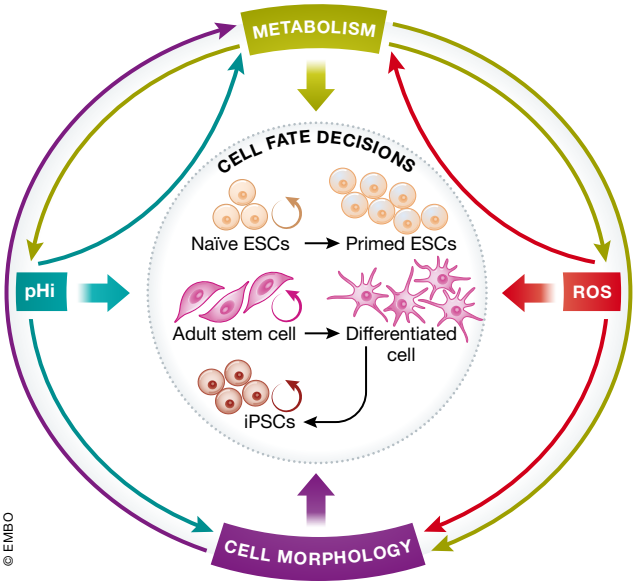

Figure 4. Network of cell biological cues that instruct cell fate decisions. Examples of cell fate decisions include naïve embryonic stem cells (ESC) undergoing self-renewal (curved arrow) or differentiating into a primed ESC (straight arrow); adult stem cells self-renewing (curved arrow) or becoming differentiated cells (straight arrow); and reprogramming of differentiated cells to form induced pluripotent stem cells (iPSCs). Cell fate decisions are directly influenced by cell biological cues like metabolism, intracellular pH (pHi), reactive oxygen species (ROS), and cell morphology (short colored arrows). Additionally, since these cell biological cues can affect each other (long colored arrows), these cues also affect cell fate decisions indirectly. Metabolism can affect pHi, ROS and cell morphological changes. pHi and ROS can influence cellular metabolism and morphology. Cell morphological changes can also affect metabolic changes. Therefore, the interaction between these cell biological cues forms a network of cues that instruct cell fate decisions.

response to intestinal epithelia damage [144,145] and also functions downstream of hedgehog signaling to promote proliferation of follicle stem cells [146]. In the mouse intestine, Yap activity contributes to the downregulation Wnt signaling, which is the key ISC self-renewal signal, and overexpression of Yap causes ISC loss whereas knockout of Yap causes an increase in the number of ISCs and Paneth cells. In contrast, overexpression of Yap in the epidermis has the opposite effect, causing an expansion of the stem cell pool and the formation of squamous cell-like carcinomas. However, knockout of the upstream negative regulator, Mst1/2, does not have the same effect, suggesting that Yap is activated by a non-canonical mechanism in this tissue.

Myocardin-related transcription factor is another link between actin remodeling and transcriptional regulation. In contrast to nuclear translocation of YAP/TAZ in response to cell-substrate signals, MRTF is translocated from the cytosol to the nucleus in response to increased actin polymerization [147,148]. In the nucleus, MRTF is a cofactor for transcriptional regulation by SRF to induce expression of over 200 transcripts, mostly related to actin dynamics, cell motility, muscle-specific genes, and miRNAs (reviewed in [149]). Although a role for MRTF in ESCs remains undetermined, it is important for adult MSC differentiation. Specifically, the degree of cell spreading in a precursor of the adipogenic and osteogenic lineages increases actin polymerization, and MRTF is translocated to the nucleus to promote osteogenic gene expression programs [150–154].

Despite recent advances, further understanding of how cell shape, adhesion, and actin filament dynamics contribute to stem cell differentiation is needed to inform how directed *in vitro* differentiation protocols are optimized for regenerative medicine applications. For example, Gilbert *et al* [155] showed that differentiating muscle cells reorganizes their actin cytoskeleton to match their cultured substrate stiffness, significantly improving the cell's ability to engraft and properly heal after implantation when the cultured substrate stiffness matched that of the *in vivo* niche. Additionally, Myers *et al* [156] found that cell colony geometry is a driver of stem cell fate decisions in 2D culture systems: patterning of colonies according to uniform size, density, and shape resulted in improved homogeneity and yield of human iPSC-derived cardiomyocytes. Zoldan *et al* [125] found that culturing hESCs on variable scaffold stiffnesses was sufficient to induce lineage-specific gene expression. As the field of regenerative medicine continues to develop *in vitro*-derived cell replacement therapeutics, knowledge of the underlying cell biology of stem cell shape and adhesion as it pertains to both *in vivo* development and *in vitro* differentiation will greatly inform future studies.

Conclusion

Our review highlights the diversity of mechanisms used to regulate cell fate decisions. Assuming that a more robust cell fate determination process provides an evolutionary advantage, it seems likely that different cells are regulated by multiple and sometimes distinct cues. Extracellular chemical and mechanical cues integrate with intracellular protein- and metabolite-based signaling for the complex control of cell fate decisions (Fig 4). Changes in metabolic state directly impact protein-based regulation of cell fate decisions by, for example, shifting the availability of metabolites that are required for epigenetic modifications and regulating metabolic sensors, such as AMPK and sirtuins [24]. Metabolic changes are also the primary causes of changes in ROS concentrations, which contribute to cell fate decisions through the JNK and p38 MAPK pathways [66].

Box 1: In need of answers

(i) What roles do metabolic pathways other than glycolysis and the TCA cycle play in cellular differentiation?
(ii) Do changes in S-adenosyl methionine concentrations regulate adult stem cell self-renewal and/or differentiation?
(iii) Do the ROS signals that contribute to the regulation of cellular differentiation promote aging?
(iv) What are the key pH-sensing proteins that mediate effects of pHi dynamics in regulating cell fate?
(v) Do changes in pHi affect cellular differentiation by influencing metabolism, reactive oxygen species, or the cytoskeleton?
(vi) Despite considerable work, there is still a lack of a comprehensive understanding about the role of adherens junctions in ESC pluripotency.
(vii) How do changes in actin dynamics, cell shape, and cell adhesion regulate cellular differentiation, particularly with regard to epigenetic and transcriptional effects?

Increased ROS levels feedback to regulate metabolism by activating transcription factors such as FoxO family members that regulate metabolism, and can promote the activity of Rho-associated protein kinase (ROCK), which regulates cytoskeletal-associated proteins such as myosin, talin, and cofilin [157]. Additionally, Hippo signaling, which responds to mechanical cues, can induce changes in metabolism [158], and metabolism can affect cell shape and cytoskeletal dynamics through the effects of AMPK on cell polarity proteins and myosin regulatory light chain [159–161]. Metabolic changes that shift in the balance of energy and biomass production from glycolysis versus oxidative phosphorylation also affect pHi by changing the redox state of the cell. Changes in pHi can also feedback to regulate metabolism by, for example, affecting the activity of pH-sensitive enzymes such as phosphofructokinase-1 [162,163]. Finally, changes in pHi can also impact cell shape and mechanical cues through effects on pH-sensing actin regulatory proteins, such as cofilin and talin [164,165].

Collectively, the studies summarized here demonstrate the extensive contribution of cell biological regulators to the mechanisms that govern cell fate decisions. Nevertheless, many open questions remains (Box 1). However, the field is still relatively new, and the increasing interest combined with new methods for studying the cell biology of cell fate decisions *in vivo* is likely to lead to more insights into this significant area of developmental biology.

Conflict of interest

The authors declare that they have no conflict of interest.

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