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### ► To cite this version:

Guotian Luo, Pauline Wosinski, Giuliana Salazar-Noratto, Morad Bensidhoum, Rena Bizios, et al.. Glucose metabolism: optimizing regenerative functionalities of mesenchymal stromal cells post-implantation. *Tissue Engineering Part B: Reviews*, 2023, 29 (1), pp.47-61. 10.1089/ten.TEB.2022.0063 . hal-03836581

**HAL Id: hal-03836581**

**<https://hal.science/hal-03836581v1>**

Submitted on 8 Nov 2022

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# Glucose metabolism: optimizing regenerative functionalities of mesenchymal stromal cells post-implantation

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**Key words:** Mesenchymal stromal cells, Glucose, Metabolism, Cell survival, Cell proliferation, Immunosuppression, Angiogenesis, *In silico* models

*To cite this article:* Luo G, Wosinski P, Salazar-Noratto GE, Bensidhoum M, Bizios R, Marashi SA, Potier E, Sheng P, Petite H. Glucose metabolism: optimizing regenerative functionalities of mesenchymal stromal cells post-implantation. Tissue Eng Part B Rev. 2022 Jun 25. doi: 10.1089/ten.TEB.2022.0063. Epub ahead of print. PMID: 35754335.

*Document Version:* Accepted manuscript including changes made at the peer-review stage.

## Abstract

Mesenchymal stromal cells (MSCs) are considered promising candidates for regenerative medicine applications. Their clinical performance post-implantation, however, has been disappointing. This lack of therapeutic efficacy is most likely due to suboptimal formulations of MSC-containing material constructs. Tissue engineers, therefore, have developed strategies addressing/incorporating optimized cell-, microenvironmental-, biochemical, - and biophysical- cues/stimuli to enhance MSC-containing construct performance. Such approaches have had limited success because they overlooked that maintenance of MSC viability after implantation for a sufficient time is necessary for MSCs to develop their regenerative functionalities fully. Following a brief overview of glucose metabolism and regulation in MSCs, the present literature review includes recent pertinent findings that challenge old paradigms and notions. We hereby report that glucose is the primary energy substrate for MSCs, provides precursors for biomass generation, and regulates MSC functions, including proliferation and immunosuppressive properties. More importantly, glucose metabolism is central in controlling *in vitro* MSC expansion, *in vivo* MSC viability, and MSC-mediated angiogenesis post-implantation when addressing MSC-based therapies. Meanwhile, *in silico* models are highlighted for predicting glucose needs of MSCs in specific regenerative medicine settings, which will eventually enable tissue engineers to design viable and potent tissue constructs. This new knowledge should be incorporated into developing novel effective MSC-based therapies.

## Impact statement

The clinical use of mesenchymal stromal cells (MSCs) has been unsatisfactory due to the inability of MSCs to survive and be functional after implantation for sufficient periods to mediate directly or indirectly a successful regenerative tissue response. The present review summarizes the endeavors in the past but, most importantly, reports the latest findings that elucidate underlying mechanisms and identify glucose metabolism as the crucial parameter in MSC survival and subsequent functions pertinent to new tissue formation of importance in tissue regeneration applications. These latest findings justify further basic research and the impetus for developing new strategies to improve the modalities and efficacy of MSC-based therapies.

## 1. Introduction

Mesenchymal stromal cells (MSCs) are promising candidates for regenerative medicine applications because they can proliferate and differentiate into other cell types (including osteoblasts, chondrocytes, and adipocytes) and induce a regenerative microenvironment by secreting bioactive chemical compounds<sup>1-3</sup>. The functionality of MSC-containing constructs is a joint action of MSCs and their microenvironment in which they are implanted<sup>4,5</sup>. Tissue engineers have recognized the crucial role of cellular characteristic aspects (such as cell source, passage number, and cell differentiation state)<sup>6-8</sup> on the ultimate efficacy of engineered tissues. These research endeavors have also demonstrated that biochemical stimuli (*e.g.*, cytokines<sup>9,10</sup> and extracellular matrix proteins<sup>10,11</sup>) and biophysical cues (such as stiffness and topography of the substrate material scaffold<sup>12</sup>, mechanical stresses and strains exerted on cells<sup>13,14</sup>) are critical aspects that regulate MSC functions pertinent to tissue regeneration.

Cellular metabolism, *i.e.*, the sum of all chemical reactions catalyzed by enzymes within cells, is gradually emerging as the central regulator of all cellular functions<sup>15</sup>. It provides energy that powers cellular activities and the building blocks for cell growth and regulates cell fate decisions<sup>16</sup>. MSCs transplanted within damaged tissues lacking vascularization require catabolism (to fuel their high energy demand for survival) and anabolism, to provide adequate intracellular biomass (such as proteins, lipids, and DNA) postimplantation<sup>3</sup>. Thus, transplanted MSC metabolism regulates cell survival, proliferation, differentiation and paracrine functions during tissue regeneration.

Historically, due to the low oxygen tension in damaged tissues<sup>17</sup>, researchers have focused on the role of oxygen in cellular metabolism and investigated oxygen-delivery materials for improving MSC survival and functions during tissue regeneration<sup>18,19</sup>. However, oxygen deprivation is not the only feature affecting MSC metabolism; the availability of nutrients (such as glucose and amino acids) as metabolic substrates is another critical component affecting MSC metabolism and ultimately dictating cell fate outcomes.

Although there has been less focus on nutrients, the roles of glucose have progressively emerged in MSC-mediated regenerative response<sup>3,20,21</sup>. In fact, glucose is the primary energy source to fuel most mammalian cells. Additionally, glucose provides carbon to generate biomass precursors, including phospholipids, amino acids, and nucleotides, to support cell division and other functions<sup>22,23</sup>. In addition, glucose metabolism through anaerobic glycolysis, oxidative phosphorylation (OXPHOS), and interlinked side pathways provides precursors for protein modifications and epigenetic signatures (Figure 1)<sup>24,25</sup>; such outcomes exert regulatory effects on stem cell functionalities<sup>26-28</sup>.

Several comprehensive reviews have highlighted how glucose metabolism affects the biology of various stem cells, including embryonic, pluripotent, and hematopoietic stem cells<sup>29-31</sup>. The present review focuses on the pivotal role of glucose in the regenerative functionalities of MSCs. Although MSCs can be isolated from various tissues, we focus on bone marrow-derived MSCs, which have generated a great deal of interest in many clinical settings, including tissue engineering, immunomodulation, and regenerative medicine. Unless otherwise stated, the term "MSCs" in this review refers to bone marrow-derived MSCs.

Following a brief overview of glucose metabolism in MSCs, the current fundamental knowledge about the role of glucose in MSC proliferation and MSC-mediated immunosuppressive properties, as well as the applications of glucose in optimizing *in vitro* MSC expansion and improving post-implantation MSC viability and therapeutic angiogenesis from regenerative medicine perspectives, are reviewed. Meanwhile, the relevance of *in silico* models are highlighted for predicting glucose needs of MSCs in specific regenerative medicine settings, which will eventually enable tissue engineers to design viable and potent tissue constructs.

## 2. Fundamental aspects

### *An overview of glucose metabolism in MSCs*

**Glucose uptake from the extracellular milieu.** Glucose is a small hydrophilic molecule that circulates dissolved in blood plasma but cannot cross the cell-plasma membrane. Instead, it is uptaken through either endolysosomal pathways<sup>32,33</sup> or facilitative diffusion by adenosine 5'-triphosphate (ATP)-independent, cell-surface, nutrient transporters referred to as Glucose Transporters (GLUT1 to 14)<sup>34</sup>. GLUT1, GLUT3, and GLUT4 were identified for the glucose uptake in human placental amnion-derived MSCs (h-PA-MSCs)<sup>35</sup>. Their high affinity for glucose permits its uptake at a high rate under physiological conditions. However, h-PA-MSCs cannot efficiently downregulate glucose transporters, and thus glucose intake, in the presence of excess glucose<sup>35</sup>. GLUT1, which expresses ubiquitously, is considered responsible for basal glucose uptake<sup>36</sup>. Oxygen tension, nutrient deprivation, and the PI3K/AKT pathway regulate GLUT1 expression<sup>37-39</sup>. In mouse MSCs (mMSCs), GLUT1 is needed for osteoblast differentiation and new bone formation by regulating both master osteogenic transcription factor RUNX2 and nutrient sensor 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK)<sup>40,41</sup>. Increased GLUT1 expression was reported in MSC-mediated immunomodulation<sup>42,43</sup>. GLUT4 is insulin-sensitive and is sequestered intracellularly in GLUT4 storage vesicles, which undergo exocytosis to cell plasma membrane upon insulin binding to its receptor and activation of the PI3K/AKT signaling cascade<sup>44</sup>. This GLUT4 translocation results in a 10-to-20-fold increase in glucose uptake<sup>36</sup>. To the author's knowledge, the precise role of GLUT3 and GLUT4 in MSC biology remains unknown. Last but not least, glucose uptake in mammalian cells surrounded by nutrient-rich body fluids is predominantly placed under the control of growth factors acting as cell-extrinsic regulators of nutrient acquisition and usage. Glucose uptake can be secondarily regulated by intrinsic nutrient sensors such as AMPK<sup>45-47</sup>. This tight regulation of glucose uptake prevents excessive nutrient consumption and abnormal cell proliferation. Despite the importance of growth factors as cell-extrinsic regulators of glucose uptake and use, it lacks studies seeking to identify the specific growth factors responsible for modulating glucose uptake in MSCs.

**MSCs' metabolic flexibility.** MSCs respond and adapt to conditional changes in metabolic demand (Figure 2). For example, when cultured *in vitro* at 21% oxygen, hMSCs progressively switch from a highly glycolytic to an OXPHOS phenotype<sup>48</sup>. Along similar lines, hMSCs cultured under chondrogenic conditions prefer glycolysis<sup>49</sup>, whereas hMSCs cultured under osteogenic conditions prefer OXPHOS<sup>50</sup>. Hypoxia-inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ), known for its ability to sense oxygen tension<sup>51</sup>, is central to the metabolic reprogramming of stem cells<sup>52-56</sup>. HIF-1 is a heterodimeric transcription factor, including an O<sub>2</sub>-regulated

HIF-1 $\alpha$  subunit and a constitutively expressed aryl hydrocarbon nuclear translocator HIF-1 $\beta$  subunit. Under sufficient oxygen conditions, HIF-1 $\alpha$  protein is primed for ubiquitination and degradation in proteasomes. HIF-1 $\alpha$ , however, is more stable under hypoxia, translocates to the cell nucleus, and dimerizes with HIF-1 $\beta$ . The heterodimer HIF-1 $\alpha$ /HIF-1 $\beta$  binds to the hypoxia response element site on DNA and thus regulate the expression of genes involved in glucose metabolism and angiogenesis<sup>57–60</sup>, thereby facilitating cell adaptation to hypoxia. HIF-1 $\alpha$  stability is also affected by metabolites, including lactate and  $\alpha$ -ketoglutarate<sup>61–63</sup>. In hMSCs, HIF-1 promotes glycolysis by directly upregulating the expression of several glycolytic enzymes, including hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase; the result is an increased production of ATP in the cytosol from the breakdown of glucose to pyruvate<sup>64,65</sup>. Moreover, in response to both oxygen and nutrient availability and level, HIF1 positively regulates lactate production and NAD<sup>+</sup> regeneration and negatively regulates the rate of pyruvate entry into the mitochondria<sup>63,66</sup>. Last but not least, HIF-1 regulates GLUT1 expression<sup>39</sup>.

**The main glucose signaling networks.** Several nutrient sensors (such as AMPK and mechanistic target of rapamycin kinase (mTOR)) are critical in coordinating changes between the local metabolic milieu and MSC biology by inducing signals inside cells in response to changes in the extracellular environment<sup>28</sup>. These nutrient sensors do not belong to strictly linear signaling pathways. In fact, they act interdependently both antagonists and agonists of each other's activity to collectively determine the response of a given cell type to extracellular signals<sup>67</sup> (Figure 3). Briefly, AMPK is of utmost importance because it enables cells to sense cellular energy levels and nutrient availability and respond to changes in their milieu. The underlying mechanism involves allosteric activation of AMPK by increased AMP<sup>67</sup>. Upon activating its catalytic alpha subunit, AMPK phosphorylates pertinent substrates to restore cellular bioenergetics homeostasis. AMPK activation directly impacts glucose homeostasis by increasing expression and activity of GLUTs<sup>28,68–70</sup>. AMPK regulates MSC proliferation<sup>71,72</sup>, viability<sup>20,73</sup>, differentiation<sup>74–76</sup>, and immunosuppressive properties<sup>77</sup>.

Another important signaling pathway is mTOR integrating metabolic regulation of growth factors and nutrients. mTOR includes two complexes (mTORC1 and mTORC2) with divergent functions. mTORC1 responds to cellular energy status and cellular oxygen, amino acids, and glucose levels. Specifically, even in the presence of growth factors (e.g., insulin and insulin-like growth factors (IGF)), mTORC1 is inhibited in cells that are either energetically stressed or have depleted oxygen or amino acid levels<sup>78</sup>. Under these conditions, mTORC1 suppression represses anabolic pathways and induces autophagy and lysosomal biogenesis<sup>78,79</sup>. Conversely, in the presence of ample nutrients and growth factors, mTORC1 activation upregulates glycolysis, protein and lipid synthesis, and mitochondrial metabolism<sup>80</sup>. Changes in mTORC1 signaling affect hMSCs survival<sup>81</sup>, osteogenic differentiation<sup>82,83</sup>, and immunosuppressive properties<sup>84</sup>. Unlike mTORC1, mTORC2 is insensitive to the presence of nutrients but primarily responds to critical growth factors (e.g., insulin and IGF)<sup>85</sup>.

AMPK and mTOR are not only regulated by metabolite levels but also by growth-factor-related signaling pathways. For example, AKT (an anti-apoptotic factor) activates mTORC1 but antagonizes

AMPK activation. Moreover, AKT activation does not inhibit cell death but instead renders cells more sensitive to metabolic stress<sup>86–89</sup>. AKT regulates glucose metabolism by increasing GLUT1 trafficking to the mammalian cell surface and activating HK and PFK, thus promoting glycolysis<sup>86,88</sup>. Some other studies reported that antiapoptosis capacity of AKT is coupled with glucose metabolism<sup>86,87,90,91</sup>. Regarding the role of AKT on MSCs, literature reports indicate that AKT is involved in regulating mMSC survival in ischemic tissues<sup>92</sup>, rat MSC (rMSC) senescence (induced by high glucose<sup>93</sup>), and hMSC osteogenesis<sup>94</sup>.

### *The role of glucose on MSC proliferation*

**Contribution of anaerobic glycolysis to MSC expansion.** In proliferating hMSCs, glycolysis contributes between 67%<sup>49</sup> and 97%<sup>95</sup> ATP production. To match their metabolic needs, proliferating MSCs switch from glycolytic metabolism state (of quiescent MSCs) to mixed metabolism of OXPHOS and glycolysis (of proliferating MSCs). During MSC expansion, this metabolic “switch” is evidenced by the decrease in the yield of lactate from glucose (Ylac/glc) from 2 mol/mol (suggesting anaerobic glycolysis) at the start of cell culture (days 0–3) to 1 mol/mol (indicating a combination of aerobic glycolysis and OXPHOS) at later time points (days 3–15)<sup>96</sup>.

**Computational modeling of metabolism during MSC expansion.** In recent years, genome-scale metabolic network models (GEMs) have been extensively used to model human cell metabolism<sup>97–99</sup>. A quick overview of these models is further presented in Section 4. Such models are valuable computational tools because they integrate all known biochemical genes, enzymes, metabolites, and reactions for specific cell types and simulate metabolic phenotypes *in silico*. MSC-specific GEMs have been successfully used to study a variety of MSC metabolic phenotypes, e.g., during cell proliferation<sup>100–102</sup>. For example, a GEM of hMSC called iMSC1255 can acceptably predict the growth rate of hMSCs in the growth medium and simulate quantitatively the consumption profiles of various nutrients in expanding MSCs, including amino acids and glucose<sup>100</sup>. This hMSC model is further validated by comparing the experimentally measured metabolic fluxes with the *in silico* ones under either normoxia or hypoxia conditions<sup>101</sup>. More specifically, the GEM-based simulations demonstrate that, in a low glucose medium, hypoxia notably decreases the pyruvate transport to the mitochondrion, despite the increased glucose uptake<sup>101</sup>. Flux balance analysis (FBA) of iMSC1255 shows that glucose availability directly influences the biomass production rate, while oxygen availability hardly affects biomass production. This observation implies that glucose availability directly affects cell proliferation and oxygen availability plays a minor effect in cell proliferation<sup>103</sup>. Altogether, MSC-specific GEMs predict that sufficient glucose concentrations should be available in the cell microenvironment to support MSC expansion.

### *The role of glucose on MSC immunosuppressive properties*

MSCs possess broad immunosuppressive properties that affect innate and adaptive immunity. For example, MSCs favor macrophage polarization to anti-inflammatory phenotype, reduce lymphocyte B cell proliferation, and inhibit conventional lymphocyte T cell proliferation, but induce regulatory lymphocyte T cell expansion<sup>104,105</sup>. These immunosuppressive properties are not constitutively expressed but depend on proinflammatory stimuli in a process known as ‘licensing’<sup>104</sup>. Licensed MSCs then acquire immunosuppressive properties by releasing several soluble

chemical compounds including indoleamine 2,3-dioxygenase (IDO), prostaglandin E2, and programmed death ligand-1 (PD-L1)<sup>104,106–108</sup>.

Like MSCs requiring metabolic reprogramming for proliferation, MSCs undergo a metabolic shift to express immunosuppressive properties upon licensing<sup>42,109–111</sup>. An increase in glycolysis is evidenced by increased surface expression of GLUT1, increased mRNA expression of several glycolytic genes (including enolase1 and HK2), higher glucose consumption, and higher ECAR<sup>42,110</sup>. The fact that extracellular glucose concentration directly affects the ability of MSCs to inhibit T cell proliferation<sup>112</sup> and IDO<sup>42</sup> expression illustrates the interrelationship between MSC metabolism and immunosuppressive properties.

One possible pathway for integrating MSC metabolism and immunosuppressive properties is protein glycosylation, which diverts 2–5% of all glucose metabolism<sup>113</sup>. In this process, sugar residues are covalently attached to the amide nitrogens of asparagine (N-linked glycosylation) or the hydroxyl groups of serine or threonine (O-linked glycosylation), usually as branched chains. Protein glycosylation occurs within the endoplasmic reticulum and is critical for molecular trafficking and clearance, receptor activation, and signal transduction. Protein glycosylation regulates the immunosuppressive properties of MSCs in at least two instances. First, N-glycosylation controls PD-L1 transport to hMSC surface and its subsequent release in response to MSC licensing<sup>111</sup>. Second, O-GlcNAcylation of signal transducer and activator of transcription 1 controlled the IDO mRNA and protein expression in licensed hMSCs<sup>110</sup>. Overall, manipulating MSC metabolism represents a strategy for enhancing MSC-mediated immunosuppressive properties.

### 3. Glucose and MSC-based tissue engineering

There have been comparatively few research endeavors focusing on the role of glucose in MSC-mediated regenerative responses and directly using glucose for tissue engineering applications. Nonetheless, the critical roles of glucose in MSCs, its ready availability, and low cost make glucose supply a promising approach for investigating several applications pertinent to MSCs-mediated tissue engineering.

#### *In vitro* MSC expansion

Independent of the adult human tissue source from which MSCs are isolated, MSCs are rare (less than one MSC per 1,000 aspirated nucleated cells<sup>114</sup>) and, large-scale expansion of MSCs is necessary. Glucose metabolism is a critical parameter that must be closely monitored and controlled during MSC expansion. Thus, MSC glucose consumption depends on the biochemical and biophysical milieu parameters which MSCs are expanded including choice of basal culture media<sup>115</sup>, oxygen tension<sup>116,117</sup>, and a three-dimensional environment<sup>96</sup>. An additional essential aspect of *in vitro* MSC expansion is that high glucose concentration negatively impacts MSC proliferation<sup>118,119</sup>.

#### *MSC viability post-implantation*

The observed massive MSC death post-implantation prevents achieving the full potential of MSC-based therapy<sup>120–124</sup>. Excellent review articles<sup>3,125–127</sup> were published describing various aspects, such as nutrient deprivation and hypoxia, increased cytokine production, and oxidative stress, involved in MSC death post-

implantation. Several approaches, including genetic manipulation (e.g., Akt-modified rMSCs<sup>128</sup>) and recombinant growth factor delivery (e.g., epidermal growth factor<sup>129</sup>, platelet-derived growth factor<sup>130</sup>), protect rMSCs and mMSCs against cell death in infarcted hearts. rMSCs modified with constitutively active Akt gene, however, may increase the risk of tumorigenesis<sup>128</sup>. Additionally, recombinant growth factors are challenging to deliver in a controlled spatiotemporal manner. More importantly, neither one of these approaches attempts to address the bioenergetics collapse of MSCs post-implantation. Thus, it is crucial to develop novel approaches focusing on aspects related to the metabolic needs of MSCs postimplantation.

Post-implantation, MSCs encounter an ischemic environment characterized by hypoxia and either low concentration or lack of nutrients<sup>120</sup>. Historically, hypoxia has been considered the main culprit in MSC death<sup>131–133</sup>. Recent research, however, provided evidence supporting a shift of paradigm and demonstrated that lacking glucose is responsible for the observed massive MSC death post-implantation<sup>20,21,134–136</sup>. Moreover, supplying glucose to hMSCs improved their survival post-implantation<sup>20,21</sup>.

*In vitro* studies delineating the pivotal role of glucose metabolism on hMSC<sup>20</sup> and sheep MSC survival<sup>135</sup> were carried out under near-anoxia ( $pO_2 \leq 0.1\%$ ), which simulates the milieu faced by hMSCs post-implantation<sup>21</sup>. In this model, glucose deprivation induced massive sheep MSCs death; in contrast, sheep MSC retained their viability for up to day 12 of culture with glucose<sup>135</sup>. These results were confirmed with hMSCs for up to 21 days *in vitro*<sup>20,21</sup>. The addition of either glutamine or serine resulted in massive hMSC death in near anoxia as early as day 3 *in vitro*, underscoring the glucose needs for hMSC survival<sup>21</sup>. The crucial role of glucose was further confirmed by (i) gene expression profiles of metabolic enzymes<sup>21</sup>, which revealed that glycolysis is the only energy-related pathway involved in hMSC survival under near-anoxia<sup>21</sup>, and (ii) the addition of glycolysis inhibitors (either 2-deoxy-D-glucose or sodium oxamate) which induced a rapid and drastic decrease in cell viability<sup>21</sup>.

*In vivo* studies revealed a four-to-five-fold increase of viable hMSCs in glucose-enriched constructs (either hyaluronic acid or fibrin gel loaded with 56 mM glucose), compared to glucose-free constructs, 14 days after implantation in an ectopic mouse model<sup>20,21</sup> (Figure 4). Moreover, hMSCs have minimal glycolytic reserves. hMSCs loaded into fibrin hydrogels and implanted subcutaneously in nude mice exhibited a rapid decrease (approximately 66%) of their glycolytic resources during the first 24 hours post-implantation<sup>21</sup> paralleled by a rapid reduction in the ATP content of hMSCs: specifically, 40% and 63% of the ATP stock was lost after 24 hours and 3 days, respectively<sup>21</sup>. These results are consistent with the observations that either hypoxia- or serum-deprivation preconditioning of hMSCs and mMSCs induced metabolic adaptation by either decreased glucose consumption or increased glucose stock<sup>81,92,137</sup>, possibly resulting in improving cell retention *in vivo* due to glucose being available for more extended periods.

The results above provide compelling evidence that hMSCs can withstand exposure to severe, continuous near-anoxia conditions, providing that glucose is available to fuel ATP production via glycolysis to satisfy the high energy demands of hMSCs (Figure 4). Taken together, these data establish that the presence of glucose in MSC-containing constructs is an essential but for a long time missing) condition for MSC survival post-

implantation. This compelling evidence justifies and prompts the development of novel, glucose-enriched scaffolds that enable MSCs to cross the bioenergetic "valley of death" they encounter between the time of implantation and when much-needed nutrients will be available through the newly formed blood vessels.

#### *MSC-mediated angiogenesis*

Angiogenesis and formation of an interconnected network of functional blood vessels are critical for successful MSC-mediated tissue repair. The new vascular growth provides circulating blood and, thus, supply of oxygen and nutrients as well as simultaneous removal of metabolic waste. It is well-established that secretion of biologically active molecules collectively referred to as the "secretome" is responsible for MSC-mediated angiogenesis. Supernatant conditioned media (CM) collected from MSCs improved the healing of ischemic tissue via bioactive, pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF)<sup>138–140</sup>, basic fibroblast growth factor<sup>138,140,141</sup>, and the monocyte chemoattractant protein 1 (MCP-1)<sup>141</sup>.

Several studies by our research team demonstrated that glucose promotes MSC-mediated angiogenesis<sup>142,143</sup>, suggesting that supplying glucose to MSCs is an alternative strategy for achieving this outcome. Specifically, supernatant CM (collected from hMSCs cultured with either 5.6 or 33 mM glucose under 0.1% pO<sub>2</sub> conditions for 3 days) increased human umbilical vein endothelial cell (HUVEC) migration for up to 36 hours when compared to results obtained using supernatant CM collected from hMSCs cultured without glucose<sup>142</sup>. The increased HUVEC migration was corroborated by increased amounts of several pro-angiogenic factors in CM<sup>142</sup>. Moreover, hMSC-containing hydrogels loaded with glucose were implanted into nude mice. They exhibited an increased volume of newly-formed blood vessels compared to results obtained with respective controls of hMSC-containing hydrogels without glucose at day 21 post-implantation<sup>142</sup>. The volume of newly-formed blood vessels in MSC-containing hydrogels without glucose and in MSC-free hydrogels loaded with glucose were similar and minimal, indicating that the observed angiogenesis was MSC-driven but minimal in the absence of glucose<sup>142</sup>. In addition, glucose delivery to hMSCs via hydrolysis of starch significantly ( $p < 0.05$ ) increased MSC-mediated angiogenesis, as evidenced by enhanced HUVEC migration and tubular structure formation *in vitro* and by higher volume of newly-formed blood vessels *in vivo*<sup>143</sup>.

To date, the underlying mechanism regarding how glucose regulates MSC-mediated angiogenesis remains unclear. Glucose regulates HIF-1 $\alpha$  expression and/or stability in several cell types, including endothelial cells<sup>144,145</sup>, primary cortical neurons<sup>146</sup>, and cancer cells<sup>147–150</sup>. Glucose also increased HIF-1 $\alpha$  expression and bioactivity in hMSCs cultured under 0.1% pO<sub>2</sub><sup>20</sup>. Such regulation of HIF-1 $\alpha$  is a plausible molecular mechanism for explaining the role of glucose on hMSC-mediated angiogenesis. Glucose transfer via gap junction-mediated cell-cell interaction was reported as an alternative mechanism in angiogenesis<sup>145,151</sup>. mMSCs uptake glucose mainly from their extracellular milieu, while the uptaken glucose in HUVEC is transferred to mMSCs via gap-junction-mediated cell-cell interaction<sup>151</sup>. The uptaken glucose in bone marrow mononuclear cells was transferred to HUVECs via gap-junction-mediated cell-cell interaction with increased HIF-1 $\alpha$  activation and VEGF uptake, resulting in increased angiogenesis at ischemic tissue locations<sup>145</sup>.

Collectively, these intriguing data justify further investigation to determine whether the transferred glucose between HUVEC and MSCs regulates MSC-mediated angiogenesis through HIF-1 $\alpha$  activation. In addition, several other pertinent questions (such as whether glucose regulates MSC-mediated angiogenesis directly or indirectly through improved MSC survival followed by increased release of the pro-angiogenic secretome) require further research to be answered definitively.

#### **4. *In silico* models to predict the glucose needs of MSCs for regenerative functionalities**

The present review has described different facets of glucose metabolism that modulate the regenerative functionalities of MSCs. However, the effects of glucose are context-specific, making it difficult to predict its role in a particular regenerative medicine condition without resorting to costly wet-lab experimentation. To overcome this issue, *in silico* studies using genome-scale metabolic models appear to be time- and cost-efficient alternatives to wet-lab studies. They will be soon able to assist tissue engineers in designing viable and clinically potent tissue constructs.

A number of generic genome-scale metabolic reconstructions for human cells have been previously published<sup>152,153</sup>, which include comprehensive data on gene-protein-reaction relations in human metabolism. Based on such a reconstruction, it is possible to obtain a generic mathematical model of cell metabolism, or a GEM (Figure 5A). An MSC-specific reconstruction can be obtained by considering only the active reactions of MSC. The MSC-specific GEM like iMSC1255 is, therefore, a more constrained model compared to the generic GEM (Figure 5B). Let  $v = *v_1, v_2, \dots, v_n +$  be the metabolic flux vector, where every element  $v_i$  represents the cellular biochemical flux of reaction  $i$  of the MSC metabolism. In GEM-based modeling, our goal is to predict  $v$ , under a certain physiological condition (*e.g.*, in  $\alpha$ -MEM under normoxia). Every cell is evolved to achieve a certain biological objective at its optimal level. For single-celled organisms, this objective is often to maximize biomass production rate, while other cells may have more complex objectives. Given the objective function of the cell, *i.e.*, biomass production rate in expanding MSCs, one can find the metabolic flux vector for which the objective function is maximized (Figure 5C). Finding the maximum value for the objective function is usually formulated as a linear optimization problem, which is referred to as flux balance analysis, or FBA<sup>154</sup>. Particularly, the solution to this problem is the flux distribution for which the MSC grows maximally. Thus, the application of FBA on the GEM of iMSC1255 can simulate the metabolic phenotypes of MSCs, including growth rate and glucose consumption rate. Other related methods, including dynamic FBA (dFBA) and unsteady-state FBA (uFBA), further expand the applicability of GEMs for simulating metabolic fluxes in changing environments<sup>155,156</sup>, which is a more realistic assumption especially in tissue engineering.

GEMs can currently computationally predict the metabolic alterations required to optimize MSC proliferation<sup>101,103</sup>. Modeling metabolism during cell differentiation and senescence is a more challenging problem, as metabolic constraints are likely to change. However, some computational approaches are available to overcome this issue<sup>97</sup>. On the other hand, current *in silico* models cannot accurately predict MSC metabolic parameters favoring their survival post-implantation. This goal requires additional knowledge about (i) the metabolic strategy implemented by MSCs

to adapt to the stress of implantation and (ii) a better characterization of the metabolic requirements (including glucose fluxes) of MSCs post-implantation and the biochemical characteristics of the implantation medium.

Intrinsic (e.g., cell-to-cell molecular heterogeneity) and extrinsic (e.g., external metabolites and hormones) factors potentially affect the status and fate of MSCs within implants. Understanding these factors enables GEMs to simulate MSC metabolism within implants with temporal and spatial resolution<sup>157</sup>. Additionally, it allows one to simulate the effects of glucose (and other metabolites) on cell survival, proliferation, differentiation, and senescence. With the increasing availability of 'omic'-scale single-cell data<sup>158,159</sup>, such a goal seems to be closer than ever before. Hopefully, such models will enable us to determine the metabolic requirements of MSCs precisely under *ex vivo* and *in vivo* conditions and, ultimately, tailor tissue construct design and implantation procedure to enhance MSC regenerative potential.

## Conclusions and future directions

All studies mentioned above provide evidence that glucose is central to the fulfillment of energetic and biosynthetic needs for the maintenance of MSCs. The crucial aspect that needs to be determined to achieve optimal MSC transplantation is whether MSCs can get glucose at a level that meets their requirements at the transplantation site prior to revascularization. In this regard, all modalities (including MSC numbers, delivery route, single versus multiple injections, and site vascularity) that affect local glucose availability need to be examined by tissue engineers to optimize MSC delivery. However, we cannot definitively exclude the possibility, based on the studies mentioned above, that the inability of hMSCs to achieve intracellular glucose amounts that match their needs does not originate, at least partially, from a deficiency in glucose transporters.

Relying exclusively on the capacity of the tissues surrounding the lesion to supply glucose at a level that meets the MSC needs may not be sufficient when large numbers of cells is administered at one time. For this reason, the development of nutritive delivery vehicles capable of supplying physiological glucose concentrations to MSCs would be a most critical breakthrough in tissue engineering. Such a nutritive delivery vehicle will meet MSC metabolic needs independent of the local glucose availability. The metabolic autonomy of MSCs provided by such nutritive scaffolds should last until lesion revascularization is completed to ensure that MSCs successfully cross the bioenergetics “valley of death” between the time of implantation and availability of a functional, blood supplying network. Alternatively, the enhancement of glucose transports via the overexpression of GLUTs or gap junctions<sup>145,151</sup> may represent complementary strategies that prove beneficial. Interestingly, because more MSCs survive when glucose is supplied, this may decrease the numbers of MSCs required for clinical applications, leading to shorter *in vitro* expansion times and younger MSCs utilization, ultimately resulting in improved MSC functions. Meanwhile, the need for a limited number of cells will reduce the risk of cell senescence and decrease the cost of the *in vitro* cell expansion.

Although taking into account the MSC metabolic needs are expected to prevent the hMSC bioenergetic collapse<sup>20</sup> and allow long-term hMSC viability, further research is needed to elucidate all the parameters which are pertinent, necessary, and sufficient to achieve also long-term hMSC functions pertinent to tissue repair

and, ultimately, hMSC engraftment post-implantation. Moreover, the demonstration that glucose, but not oxygen, is critical for neural cells<sup>160</sup>, adipose-derived MSCs<sup>143,161</sup>, myoblasts<sup>143,161</sup> and osteocytes<sup>162</sup> survival in near low oxygen environments suggest that the concept of nutritive scaffolds for delivering glucose at level that matches transplanted cell needs may extend to a vast myriad of other cell types being considered for cell transplantation.

In conclusion, it is hoped that just as understanding the role of metabolism in regulating immune cell responses has paved the way for new therapies for infections, cancer, autoimmunity, and obesity, understanding the metabolism of MSCs (specifically, pertinent glucose metabolism) will contribute in developing transplantation conditions and nutritive delivery vehicles for MSCs that will solve some critical issues associated with MSC transplantation: the massive cell death, the low engraftment efficiency, and the lack of control over the MSC fate post-implantation.

## Acknowledgements

The authors would like to acknowledge their funding sources from L'Agence Nationale de la Recherche (ANR-16-ASTR-0012-01, ANR-18-CE18-0004-01, and ANR-20-CE19-0002), Gueules Cassées 67-2020, Fondation de l'avenir (AP-RM-17-006 and AP-RM-18-029), and the China Scholarship Council (No. 201600160067).

## Disclosure Statement

H.P. claimed ownership of the patent "Time-controlled glucose releasing hydrogels and applications" (EP3011952A1, 2014). The remaining authors stated no potential conflicts of interest.

## Authors' Contributions

G.L., P.W., G.E.S.-N., and E.P.: conception and design, manuscript writing; M.B., R.B., S.-A.M., and P.S.: manuscript writing; H.P.: conception and design, manuscript writing, final approval of the manuscript.

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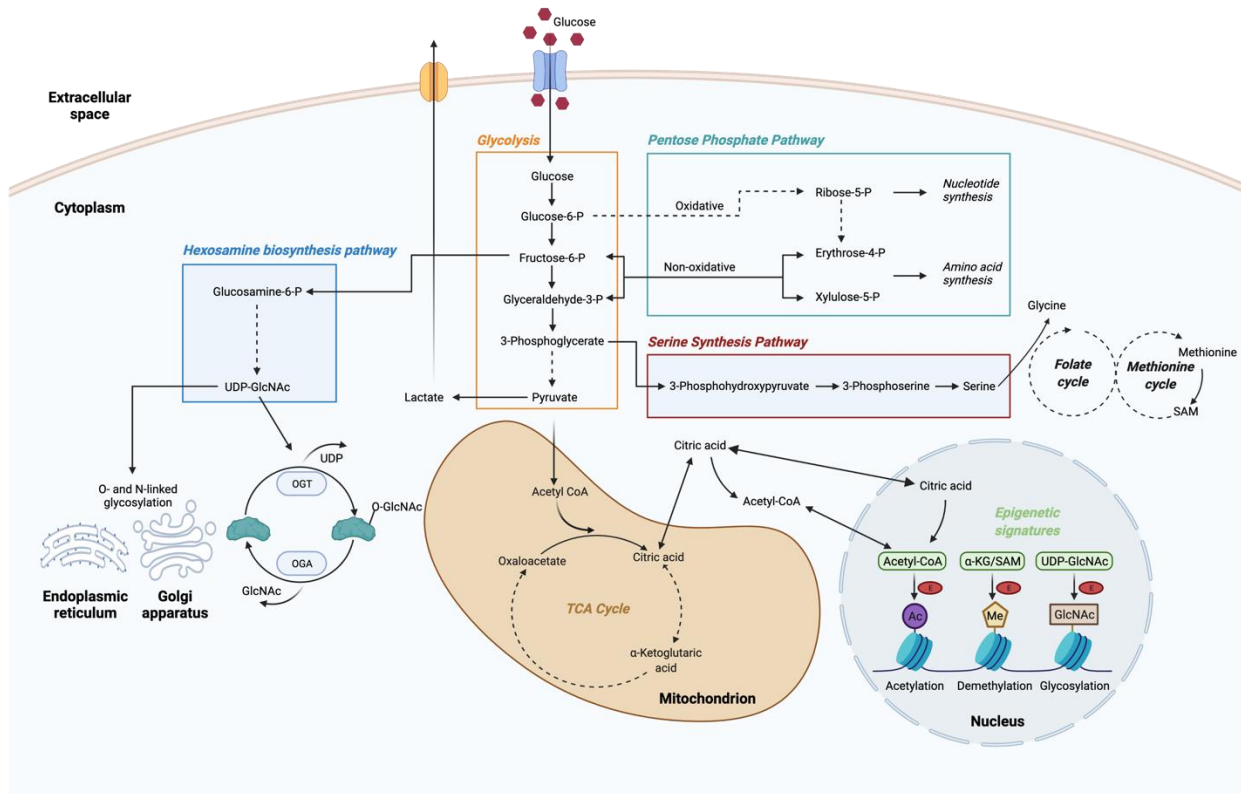
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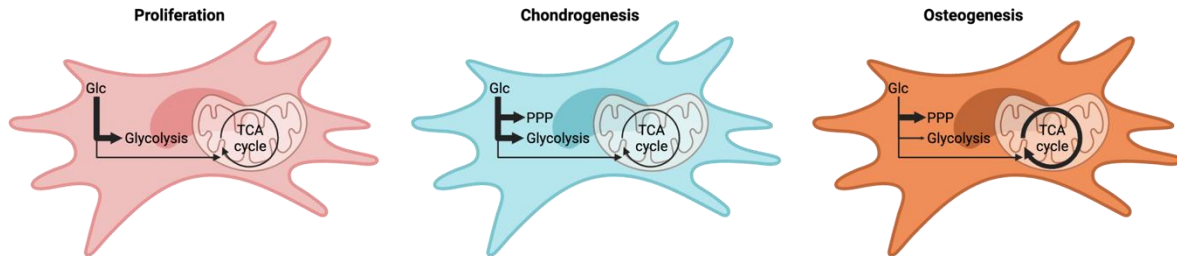
**Figure 1. Overview of the glucose metabolic pathways in mesenchymal stromal cells (MSCs) discussed in the present review<sup>22–25</sup>.**

Glucose is metabolized through glycolysis to generate various metabolites that serve as substrates for multiple metabolic pathways. Pyruvate, generated from glycolysis, can be either converted to lactate and excreted from the MSCs or transported into the mitochondria and oxidized to acetyl-CoA to feed the Tricarboxylic acid (TCA) cycle and drive oxidative phosphorylation (OXPHOS) in the presence of sufficient oxygen. The pentose phosphate pathway and the serine synthesis pathway branch from glycolysis and contribute to nucleotide, amino acid, and lipid biosynthesis. Serine is incorporated into the one-carbon metabolism, which comprises a network of interlinking metabolic pathways including the folate cycle and methionine cycle. The hexosamine biosynthesis pathway is another pathway branched on glycolysis that contributes to production of UDP-GlcNAc, a key intermediate for post-translation modification of serine and threonine residues. Cellular metabolism and epigenetic modification are tightly linked and reciprocally regulate each other. Many intermediate metabolites serve as substrates or cofactors for regulating epigenetic signatures and various enzymes (E) are involved in epigenetic modifications. These metabolites used for epigenetic modifications in the cell nucleus, include acetyl-CoA for histone acetylation, Sadenosylmethionine (SAM) for histone and chromatin methylation, and UDP-GlcNAc for histone glycosylation. Alpha-ketoglutarate ( $\alpha$ -KG) serves as a co-substrate required for the activity of certain demethylases that remove methyl groups from histones and chromatin. (Created in biorender.com)



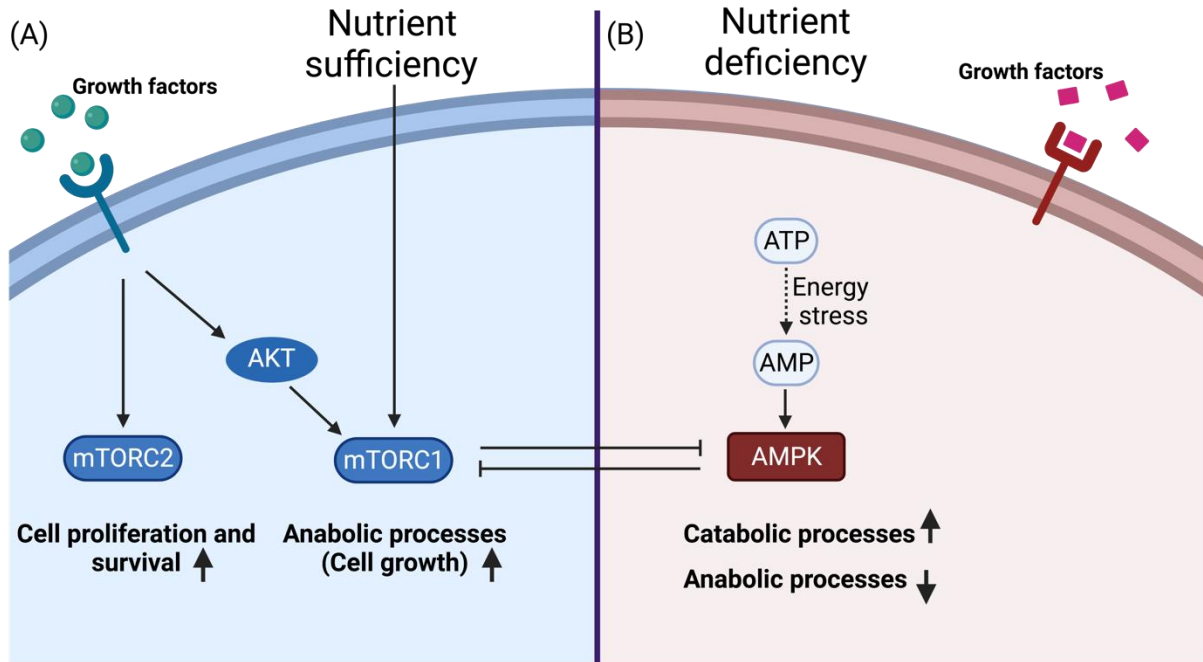
**Figure 2. Metabolic flexibility of mesenchymal stromal cells (MSCs) under various conditions<sup>49–52</sup>.**

The thickness of the arrows in these schematic illustrations of cells indicates the activity level of the respective metabolic pathways. PPP: Pentose phosphate pathway; Glc: Glucose; TCA cycle: Tricarboxylic acid cycle (Created in biorender.com).



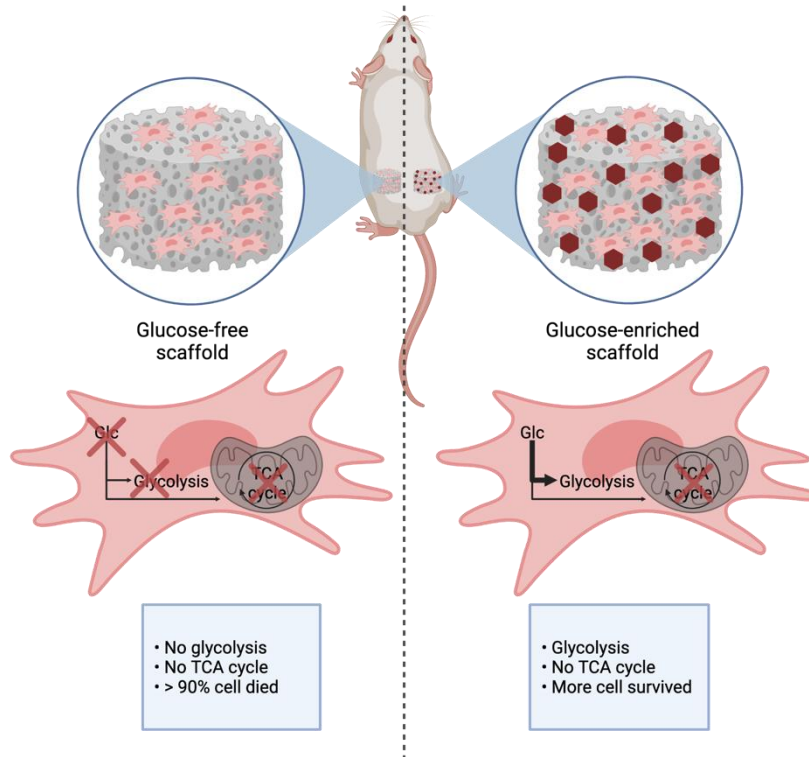
**Figure 3. Schematic illustration of the mTOR/AMPK nutrient sensor and growth factor signaling pathways<sup>80–82,87–91</sup>.**

(A) Under nutrient sufficient conditions, mTORC1 is activated. Activation of mTORC1 is also regulated by the growth factor signaling pathway via AKT. The activated mTORC1 phosphorylates many downstream targets to increase anabolic processes and thus increases m-TORC1-driven cell growth. In contrast to mTORC1, mTORC2 is primarily activated by growth factor signaling, and thus controls cell proliferation and survival. (B) Under nutrient deficient conditions (which induce energy stress), AMPK directly senses increases of AMP, leading to its activation. Once activated, AMPK participates in many pathways and cellular processes, including increase of catabolic processes and inhibition of anabolic processes. In addition, this schematic illustrates the interplay between the mTOR and AMPK pathways; mTORC1 activation inhibits AMPK while AMPK activation switches off the mTORC1 complex. (Created in biorender.com).



**Figure 4. Schematic illustration of the positive effects of glucose on mesenchymal stromal cell (MSC) survival post implantation<sup>20,21</sup>.**

MSCs seeded into glucose-free scaffolds encounter switched-off glycolysis and tricarboxylic acid (TCA) cycle, a condition that leads to massive and rapid cell death. In contrast, the presence of glucose in cell-containing scaffolds enhances MSC survival relying on glycolysis. Glc: Glucose, TCA cycle: Tricarboxylic acid cycle. (Created in biorender.com).





**Figure 5. Analyzing mesenchymal stromal cell (MSC) metabolism using cell-specific metabolic network models.**

(A) The genome-scale metabolic network reconstruction for human cells includes all (known) biochemical information about the human metabolism (e.g., Recon1152). Such a reconstruction can be converted to a mathematical model (i.e., a GEM). Typically, a GEM includes a set of flux constraints, including flux balance constraint ( $S \cdot v = 0$ ) and non-negativity constraint ( $v_i \geq 0$ ) for every irreversible reaction, which limit the space of feasible biochemical fluxes ( $v$ ). (B) Within the context of modeling MSC metabolism, the available data on the unexpressed genes are translated to constraints on metabolic fluxes. Each linear constraint (e.g., the plane shown in orange) can cut the flux space and make it smaller (like the polytope in green). By applying further constraints, one can narrow down the feasible flux space, which is equivalent to selecting a context-specific metabolic subnetwork, i.e., iMSC1255. (C) 'Omic'-level data are usually converted to simple flux inactivity constraints for reactions with unexpressed genes ( $v_i = 0$ ), while nutrient uptake rates can be used as constraints as well ( $lb_i \geq v_i \geq ub_i$ ). Given the objective of the cell (i.e., biomass production rate in proliferating MSCs), by using the MSC-specific GEM, one can predict the metabolic flux vector which maximizes the cellular objective (shown as the blue arrow), subject to certain physiological constraints (represented as the green polytope). This procedure is basically a linear optimization problem, which is referred to as flux balance analysis (FBA)<sup>154</sup>. The solution to this problem is a certain flux contribution (shown here as the blue dot) for which the objective function is maximized.

