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Investigating the role of glucose on mesenchymal stem cell-mediated tissue engineering

Guotian Luo

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Université de Paris

Ecole doctorale **Hématologie, Oncogénèse et Biothérapies N°561**
Laboratoire de **Biologie, Bioingénierie et Bioimagerie Ostéo-
Articulaires (B3OA)**

Investigating the Role of Glucose on Mesenchymal Stem Cell- Mediated Tissue Engineering

Par **Guotian LUO** (罗国填)

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Dirigée par **Hervé PETITE**

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“One man can be a crucial ingredient on a team, but one man cannot make a team.”

- Kareem Abdul-Jabbar

Obtaining a doctoral degree is similar to participating in a basketball game. I am yet to fight alone in the absence of a coach, teammates, audiences, and referee. I would want to take this occasion to express my gratitude to everyone who has had a significant influence on this basketball game.

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To my family again in Chinese (再次用中文致谢我的家人)

在此我也想再次用中文感激我的家人，感激我的父母多年的养育之恩，支持我多年的求学之路，尤其是身处异国求学5年间，更是无时无刻不心系我的饮食起居人身安全等问题，即使他们每一次交待的事情永远也就那么几句连语气都一样的繁琐小事；也感激我的弟弟妹妹们对我学业的支持，是你们的一举一动让我能坚定地往前迈步。



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DECLARATION

I declare that this thesis entitled ‘Investigating the Role of Glucose on Mesenchymal Stem Cell-Mediated Tissue Engineering’ is my original work. The manuscript of this thesis has been written by me in its entirety. I have appropriately credited all sources of material utilized in my thesis, including the reproduction permission of John Wiley and Sons for the literature review article.

This thesis has never been presented for any other academic degree at any other university.

ABSTRACT

Mesenchymal stem cells (MSCs), also known as multipotent stromal cells, are promising candidates for tissue engineering applications. However, mounting evidence indicates that their therapeutic efficacy has fallen short of their initial promise and hype due to observed poor MSC survival and engraftment post-implantation. The hostile ischemic microenvironment, primarily characterized by oxygen and nutrient deprivation, is responsible for the rapid and massive death of MSCs post-implantation. Previously published research from the B3OA lab established that the absence of glucose (but not oxygen) is responsible for MSC death. The central hypothesis of this thesis is that glucose metabolism, a critical regulator of stem cell activity, enables MSCs to improve their survival and functional properties after transplantation, thereby increasing their therapeutic efficacy.

As a first step toward engineering a glucose-supplying construct, we propose investigating whether and how glucose affects the MSCs-mediated angiogenesis and then to developing an enzyme-controlled, nutritive hydrogel with an inbuilt system of glucose delivery to enhance MSC survival and functionalities post-implantation.

To accomplish our first objective, we discovered that supernatant conditioned media (CM) derived from human MSCs (hMSCs) cultured in the presence of glucose under near-anoxia (0.1% O₂) exhibited significantly greater angiogenic potential than CM derived from hMSCs cultured in the absence of glucose. Additionally, compared to hMSCs cultured in the presence of glucose under near-anoxia, hMSCs cultured in the absence of glucose exhibited a higher level of endoplasmic reticulum (ER) stress as evidenced by decreased nascent protein biosynthesis, and increased PERK pathway activity. Most importantly, when hMSC-containing hydrogels were implanted with glucose, the volume of newly formed blood vessels increased significantly compared to hMSC-containing hydrogels without glucose.

To accomplish our second objective, we developed a novel fibrin hydrogel composed of starch (a glucose polymer) and amyloglucosidase (AMG, an enzyme that degrades starch) to supply physiological levels of glucose to MSCs via glycolysis. Up to 14 days *in vitro*, hMSCs loaded in this novel starch/AMG hydrogel demonstrated improved cell survival and paracrine functions. Additionally, this novel nutritive hydrogel enhanced

survival and angiogenesis of MSCs when subcutaneously implanted in nude mice. However, the long-term sustainability of glucose production within the hydrogel and the complete resorption of this novel nutritive hydrogel require additional investigation.

Taken together, our findings demonstrate the beneficial effects of exogenous glucose on enhancing hMSCs-mediated angiogenesis and regulating hMSC ER stress under near-anoxia, as well as establishing proof of concept that a novel inbuilt glucose delivery system improves MSC survival and angiogenesis post-implantation. These findings motivate the field to pursue a glucose-supply strategy to increase the therapeutic efficacy of MSC-mediated tissue engineering.

Keywords: Mesenchymal stem cells(MSCs), glucose, hydrogel, starch, cell survival, angiogenesis, endoplasmic reticulum stress

RÉSUMÉ

Les CSMs ont été identifiées pour la première fois par Alexander Friedenstein 1966, qui les a isolées à partir de la moelle osseuse grâce à leur propension à adhérer au plastique. Ces cellules prolifèrent de façon importante (mais limitée) in vitro et forment des colonies cellulaires semblables à des fibroblastes. Pour ces raisons, ces cellules ont été initialement appelées "fibroblastes formant des colonies" (Colony Forming Units-Fibroblasts ou CFU-F). Le terme « cellules souches mésenchymateuse » (CSM) a été suggéré par Arnold Caplan en 1991. D'autres dénominations sont également utilisées dans la littérature pour mettre en exergue certaines caractéristiques de ces cellules telles que : (i) "cellules souches squelettiques" proposées par P. Bianco parce que ces cellules peuvent se différencier dans tous les types de cellules squelettiques; (ii) "cellules stromales de la moelle osseuse" parce que cette population de cellules supportent également l'hématopoïèse; (iii) "fibroblastes du stroma de moelle osseuse" en raison de leur morphologie fibroblastique ; et plus récemment « Cellules Stromales Multipotentielles » en raison de leur multipotentialité. Les CSMs représentent 0,001 à 0,01% de la population totale de cellules mononucléées de la moelle osseuse. En réponse à des stimuli biochimiques et biophysiques spécifiques, les CSMs se différencient en cellules de l'os, du cartilage, du tissu adipeux ou en cellules stromales de soutien à l'hématopoïèse. Ces cellules sont des candidates prometteuses pour les applications d'ingénierie tissulaire. En effet, elles ont non seulement (i) la capacité de proliférer de façon importante mais limitée et (ii) de se différencier en une variété de tissus mésodermiques mais aussi (iii) une capacité à sécréter un ensemble de facteurs bioactifs induisant un milieu favorable à la réparation tissulaire favorisant l'angiogenèse et la régénération tissulaire tout en inhibant l'apoptose, la fibrose et l'inflammation. Cependant, leur efficacité thérapeutique réelle est de plus en plus remise en cause. Les CSM ne semblent pas être à la hauteur des espérances initiales fondé sur leur potentiel de régénération évalué dans des modèles in vitro ou des modèles in vivo partiellement représentatifs de la réalité clinique. Au final, l'efficacité thérapeutique des CSMs est faible et ceux en raison du faible de taux de survie des CSMs au sein des greffons et de leur persistance limitée à long-terme. Le microenvironnement ischémique hostile, principalement caractérisé par la privation d'oxygène et de nutriments, est responsable au

moins en partie de la mort rapide et massive des CSM après leur implantation. Des recherches précédemment publiées par le laboratoire B3OA ont établi que l'absence de glucose (mais pas d'oxygène) est responsable de la mort massive des CSMs *in vitro*. Ces résultats ont été confirmés *in vivo* en montrant que l'addition de glucose au sein de produits d'ingénierie tissulaire augmentait la survie des CSMs par rapport au groupe témoin dans un modèle ectopique murin. L'hypothèse centrale de cette thèse est que le glucose, un régulateur critique de l'activité des cellules souches, permet de favoriser la survie des CSMs ainsi que leurs propriétés fonctionnelles après transplantation. Délivrer de façon adéquate à des concentrations physiologiques, il devrait donc augmenter l'efficacité thérapeutique des CSM.

Dans un premier temps, nous proposons d'étudier si et comment le glucose affecte l'angiogenèse médiée par les CSM. Dans un second temps, nous développerons un hydrogel nutritif doté d'un système intégré d'apport de glucose contrôlé par des enzymes dans l'idée d'améliorer la survie et les fonctionnalités des CSM après leur implantation.

Pour atteindre notre premier objectif, nous avons étudié l'angiogenèse médiée par les CSMh. Nous avons tout d'abord évalué l'influence de milieux conditionnés provenant de surnageants de culture de CSM humaines (CSMh) cultivées sous quasi-anoxie (0,1% O₂) en présence ou pas de glucose sur la migration et la formation de structures tubulaires par les cellules endothéliales de la veine ombilicale humaine (HUVEC). Nous avons ainsi pu montrer que les milieux conditionnés dérivés de CSMh cultivées en présence de glucose (1 ou 5 g/L) augmentaient non seulement la migration des HUVEC de manière significative ($p < 0,05$) mais aussi la longueur totale des structures tubulaires de manière significative ($p < 0,05$) par rapport au milieu conditionné surnageant dérivé de CSMh cultivées en l'absence de glucose. Cette observation a été corroborée par la caractérisation des milieux conditionnés par ELISA qui a montré une augmentation significative ($p < 0,05$) de la quantité de facteurs de croissance pro-angiogéniques (Angiogénine, VEGF-A, VEGF-C, Angiopoïétine-1, Endostatine, et CCL2) libérés par les CSMh cultivées en présence de glucose par rapport aux résultats obtenus à partir des CSMh cultivées en absence de glucose. En outre, par rapport aux CSMh cultivées en présence de glucose en quasi-anoxie, les CSMh cultivées en l'absence de glucose ont présenté un niveau plus élevé de stress du réticulum endoplasmique (RE). Témoignant de ce stress, nous avons observé (i) une diminution de la biosynthèse des protéines nouvellement formées et (ii) une augmentation de l'activité de la voie PERK (niveau significativement ($p < 0,05$) plus élevé d'expression des ARNm CHOP et GRP78, d'eIF2 α phosphorylé et d'expression de

la protéine CHOP). Plus important encore, lorsque des hydrogels chargés ou pas en glucose contenant des CSMh ont été implantés en sous cutanée chez la souris Nude, on notait (i) une amélioration de la survie des CSMh et (ii) une augmentation significative du volume des vaisseaux sanguins nouvellement formés dans les hydrogels chargés en glucose et CSMs par rapport aux hydrogels sans glucose contenant des CSMh ou aux hydrogels sans cellules chargés seulement en glucose.

Pour atteindre notre deuxième objectif, nous avons d'abord développé un nouvel hydrogel composé de fibrine, d'amidon (un polymère de glucose) et d'amyloglucosidase (AMG, une enzyme hydrolysant les liaisons glucosidiques reliant les glucoses entre eux au sein de l'amidon). Cette stratégie visait à fournir des niveaux physiologiques de glucose aux CSMs durant la période comprise entre le moment de la transplantation et la revascularisation de la lésion. Les hydrogels nutritifs ont été ensemencés avec des CSMh et la survie des CSMh dans ces hydrogels a été évaluée in vitro et in vivo. Les résultats in vitro ont montré que les CSMh chargées dans ce nouvel hydrogel de fibrine/amidon/AMG et exposées à une déplétion en oxygène et glucose présentaient un taux de survie cellulaire significativement amélioré par rapport aux mêmes CSMh chargées dans des hydrogels de fibrine sans glucose après 14 jours. L'amélioration de la survie cellulaire dans ces hydrogels de fibrine/amidon/AMG a également été observée avec des cellules souches dérivées de tissus adipeux et des myoblastes. De plus, après 14 jours d'implantation en site sous-cutané chez la souris Nude, les CSMh ensemencées dans l'hydrogel de fibrine/amidon/AMG présentaient un taux de survie significativement plus élevé ($p < 0,05$) que les CSMh chargées dans les hydrogels de fibrine sans glucose. D'autre part, nous avons évalué les propriétés chimiotactiques et pro-angiogéniques des CSMh chargées dans des hydrogels de fibrine/amidon/AMG dans des conditions de quasi-anoxie sans sérum ni glucose in vitro et in vivo. Nous avons découvert que les surnageants de milieux conditionnés provenant de CSMh chargées dans des hydrogels de fibrine/amidon/AMG augmentaient significativement ($p < 0,05$) le potentiel chimiotactique des CSMh vers les CSMh et les HUVECs en comparaison des résultats obtenus en utilisant les hydrogels de fibrine sans glucose ou avec 5,5 mM de glucose. Ces données étaient cohérentes avec les concentrations significativement ($p < 0,05$) plus élevées de facteurs de croissance chimiotactiques (MCP-1, MIF, MIP-2, ENA 78 et IP-10) et de facteurs de croissance pro-angiogéniques (VEGF-A, VEGF-C, VEGF-D, Angiopoïétine-1, Angiopoïétine-2, Angiogénine et FGF-b) retrouvés dans les surnageants obtenus à partir des milieux conditionnés obtenus à partir des hydrogels de

fibrine/amidon/AMG/CSMh par rapport aux résultats des hydrogels contrôles fibrine/CSMh sans glucose ou Fibrine/5,5 mM de glucose sans cellules. Nous avons également démontré que le volume des vaisseaux sanguins nouvellement formés augmentait de manière significative ($p < 0,05$) à la périphérie des hydrogels de fibrine/amidon/AMG contenant des CSMh par rapport aux résultats obtenus avec des hydrogels contrôles fibrine/CSMh sans glucose ou fibrine/5,5 mM de glucose sans cellules.

Dans l'ensemble, nos résultats démontrent les effets bénéfiques de l'apport de glucose exogène sur l'angiogenèse médiée par les CSMh ainsi que sur la régulation du stress du RE des CSMh dans des conditions de quasi-anoxie. Nous avons aussi établi pour la première fois la preuve de concept qu'un système d'hydrogel nutritif de fibrine/amidon/AMG produisant du glucose permettait d'améliorer la survie des CSMh et l'angiogenèse médiée par les CSMs après implantation. Ces résultats incitent à poursuivre une stratégie d'approvisionnement en glucose afin d'augmenter l'efficacité thérapeutique des CSMs dans le cadre de l'ingénierie tissulaire.

Les résultats intéressants de la présente étude justifient (i) la poursuite de recherches afin d'élucider le(s) mécanisme(s) sous-jacent(s) concernant le rôle du glucose dans la survie et les fonctionnalités des CSM après leur implantation et (ii) la poursuite de recherches dans le but d'optimiser le nouvel hydrogel nutritif en tant que substrat tridimensionnel pour diverses applications des CSM. En particulier, la cinétique de délivrance du glucose doit être améliorée ainsi que la biodégradabilité de l'hydrogel. Outre la privation de nutriments, la réponse inflammatoire et le stress oxydatif jouent un rôle important dans les processus de réparation tissulaire *in vivo*. Étant donné le rôle primordial de l'immunomodulation dans ces processus, il est crucial de mieux comprendre le rôle du glucose sur les CSMs dans ce phénomène ainsi que dans l'homéostasie redox afin d'optimiser les effets bénéfiques de l'apport de glucose dans les thérapies à base de CSM. En ce qui concerne les nouveaux hydrogels nutritifs, les principaux aspects qui doivent être pris en considération pour optimiser leur utilisation dans les applications d'ingénierie tissulaire comprennent (sans s'y limiter) les propriétés biophysiques de l'hydrogel, le choix du polymère de glucose (par exemple, amidon ou maltodextrine), le choix de l'enzyme (par exemple, une seule enzyme ou une combinaison de plusieurs enzymes dégradant l'amidon), l'injectabilité, la biocompatibilité et le mode d'administration.

Pertinence de la thèse :

Les CSMs ont été identifiées pour la première fois par Alexander Friedenstein 1966, qui les a isolées à partir de la moelle osseuse grâce à leur propension à adhérer au plastique. Ces cellules sont des candidates prometteuses pour les applications d'ingénierie tissulaire notamment la régénération osseuse, la réparation du cartilage, la cicatrisation des plaies, etc. En effet, elles ont la capacité de proliférer de façon importante mais limitée, de se différencier en une variété de tissus mésodermiques et de sécréter un ensemble de facteurs bioactifs induisant un milieu favorable à la réparation tissulaire. Hélas, la mort cellulaire rapide et massive des CSMh observée dans le microenvironnement ischémique limite fortement leur efficacité thérapeutique. C'est, en fait, le principal obstacle à leur utilisation en clinique. Cette thèse comporte un aspect fondamental à la résolution du défi « mort des CSMs après implantation » en proposant des éléments permettant une meilleure compréhension du rôle des nutriments (et plus particulièrement du glucose) dans la survie et l'expression des fonctionnalités des CSMs post-implantation. Cette thèse comporte aussi un aspect appliqué en établissant la preuve du concept de l'intérêt d'hydrogels nutritifs la délivrance des CSMh en ingénierie tissulaire.

Mots clefs : Cellules Souches Mésenchymateuses (CSM), glucose, hydrogel, amidon, survie cellulaire, angiogenèse, stress du réticulum endoplasmique

LIST OF ABBREVIATIONS

AMG	Amyloglucosidase
2-DG	2-Deoxyglucose
ACS	Adult Stem Cell
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein Kinase
apoEVs	Apoptotic Extracellular Vesicles
ASK1	Apoptosis Signal-regulating Kinase 1
ATF4	Activating Transcription Factors 4
ATF6	Activating Transcription Factor 6
ATP	Adenosine Triphosphate
BLI	Bioluminescence Imaging
BM	Bone Marrow
BMPs	Bone Morphogenetic Proteins
bZIP	Basic leucine zipper
C/EBPs	CCAAT/Enhancer-Binding Proteins
CCL2	Chemokine (C-C Motif) Ligand 2
CFU-F	Colony-Forming Unit-Fibroblast
CHOP	C/EBP-Homologous Protein
CM	Conditioned Media
DNP	2,4-Dinitrophenol
EC	Endothelial Cell
ECAR	Extracellular Acidification Rate
ECM	Extracellular Matrix
e-IF2α	Eukaryotic translation Initiator Factor-2
ENO1	Enolase 1
ER	Endoplasmic Reticulum
ESC	Embryonic Stem Cell
ETC	Electron Transport Chain
FCFC	Fibroblast Colony-Forming Cell
FGF-b	Fibroblast Growth Factor Basic

FGFs	Fibroblast Growth Factors
GADD34	Growth Arrest and DNA Damage-inducible 34
GAGs	Glycosaminoglycans
Gluc	Gaussia Luciferase
GLUT	Glucose Transporter
GPI	Glucophosphate Isomerase
GRP78	Glucose-Regulated Protein 78
HBP	Hexosamine Biosynthesis Pathway
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-Inducible Factor
HK	Hexokinase
hMSC	Human Mesenchymal Stem Cell
HSC	Hematopoietic Stem Cell
HUVEC	Human Umbilical Vein Endothelial Cell
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
iPSC	Induced Pluripotent Stem Cell
IRE1α	Inositol-Requiring Enzyme-1 α
ISCT	International Society For Cellular Therapy
JNK	JUN N-terminal Kinase
LDH	Lactate Deshydrogenase
MCP-1	Monocyte Chemoattractant Protein 1
MDSC	Marrow-Derived Stromal Cell
MMP	Metalloproteinase
mMSC	Mouse Mesenchymal Stem Cells
MSC	Mesenchymal Stem Cell
mTOR	Mechanistic Target of Rapamycin
mTORC	Mechanistic Target of Rapamycin Complex
NO	Nitric Oxide
O-GlcNAcylation	O-Linked N-Acetylglucosamine
OGT	O-GlcnaC Transferase
OXPHOS	Oxidative Phosphorylation

PDGF	Platelet-Derived Growth Factor
PDK	Pyruvate Dehydrogenase Kinase
PD-L1	Programmed Death Ligand-1
PERK	Protein kinase RNA-like ER Kinase
PFK	Phosphofructokinase
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-Kinase
PKM	Pyruvate Kinase M
PP1C	Protein Phosphatase 1C
PPAR2	Peroxisome Proliferation-Activated Receptor 2
PPP	Pentose Phosphate Pathway
qGlc	Cell-Specific Consumption Rate of Glucose
RIDD	Regulated IRE1 – Dependent Decay
rMSC	Rat Mesenchymal Stem Cells
Runx2	Runt-Related Transcription Factor 2
S1P	Site-1 Protease
S2P	Site-2 Protease
SDF-1	Stromal Cell-Derived Factors 1
SSEA-4	Stage-Specific Embryonic Antigen-4
STAT	Signal Transducer and Activator of Transcription
TCA cycle	Tricarboxylic Acid Cycle
TE	Tissue Engineering
TGF-β	Transforming Growth Factor-B
TNF	Tumor Necrosis Factor
TRAF2	Tumor Necrosis Factor Receptor-Associated Factor2
UPR	Unfolded Protein Response
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless/Integrated
XBP1	X-Box Binding Protein 1

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INTRODUCTION

*If only damaged bodies could be restored, life could go on for loved ones,
as though tragedy had not intervened.*

- Joseph Vacanti and Charles A. Vacanti

1 Tissue Engineering

1.1 Definition of Tissue Engineering

The Massachusetts Institute of Technology pioneered research into living skin equivalent grafts (Bell, et al. 1981) in the 1980s, paving the way for the era of tissue engineering (TE). The term 'tissue engineering' was coined at a 1987 National Science Foundation meeting; since then, it has evolved from a concept to a significant interdisciplinary field that applies 'the principles and methods of engineering and the life sciences towards the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain, or improve tissue function'. Three distinct strategies for tissue regeneration have been identified: 1) isolated cells or cell substitutes; 2) tissue-inducing substances; 3) cells placed on or within matrices (Langer and Vacanti 1993). The first approach, isolated cells or cell substitutes, involves the direct implantation of cells into damaged tissues. The most common application of this strategy is bone marrow transplantation (Hernigou, Poignard, et al. 2005) (Krupnick, et al. 2002) and autologous chondrocytes implantation (Mistry, Connock, et al. 2017) (Minas, Ogura and Bryant 2016). The second approach to tissue-inducing substances is based on the use of scaffolds that either recruit cells *in vivo* or act as carriers for signal molecules such as growth factors. The combination of osteoinductive biomaterials and bone morphogenetic protein is one method for repairing bone tissue (Kowalczewski and Saul 2018) (Sheikh, Javaid, et al. 2015). The final strategy combines cells with a matrix or scaffold, allowing growth factors and various nutrients to guide tissue development via cell proliferation, differentiation, and secretion of bioactive factors. This technique has been used to generate cartilage, bone, skin, liver, and a variety of other tissues and organs in order to replace damaged tissues and organs.

1.2 Components of TE

There is substantial evidence that TE-based therapies have been used to regenerate skin, cornea, liver, pancreas, bone, cartilage, and muscle. In essence, current methods for fabricating new and functional tissues rely on living cells that are typically associated with a porous scaffold that serves as a supporting structure for initial cell attachment and subsequent tissue ingrowth. This establishes a fundamental paradigm for TE and regenerative medicine. This is demonstrated in Figure 1-1 by the three 'pillars' of cells, scaffolds, and signals.

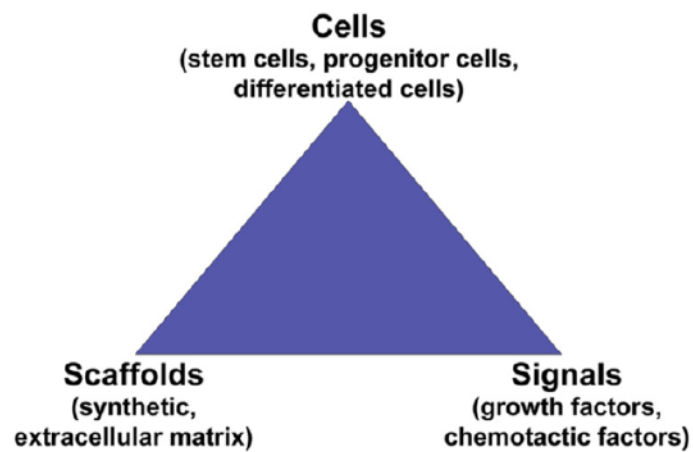


Figure 1-1 **Three 'pillars' underpin tissue engineering.** From Robert Lanza et al., Principles of Tissue Engineering, 4th Edition.

1.2.1 Biomaterials - Scaffolds as Cell-Delivery Vehicles

Scaffolds serve as an integral component of TE. They are typically composed of polymeric biomaterials (Chan and Leong 2008) that attempt to perform several or all of the following functions: i) they enhance cell-scaffold interactions, cell adhesion, and extracellular matrix (ECM) deposition; ii) they help transport of oxygen, nutrients, and regulatory factors to promote cell survival, proliferation, and differentiation; and iii) they biodegrade at a rate comparable to that of the growth of new tissue at the site of implantation, iv) to elicit the least amount of inflammation or toxicity possible *in vivo* (Dhandayuthapani, et al. 2011) (Langer and Tirrell 2004). As a result, the scaffold is defined as 'A biomaterial structure that serves as a substrate and guides for tissue repair and

regeneration' in a recent conference on biomaterials science definitions (Zhang and Williams 2018).

Polymeric scaffolds can be classified into two broad categories based on their biomaterial sources: natural and synthetic scaffolds. Natural polymeric scaffolds may closely resemble the native cellular environment because they frequently contain ECM components such as collagen, Matrigel, hydroxyapatite, and alginate (Fuchs, Nasser and Vacanti 2001). Natural polymers are typically highly biocompatible and possess inherent biological functions that promote cell attachment and growth (Hosseinzadeh, et al. 2014) (Li, et al. 2015) (He and Lu 2016). However, natural polymers have a number of drawbacks. For instance, their low physical and mechanical stability precludes them from being used in certain load-bearing applications, as does their potential immunogenicity due to allogenic or xenogeneic sources, as well as their uncontrollable degradation rate. Due to the limitations of natural biomaterials, synthetic alternatives with more tunable properties have been developed (Lee and Shin 2007). Synthetic polymers enable a greater degree of control over material properties such as degradation time, porosity, strength, and microstructure (Vacanti and Langer 1999) (Fuchs, Nasser and Vacanti 2001). Thus, synthetic polymers are widely believed to be capable of being tailored for both soft and hard tissues (Chan and Leong 2008). However, biocompatibility becomes a significant disadvantage for synthetic polymers due to their lack of cell adhesion sites for cell attachment and growth (Chan and Leong 2008) (Reddy, et al. 2021). Thus, chemical modification (Kurella and Dahotre 2005) or coating with natural biomaterials such as collagen (Brodie JC 2005) of the surface is required to enhance cell attachment. In an idealistic situation, a polymeric material would be biocompatible and bioabsorbable, would promote cell growth and differentiation, would be nonimmunogenic, and would be capable of inducing angiogenesis for TE applications (Vacanti and Langer 1999) (He and Lu 2016). The development of composite materials has resulted in an explosion of biomaterial combinations to fabricate porous scaffolds. Numerous biomaterials with disparate properties can be combined and fabricated to create custom solutions for specific applications (Chan and Leong 2008). We used a natural fibrin hydrogel in this thesis to investigate the role of glucose in TE because its high bioactivity makes it an attractive scaffold for stem cell differentiation and TE.

1.2.2 Bioactive Factors

The rationale for the use of bioactive factors and signaling molecules in TE strategies is to regulate the proliferation and differentiation of seeded cells within the scaffolds (Brochhausen, Lehmann, et al. 2009), as the commitment of stem cells to specific differentiation is a complex process that is highly controlled temporally and spatially. Numerous bioactive factors act on signaling cascades, regulating gene expression and thus influencing the differentiation process (Heasley and Petersen 2004). The evidence suggests that in many instances, such as large defect repair and impaired tissue function, endogenous bioactive factors are insufficient in kind and/or quantity to repair the injured tissue (Dang, Saunders, et al. 2018). As a consequence, exogenous factors are required for tissue repair. Indeed, it has been demonstrated that exogenous growth factors can enhance or modulate the migration, proliferation, and differentiation of cells to repair large tissue defects (Brochhausen, Lehmann, et al. 2009). In preclinical and clinical studies, bone morphogenetic proteins (BMPs), Wnts/beta-catenin, parathyroid hormone, fibroblast growth factors (FGFs), and transforming growth factor- β (TGF- β), among others, have been investigated for their ability to induce and accelerate bone regeneration (Lin and Hankenson 2011) (O'Keefe and Mao 2011).

Specifically, BMP-2 has been identified as a potent osteoinductive growth factor that can be used in bone-related TE and has been approved by the Food and Drug Administration to promote bone regeneration in a variety of applications, including anterior and lumbar interbody spine fusion and tibia nonunion (Kim and Choe 2011) (Carlisle and Fischgrund 2005) (McKay and Sandhu 2002). However, to induce osteogenesis, high doses of BMP2 are required (Walker and Wright 2002). Supraphysiologic BMP2 doses in clinical use may contribute to several emerging concerns, including adverse effects associated with ectopic bone formation (Wong, et al. 2008), reports of inflammatory responses (Smucker, et al. 2006) (Vaidya, et al. 2008), and concerns about dosage and efficacy (Zara, et al. 2011). Thus, additional research on their temporal effects on bone regeneration and complex *in vivo* kinetics is required before achieving full clinical efficacy.

1.2.3 Cell Sources

TE applications have utilized cells from various sources, including autologous, allogeneic, and xenogeneic sources. Autologous cells are those that originate from the patient, whereas allogeneic and xenogeneic cells come from other same species sources

and different species, respectively. Each of these options has several distinct advantages and disadvantages (see Table 1-1). However, off-the-shelf availability is a critical factor to consider when developing a product or treatment strategy. Regardless of the cell source, numerous types of stem cells have been identified in recent years, igniting renewed interest in the field, and the emergence of stem cell biology has resulted in the coinage of a new term, regenerative medicine. Embryonic stem cells, fetal-derived stem cells, adult stem cells, and induced pluripotent stem cells are all types of stem cells (discussed below).

Table 1-1 The sources of stem cells

Type	Source	Advantages	Disadvantages
Autologous stem cells	Patients	No immunologic issues; No infection transmission risk	Restricted availability; Cell harvesting procedural risk;
Allogeneic stem cells	Other human donors	Healthy stem cell source; Off-the-shelf availability; No risk to the host patient during cell harvesting	Immunologic issues
Xenogeneic stem cells	Different species	Off-the-shelf availability	Immunologic issues; Risk of transmission of animal virus

2 Stem Cells and Mesenchymal Stem/Stromal Cells

2.1 Stem Cells

Ernest McCulloch and James Till are widely credited with being the first scientists to rigorously define the essential properties of a stem cell in the 1960s through their pioneering work in mice. They identified the hematopoietic stem cell (HSC) as a blood-forming stem cell (Becker, McCulloch and Till 1963) (Siminovitch, McCulloch and Till 1963). Two distinguishing characteristics of stem cells set them apart from other cells in the body and make them the primary source for TE applications. The first is self-renewal, which is defined as the capacity to divide while retaining the undifferentiated cell state, thereby preserving the stem cell population. The second characteristic is potency, which refers to the capacity of cells to differentiate into mature cell types with specialized functions. Stem cells can either be pluripotent or be multipotent. Pluripotent stem cells can differentiate into any of the three germ layers (endoderm, mesoderm, or ectoderm), but not into extraembryonic tissues such as the placenta. Multipotent stem cells can differentiate into a variety of cell types but are lineage-restricted to one of the three germ layers.

Three major types of stem cells are currently being investigated in depth (Figure 2-1):

- *Embryonic stem cells (ESCs)*, which are derived from 4-5-day old blastocyst-stage embryos that have been fertilized *in vitro* and were donated for research purposes;
- *Induced pluripotent stem cells (iPSCs)*
- *Adult stem cells (ACs)*, including fetal adnexa-derived and tissue-derived ACs

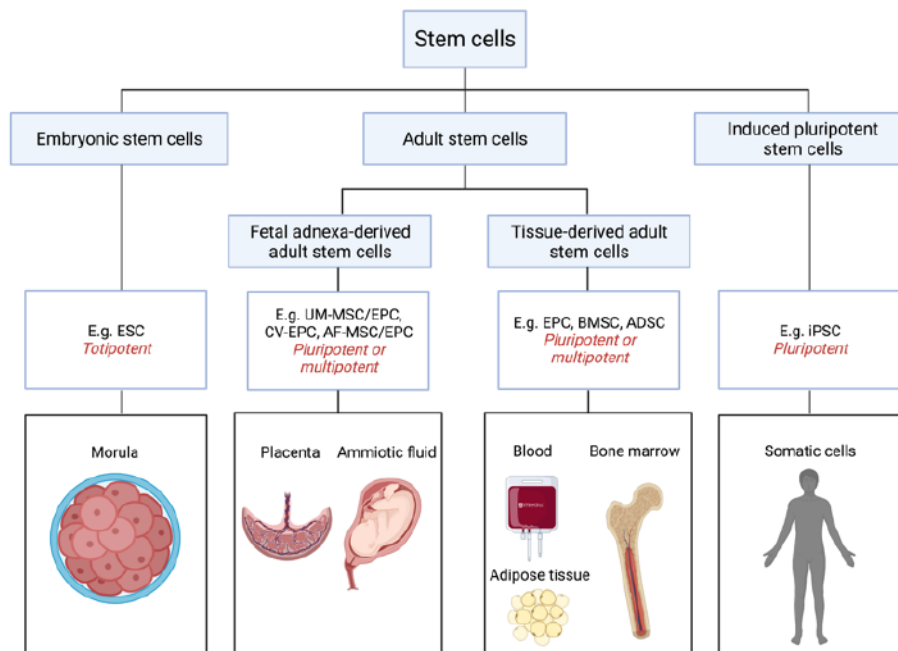


Figure 2-1 **The major types of stem cells used in tissue engineering.** Adapted from Sarah Al-Himdani et al. Front Surg 2017, (Created with biorender.com).

ESCs were initially discovered through research on mouse embryos (Evans and Kaufman 1981) (Martin 1981), and their discovery sparked a new wave of interest in stem cells, with articles reporting the isolation of the first lines of human ESCs in the late 1990s (Thomson 1998) (Vogel 1999). Human ESCs were isolated from the inner cell mass of blastocyst-stage embryos within five days of fertilization *in vitro* and donated for research purposes. Human ESCs have an unlimited capacity for self-renewal and differentiate into any cell lineage from the three embryonic germ layers. ESCs have been shown to differentiate into blood cell precursors, trophoblasts, neural progenitors, endothelial cells, hepatocytes, trophoblasts, osteogenic cells, cardiomyocytes, and keratinocytes (Pera and

Trounson 2004). While ESCs have the potential to cure a wide variety of diseases, they also present significant challenges for the field. The use of ESCs presents a moral dilemma, as it is currently impossible to isolate human ESCs without destroying the human embryo. As a result, researchers are forced to choose between their obligation to prevent or alleviate patient suffering and their obligation to respect the value of potential human life. Then, in 2007, Takahashi and Yamanaka identified four critical transcription factors, including oct-3/4, sox2, klf-4, and c-myc, required for human somatic cells such as fibroblasts to be reprogrammed into pluripotent cells known as induced pluripotent stem cells (iPSCs) (Takahashi, et al. 2007). The evidence suggests that iPSCs hold enormous potential for the future of TE. The fact that they can be obtained from patients with various diseases and possibly used as an autologous cell source to treat a specific patient is particularly intriguing. Furthermore, disease-specific iPSCs can be used for drug discovery and screening. However, the risk of developing teratomas preclude ESCs and iPSCs from being viable tools for TE.

Adult stem cells were first identified in the 1960s with the identification of HSCs from bone marrow. After that, additional adult stem cells were identified, including MSCs, epithelial stem cells, neural stem cells, and endothelial stem cells, which paved the way for the current era of TE. Adult stem cells that have been phenotypically characterized are listed in Table 2-1 (Körbling and Estrov 2003) (Slack 2000) (Blau, Brazelton and Weimann 2001). Adult stem cells are multipotent and can differentiate into specific mature cell types. They have been discovered in various tissues, including the bone marrow, blood, muscle, skin, intestine, brain, fat, and liver. Adult stem cells that are frequently studied include HSCs, MSCs, and endothelial stem cells, which can all be isolated from bone marrow. For example, HSCs generate all mature blood cells and repopulate the hematopoietic system functionally (Verfaillie 2002). Endothelial stem cells are required to maintain the integrity and function of the endothelium by replacing damaged endothelial cells and initiating endogenous neovascularization of ischemic tissues (Thijssen, et al. 2009) (Zhang, Malik and Rehman 2014). MSCs possess considerable therapeutic potential due to their capacity to differentiate into various mature cell types and induce a pro-regenerative microenvironment.

Table 2-1 Main types of adult stem cells and their primary direction of differentiation. Adapted from (Körbling and Estrov 2003)

Cell type	Tissue-specific location	Cells or tissues produced
Hematopoietic stem cells	Bone marrow, peripheral blood	Bone marrow and blood lymphohematopoietic cells
Mesenchymal stem cells	Bone marrow, peripheral blood	Bone, cartilage, tendon, adipose tissue, muscle, marrow stroma, neural cells
Neural stem cells	Ependymal cells, astrocytes (subventricular zone) of the central nervous system	Neurons, astrocytes, oligodendrocytes
Hepatic stem cells	In or near the terminal bile ductules (canals of Hering)	Oval cells that subsequently generate hepatocytes and ductular cells
Pancreatic stem cells	Intraislet, nestin-positive cells, oval cells, duct cells	Beta cells
Skeletal-muscle stem cells or satellite cells	Muscle fibers	Skeletal muscle fibers
Stem cells of the skin (keratinocytes)	Basal layer of the epidermis, bulge zone of the hair follicles	Epidermis, hair follicles
Epithelial stem cells of the lung	Tracheal basal and mucus-secreting cells, bronchiolar Clara cells, alveolar type II pneumocyte	Mucous and ciliated cells, type I and II pneumocytes
Stem cells of the intestinal epithelium	Epithelial cells located around the base of each crypt	Paneth's cells, brush-border enterocytes, mucus-secreting goblet cells, enteroendocrine cells of the villi

ASCs, particularly MSCs, are increasingly being used in TE research in place of ESCs or iPSCs due to their greater accessibility, lower ethical concerns, ease of isolation and expansion, and autologous cell sources. Table 2-2 compares the benefits and drawbacks of ASCs, ESCs, and iPSCs. As a result, the primary disadvantage of MSCs during *in vitro* expansion is culture-related senescence and spontaneous differentiation (Sherley 2002) (Sarugaser, et al. 2009).

Table 2-2 The advantages and disadvantages associated with different types of stem cells

Stem cells	Advantages	Disadvantages
Adult stem cells	Easy to obtain and expand; No ethical issues; No teratoma formation following engraftment.	Limited phenotype; Lack of specific cell markers; Culture-related senescence; Spontaneous differentiation during <i>in vitro</i> expansion.
Embryonic stem cells	Large differentiating ability; Unlimited phenotypic potential.	Ethical issues; Difficult to isolate; Concerns regarding tumorigenesis.
Induced pluripotent stem cells	Unlimited quantity; No ethical issues; Large differentiating ability; Unlimited phenotypic potential.	Concerns regarding tumorigenesis; Reprogrammed using viral vector.

2.2 Mesenchymal Stem/Stromal Cells

2.2.1 The Discovery of MSCs

Cohnheim's observation in 1867 provided the first evidence of the existence of non-hematopoietic stem cells. Cohnheim observed that most cells appearing at the site of wound repair originated in the bloodstream and were fibroblast-like cells capable of migrating to the repair site and depositing collagen fibers to facilitate repair. Then most studies focused on the role of these fibroblast-like cells in collagen formation during wound repair, while the inherent properties of these cells have not been investigated till these cells were isolated and culture *in vitro* by Friedenstein in 1970. Friedenstein and colleagues cultured stromal cells from guinea-pigs' bone marrow in culture vessels and observed a population of nonhematopoietic cells adhering to the plastic of the culture flask after a few days of culture and eventually forming large discrete colonies on days 9-12 (Friedenstein, Chailakhjan and Lalykina 1970). These cells were subsequently designated colony-forming unit-fibroblast (CFU-F) or fibroblast colony-forming cells (FCFC) with the ability to conform to the bone marrow microenvironment, thereby promoting hematopoietic cell proliferation and differentiation during hematopoiesis (Friedenstein, Chailakhjan and Lalykina 1970). Additionally, Friedenstein demonstrated that these cells from either guinea-pigs or rabbits exhibited osteogenic differentiation characteristics *in vitro* and *in vivo* (Friedenstein, et al. 1968) (Friedenstein, Chailakhjan and Lalykina 1970) (Friedenstein, et al. 1974) (Andrzejewska, Lukomska and Janowski 2019) (Figure 2-2). Numerous researchers since then have confirmed Friedenstein's findings and established that the cells isolated by Friedenstein were also present in the

human bone marrow. Additionally, they demonstrated that these cells could be differentiated *in vitro* into various mesenchymal lineage cells, including osteoblasts, chondrocytes, and adipocytes (Bianco, Robey and Simmons 2008). Thus, Caplan coined the term 'Mesenchymal stem cells' (A. I. Caplan 1991) in 1991 for cells isolated by Friedenstein as a substitute for "stromal" or "osteogenic" stem cells, and it has gained widespread popularity (Caplan 2007), despite raising doubts about the extent of their stemness (Bianco, Robey and Simmons 2008) There are currently numerous synonyms for MSCs, including multipotent stromal cells, mesenchymal stromal cells, mesodermal stem cells, marrow stromal cells, and many more. Caplan also proposed renaming these cells as 'Medical signaling cells' to more accurately reflect the mechanism of their therapeutic effects following transplantation, which is that these cells secrete bioactive factors to facilitate in tissue regeneration (Andrzejewska, Lukomska and Janowski 2019) (Caplan 2017).

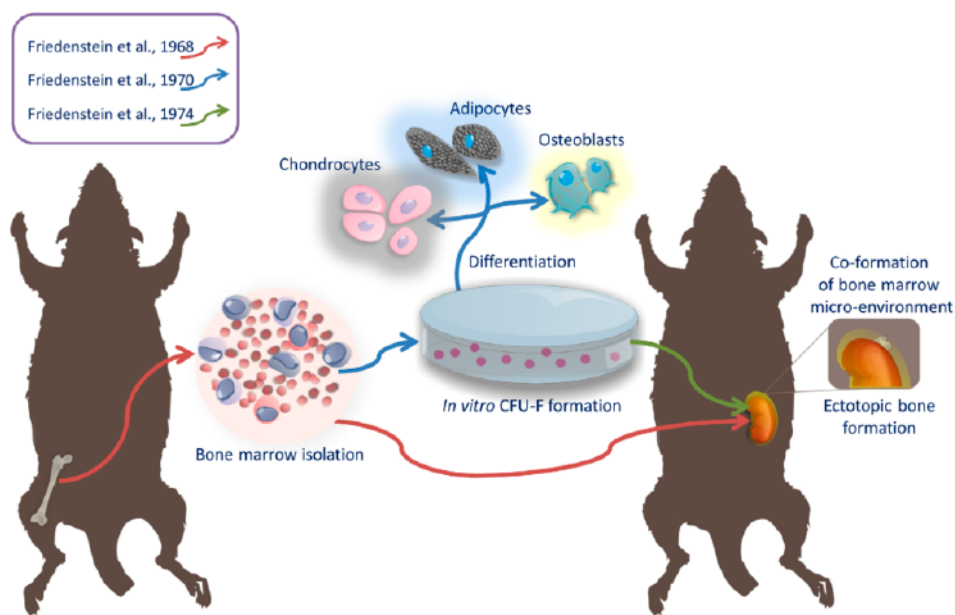


Figure 2-2 **The roots of research on bone marrow-derived stem cells** of connective tissue, which has been then named "mesenchymal stem cells." (Image from Andrzejewska, Anna, 2019, Stem cells)

2.2.2 MSCs Sources and Characterization of MSCs

Friedenstein and colleagues isolated MSCs from guinea pig and mouse bone marrow (Friedenstein, Chailakhjan and Lalykina 1970) (Friedenstein, et al. 1974) in the 1970s, followed by Haynesworth and colleagues' isolation and expansion of human bone marrow MSCs (BM-MSCs) (Haynesworth, et al. 1992) in 1992. Further studies demonstrate that MSCs can be isolated from various mesenchymal tissues, including adipose tissue,

synovial membrane, dental pulp, umbilical cord blood, and Wharton's jelly of the umbilical cord tissue exhibit *in vitro* characteristics comparable to those of BM-MSCs, the best-characterized MSCs.

Human BM-MSCs are typically isolated from the iliac crest and have been used for a long period of time (Lennon and Caplan 2006). BM-MSCs were initially described as fibroblast-like cells capable of adhering to tissue culture plastic and forming colonies of fibroblastic cells. However, subsequent research revealed that cell morphology, phenotype, and bone formation varied within cell populations and between donors. (Friedenstein, Chailakhjan and Lalykina 1970) (Friedenstein, Latzinik, et al. 1982) (Phinney 2002). Thus, the profile of cell surface proteins involved in intra- and intercellular communication is taken into account when describing and isolating MSCs. Antibodies are used to perform the profiling, which can be assessed using flow cytometry. Cells are sorted using a fluorescence- or magnetic-activated cell sorter based on the presence (positive) or absence (negative) of specific markers. A study examining the expression of the hematopoietic receptors CD14, CD34, and CD45 in MSCs and traditional marrow-derived stromal cells (MDSCs, the standard cell preparation for *in vitro* stromal cell culture) discovered that neither cell population expressed CD34. MSCs were negative for CD14 and CD45 (hematopoietic cell markers), whereas MDSCs were positive for these markers (Majumdar, et al. 1998). This finding demonstrates the intrinsic heterogeneity of MSC populations. Thus, isolating a homogeneous MSC population requires a diverse set of antigen markers associated with mesenchymal, endothelial, and epithelial cell types. There are currently various cell surface markers, such as Stro-1, CD 146, stage-specific embryonic antigen-4 (SSEA-4), CD 271, CD73, CD90, and CD105, that have been used to identify MSCs. The disparate methods of isolation and expansion, as well as the disparate approaches to characterizing MSCs, make it difficult to compare and contrast studies from various groups, impeding progress in the field (Dominici, et al. 2006). To address this issue, a definition that is unambiguous and widely accepted is necessary. Thus, the International Society for Cellular Therapy (ISCT) proposed three minimal criteria for defining an MSC for research purposes (Table 2-3) (Dominici, et al. 2006). To begin, MSC must be capable of adhering to plastics when cultured in standard tissue culture flasks. Second, $\geq 95\%$ of the MSC population must express CD105, CD73, and CD90 on the surface, while must lack expression ($\leq 2\%$ positive) of CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA class II on the surface. Thirdly, MSCs must

be capable of differentiation into osteoblasts, chondrocytes, or adipocytes under standard *in vitro* differentiation conditions (Dominici, et al. 2006).

Table 2-3 Summary of criteria to define hMSCs

1. Capable of adhering to plastic under standard culture conditions.
2. CD105, CD73, and CD90 are positive, while CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA class II are negative.
3. In vitro osteogenic, chondrogenic, and adipogenic differentiation measured by staining

2.2.3 The Potential of MSCs Differentiation

The capacity of stem cells to differentiate into mature cell types with specialized functions is a unique property. The origin of stem cells dictates their number and the types of cells into which they can differentiate. MSCs are derived from mesoderm during development. Thus, *in vitro* studies have demonstrated that MSCs can differentiate into various cell types derived primarily from the mesodermal lineages, such as osteoblasts, chondrocytes, adipocytes, and myoblasts (Figure 2-3) (Uccelli, Moretta and Pistoia 2008) (Pittenger, et al. 1999) (Majumdar, et al. 2000) (Gang, et al. 2004). However, several studies have demonstrated that MSCs can transdifferentiate into cells from unrelated germline lineages (Woodbury, et al. 2000) (Jiang, et al. 2002) (Wang, et al. 2005) (Tropel, et al. 2006), implying that they may possess a much broader differentiation potential. The developmental origin of mesenchymal tissues, which include the mesoderm and, to a lesser extent, the cranial neural crest, may explain for this transdifferentiation capacity (Takashima, et al. 2007). Additionally, there is lack of consistent transdifferentiation *in vivo* and the real contribution of this capacity for differentiation into biologically and functionally relevant tissue-specific cell types remains unknown (Phinney, Hill, et al. 2006) (Phinney and Prockop 2007).

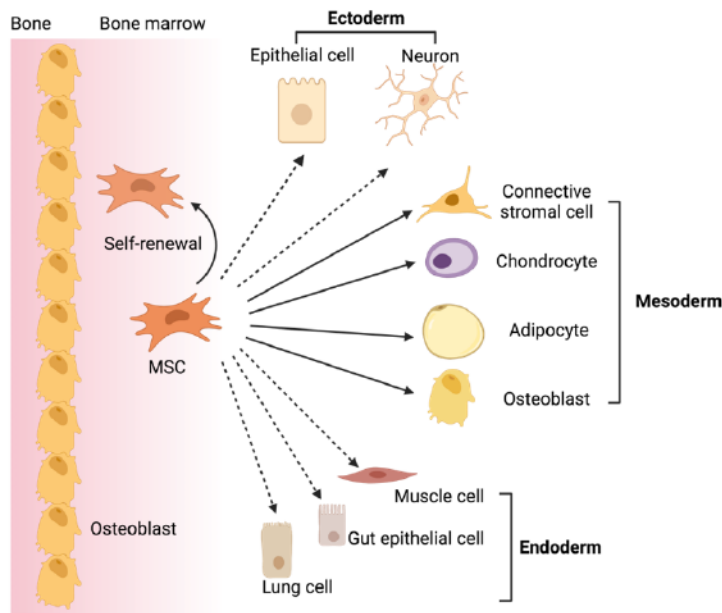


Figure 2-3 **Schematic illustration of MSCs capacity of self-renewal and differentiation.** Adapted from (Uccelli, Moretta and Pistoia 2008), (Created with biorender.com).

2.2.3.1 Osteogenic Differentiation

MSCs were first demonstrated to form bone tissue in diffusion chambers (Friedenstein, Chailakhjan and Lalykina 1970), followed by osteogenic differentiation *in vitro* using a confluent monolayer of rat bone marrow stromal cells in the presence of dexamethasone, β -glycerophosphate, and ascorbic acid. MSCs differentiate into pre-osteoblasts and then into osteoblasts during osteogenic differentiation. Osteogenic differentiation is a reciprocal and functionally coupled process encompassing three major phases of osteoblast phenotype development (proliferation, extracellular matrix development and maturation, and mineralization), each of which is tightly regulated sequentially and stringently by related gene expression, as illustrated in Figure 2-4 (Amarasekara, Kim and Rho 2021). The traditional method of osteogenic differentiation takes approximately 2-3 weeks. MSCs form nodules and increase alkaline phosphatase expression, resulting in calcium deposition over time (Chamberlain, et al. 2007). Alizarin red and von Kossa staining can be used to positively identify these bone nodules. Additionally, two families of growth factors have been identified that promote osteoblast differentiation from MSCs: the wingless/integrated (Wnt) family and the BMPs family (Fakhry, et al. 2013).

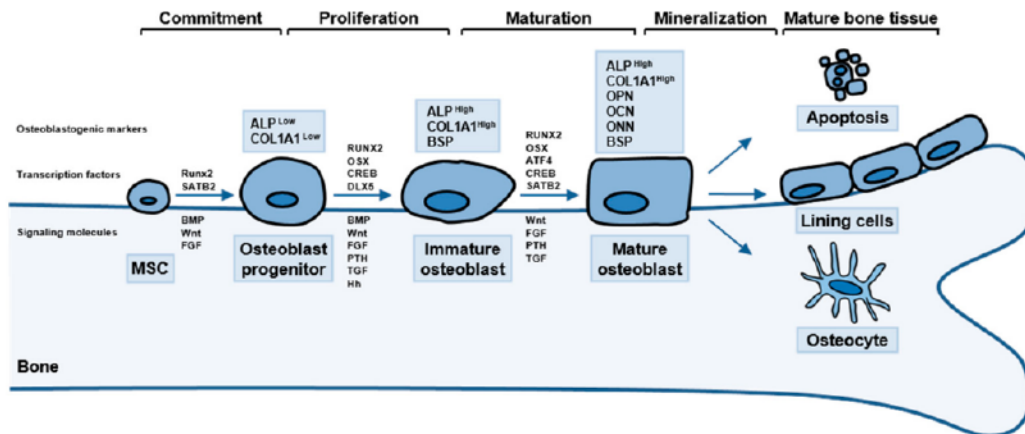


Figure 2-4 Schematic illustration of sequential action of cytokine networks and transcription factors during osteogenic differentiation from MSCs. Image from (Amarasekara, Kim and Rho 2021)

2.2.3.2 Chondrogenic Differentiation

Caplan et al. demonstrated that culture of MSC pellets in a defined medium containing bovine serum albumin, transferrin, hydrocortisone, and insulin in Eagle's minimal essential medium produced cartilage-specific ECM molecules, type II collagen, and glycosaminoglycans (GAGs) (Caplan 1991). This system is analogous to cartilage development *in vivo* during the embryological process, which involves the condensation of chondroprogenitor cells, differentiation into chondroblasts, and eventually chondrocytes. This process of chondrogenic differentiation can be divided into two distinct phases: mesenchymal condensation and subsequent differentiation processes, including chondrocyte proliferation and maturation, as well as extracellular matrix deposition. Additionally, TGF- β or BMPs treatment increased the accumulation of extracellular matrix molecules and the expression of genes associated with chondrogenesis, such as type II collagen and aggrecan. Thus, the most frequently used method for inducing chondrogenic differentiation is centrifuging MSCs to form a pelleted micromass, which is then cultured in the presence of TGF- β . These cell pellets develop a multilayered, matrix-rich morphology, and histological examination reveals strong toluidine blue staining, indicating an abundance of GAGs in the extracellular matrix (Chamberlain, et al. 2007) (Chen, et al. 2008). As illustrated in Figure 2-5, this sequential sequence of coordinated cellular events is tightly regulated by the complex interplay of numerous molecules (Green, et al. 2015).

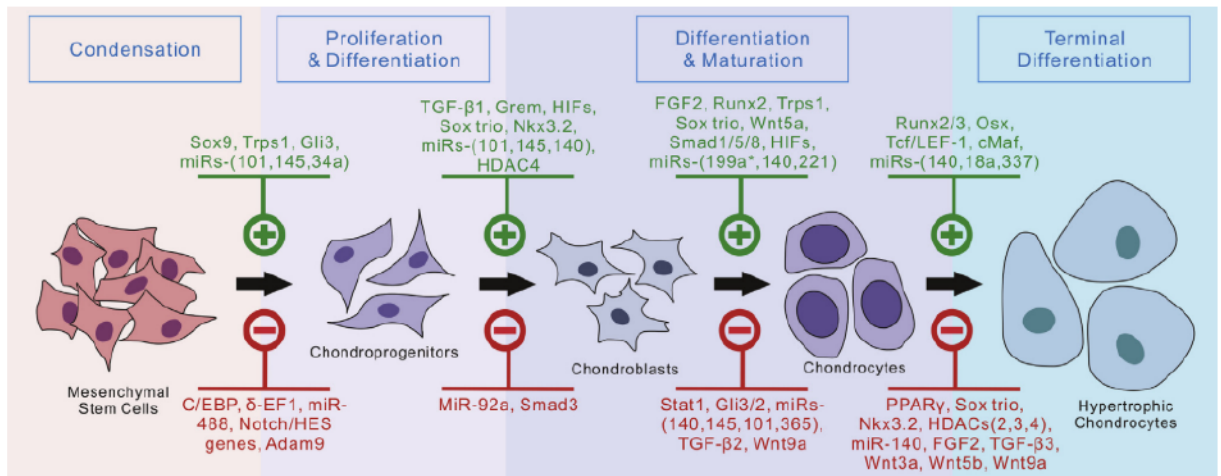


Figure 2-5 Currently identified regulators of chondrogenic differentiation from MSCs. Currently reported signaling molecules, transcription factors, microRNAs and other regulators are listed. Positive regulators are shown on the top panel in green, while the inhibitory regulators are shown in red at the bottom panel. Image from (Green, et al. 2015)

2.2.3.3 Adipogenic Differentiation

The inverse relationship between the differentiation of adipocytic and osteogenic cells established that adipogenic cells differentiate from bone marrow stromal cells (Beresford, et al. 1992) (Jaiswal, et al. 2000). When dexamethasone was added to the primary and secondary cultures, the rat bone marrow stromal cells differentiated predominantly into osteoblast. However, when dexamethasone was added exclusively to the secondary culture, these cells differentiated predominantly into adipocytes (Beresford, et al. 1992) (J.H., et al. 1991). MSCs differentiate into pre-adipocytes and then mature into lipid-containing adipocytes during adipogenic differentiation. This cellular process is divided into two distinct stages: determination and differentiation, which involve a variety of molecular events, including the expression of peroxisome proliferation-activated receptor 2 (PPAR2), CCAAT/enhancer-binding proteins (C/EBPs), lipoprotein lipase, and the fatty acid-binding protein aP2 (Lefterova and Lazar 2009) (Khalilpourfarshbafi, et al. 2019) (Lefterova and Lazar, 2009; Khalilpourfarshbafi et al., 2018; Lazar et al., 2018) (Ahmad, et al. 2020) (Fève 2005) (Figure 2-6). MSCs are cultured with dexamethasone, insulin, isobutyl methylxanthine, and indomethacin to induce adipogenic differentiation *in vitro*. Within cells, lipid vacuoles accumulate and eventually fill the cells. Thus, lipid accumulation in these vacuoles is histologically determined using oil red O staining (Ahmad, et al. 2020).

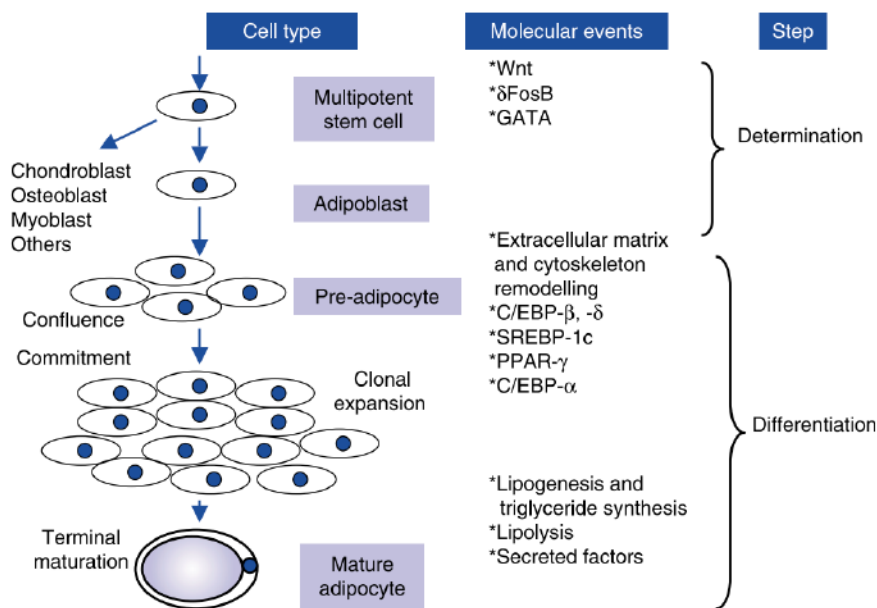


Figure 2-6 Schematic illustration of adipogenic differentiation with key molecular events. Image from (Fève 2005)

2.3 The Role of MSCs in TE

Currently, MSCs are being used in more than 1200 clinical trials for various diseases (www.clinicaltrials.gov), including bone/cartilage repair, diabetes, cardiovascular diseases, immune-related, and neurological disorders. The fact that MSCs can be differentiated into several various cell types *in vitro* and their ability to be easily isolated and expanded by various tissue culture techniques clearly make MSCs an attractive stem cell source in TE applications. When an injury is present, MSCs migrate in response to inflammatory mediators and home to injured sites where they exert powerful regulation for the progression of tissue repair/wound healing in various ways. Thus, MSCs can be applied through systemic and local injections or by TE approaches.

2.3.1 Engraftment and Integration of Differentiated Cells

Differentiation potential, the capacity of MSCs homing to injured sites, and integration as differentiated cells specific for the targeted tissue is initially considered as the primary mechanism for their role in tissue repair. The integration of differentiated cells to replace the damaged tissues has been demonstrated in several tissues—for example, MSCs repair cartilage by differentiating into chondrocytes and replacing cartilage defects

(Somoza, et al. 2014) (Goldberg, et al. 2017) (Lee and Wang 2017) (Zhang, et al. 2019). Several groups demonstrated that the employment of MSCs to repair infarcted myocardium (Barbash, et al. 2003) (Mangi, et al. 2003) (Meyer, et al. 2006). The injected MSCs could be identified within the newly-formed blood vessels near the injection sites (Hu, et al. 2008). Moreover, those transplanted MSCs have differentiated into cardiomyocytes, endothelial cells, and smooth muscle cells (Chamberlain, et al. 2007) (Phinney and Prockop 2007). Similarly, MSCs contribute to wound healing through differentiation into keratinocytes which form the epidermis (dos Santos, et al. 2019) (Sasaki, et al. 2008). Overall, these evidence indicates that MSCs can engraft into targeted tissues and can differentiate into several cell types to replace damaged tissues. However, accumulating evidence indicates that MSC engraftment is quite minimal when using labeled MSCs in several animal models. Most transplanted human MSCs died (approximate 0.44 % of initial transplanted MSCs survived) within four days after transplantation into the ischemic heart (Toma, et al. 2002). Similarly, our lab has demonstrated that massive MSCs died rapidly after transplantation either in the critical segmental femoral bone defect model or the ectopic mouse model (Manassero, et al. 2016). Luciferase-labeled human MSCs were implanted, and cell survival was evaluated by serial bioluminescence imaging (BLI) which provided evidence of rapid and continuous cell death. Specifically, the BLI signal decreased 85% over the first two weeks in both sites. Less than 2% of the initial transplanted cell number was present by four weeks post-implantation, and less than 1% of initial cell number by eight weeks post-implantation. This high rate of transplanted MSCs death is likely caused by a hostile microenvironment involving inflammatory responses, oxidative stress, and ischemic conditions. Although rapid and massive MSCs death occurred post-implantation, MSCs engrafted into myocardium and differentiation into cardiomyocytes were overserved in ischemic heart, and bone formation appeared in both orthotopic and ectopic mouse model. A study comparing allogeneic BM-MSCs with syngeneic BM-MSCs in engraftment and effect in wound healing (Chen, et al. 2009) demonstrated that the number of engrafted allogeneic and syngeneic MSCs declined as the regenerated tissue became more mature. This suggested that the direct integration of differentiated MSCs into the wound may contribute less to the progression of the wound healing process, and the changes in the wound microenvironment with the progression of the wound healing process likely cause the reduction in the number of engrafted MSCs. Thus, MSC engraftment benefits to

improve tissue repair, while its contribution may be somewhat marginal due to the low retention rate.

2.3.2 Paracrine Functions

Low MSCs retention rate but accelerating tissue repair has been readily observed, which challenged the initial mechanism of action of MSC through cell engraft and differentiation (Colletti, et al. 2009) (Quertainmont, et al. 2012) (Yang, et al. 2012). Caplan et al. first proposed the trophic effect of MSCs, also known as paracrine function, is an alternative mechanism of MSCs that exerts beneficial effects on tissue repair (Caplan and Dennis 2006). The paracrine function implies that the engrafted MSC has the capacity to release a large variety of bioactive trophic factors to regulate the activity of many surrounding cells through intercellular communication. This hypothesis is supported by the *in vitro* and *in vivo* observation that MSC-condition media (CM) improves tissue repair. Currently, researchers are increasingly integrating the paracrine functions of MSCs as a prominent feature in tissue repair. The repertoire of bioactive trophic factors secreted by MSCs is collectively referred to as the secretome, consisting of a diverse range of growth factors, cytokines, chemokines, extracellular vesicles, and probably more (Figure 2-7) (Kusuma, Carthew, et al. 2017) (Gnecchi, Danieli, et al. 2016). It has been suggested that such paracrine functions account for up to 80% of the therapeutic effect of adult MSCs (Maguire 2013) (Timmers, et al. 2008) (Kusuma, Carthew, et al. 2017). These factors contribute to tissue repair by modulating the local immune system (Aggarwal and Pittenger 2005) (Nicola, et al. 2002) (English 2013) (Klinker and Wei 2015) (Krampera, et al. 2006) (Nasef, et al. 2007) (Nasef, Mathieu, et al. 2007) (Selmani, et al. 2008) (Wang, et al. 2014), promoting angiogenesis (Sun, et al. 2007) (Spanholtz, et al. 2011) (Rehman, et al. 2004) (Kinnaird, et al. 2004) (Beckermann, et al. 2008), preventing cell apoptosis and fibrosis (da Silva Meirelles, et al. 2009) (Hocking and Gibran 2010), and stimulating survival, proliferation, cell migration (Mirotsov, et al. 2007) (Imberti, et al. 2007) (Dufourcq, et al. 2008) (Block, et al. 2009), differentiation of resident tissue-specific cells, and extracellular matrix formation (Kinnaird, et al. 2004) (Kuchroo, et al. 2015) (Kim, et al. 2007) (Andrzejewska, Lukomska and Janowski 2019) (Figure 2-8).

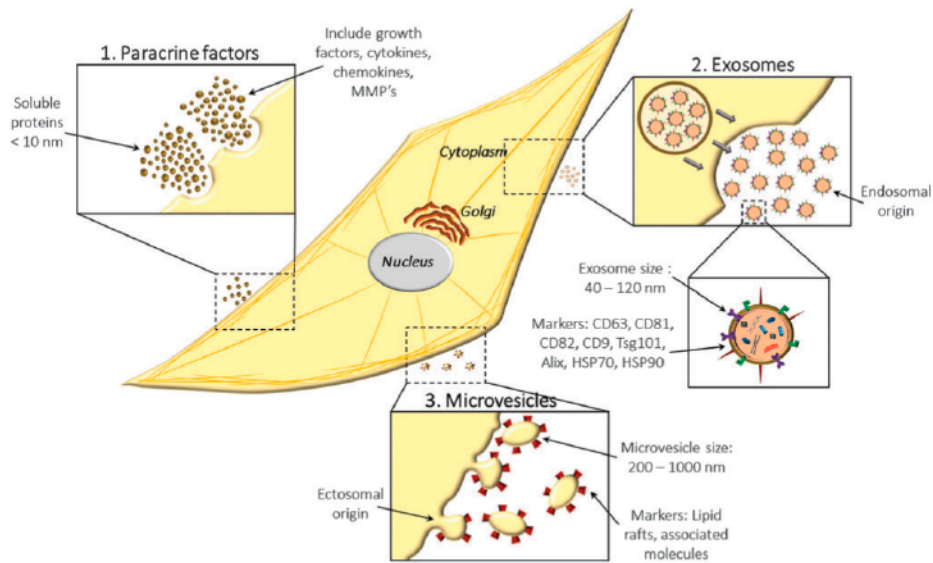


Figure 2-7 An overview of MSC secretome. Schematic representation of biologically active substances released by MSCs. Image from (Kusuma, Carthew, et al. 2017)

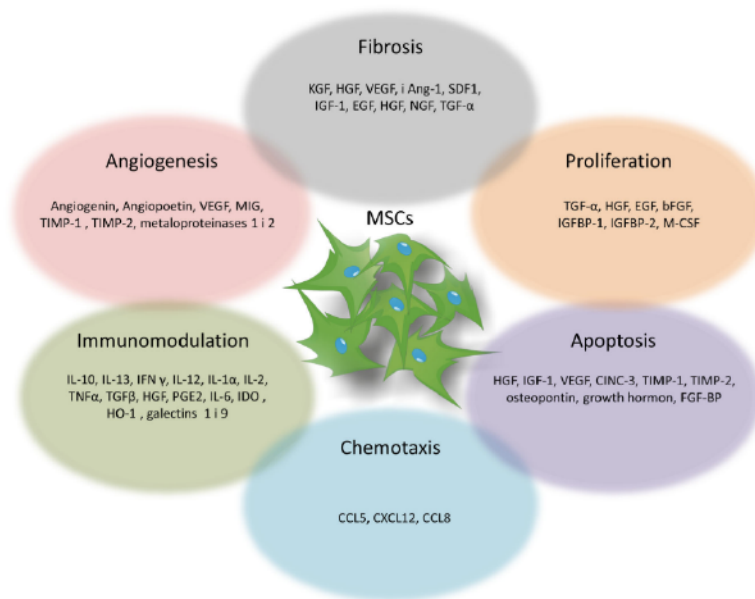


Figure 2-8 The mechanisms mediating mesenchymal stem cell-dependent trophic support. Image from (Andrzejewska, Lukomska and Janowski 2019)

2.3.2.1 Immunomodulation

Immunomodulatory properties are one of the main advantages of MSC-mediated therapies. Specifically, MSCs have exhibited immunosuppressive effects, both *in vitro* and *in vivo*, by interacting and regulating the function of the majority of effector cells, including monocytes/macrophages, dendritic cells, T cells, B cells, and natural killer cells, involved in the processes of both innate and adaptive immune response (Figure 2-9)

(Andrzejewska, Lukomska and Janowski 2019). MSCs exert their immunomodulatory effects by suppressing effector T cells proliferation and allogeneic T-cell response, reducing B cell proliferation and maturation, inhibiting proliferation and cytotoxicity of NK cells, inducing macrophage M1/M2 phenotype transformation, and promoting the generation of regulatory T cells via cell-cell contact or soluble factors mechanisms Figure 2-9 (Gao, et al. 2016) (Fu, et al. 2012) (Yagi, et al. 2010) (Chabannes, et al. 2007) (Sun, et al. 2012). For example, MSCs suppress T cell proliferation when adding to lymphocytes in a mixed lymphocyte reaction, even in the presence of T cell mitogens (Le Blanc, et al. 2003). In addition, MSCs can inhibit T cell proliferation by releasing soluble factors (Nicola, Carlo-Stella, et al. 2002) and by altering the secretion profiles of dendritic cells, effector T -cells, and natural killer cells to adopt a more anti-inflammatory profile (Aggarwal and Pittenger 2005). A large variety of soluble factors have been identified to regulate MSCs-mediated immunomodulatory properties, including indoleamine-pyrrole 2,3-dioxygenase (IDO), TGF- β 1, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), nitric oxide (NO), interleukin (IL) -10, and FGF, among many others (Song, Scholtemeijer and Shah 2020) (Li and Hua 2017) (Salgado, et al. 2010) (Gebler, Zabel and Seliger 2012). In addition, it is well-established that interferon (IFN)- γ plays an important role in the enhancement of MSCs' suppressive activity (Krampera, et al. 2006) (Zhao, Ren and Han 2016).

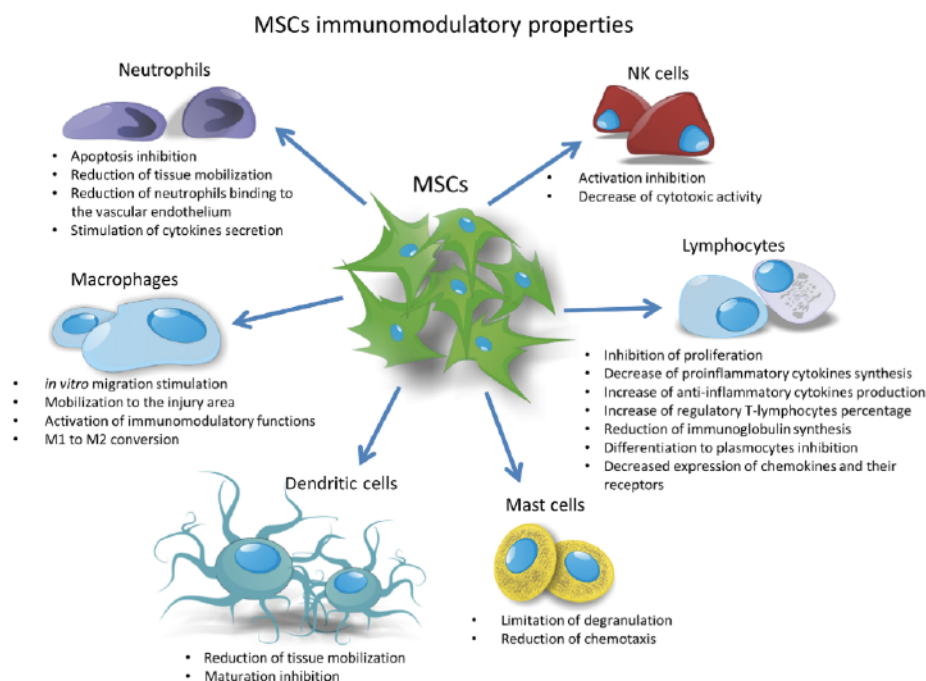


Figure 2-9 **Schematic illustration of the immunomodulatory properties of mesenchymal stem cells.** Image from (Andrzejewska, Lukomska and Janowski 2019)

2.3.2.2 Angiogenesis

The depletion of oxygen and nutrients caused by blood vessel disruption is a limit to the therapeutic efficacy of tissue engineered constructs (Mastrullo, et al. 2020). Thus angiogenesis is a fundamental biological event positively affected by MSCs in a paracrine manner during tissue repair. Reparative angiogenesis is defined as the process through which new vasculature sprouts from pre-existing blood vessels in a sprouting angiogenesis manner (Table 2-4). It intends to recreate functional and interconnected blood vessels (Mastrullo, et al. 2020), which maintains nutrient transfer, adequate tissue oxygenation, and metabolic waste removal.

Table 2-4 Main stages of sprouting angiogenesis. Adapted from (Omorphos, et al. 2021)

Stage	Key functions	Key mediators
1	Angiogenic growth factors stimulate EC proliferation and migration	VEGF and FGF-b
2	Extra-cellular proteinase mediated degradation of the capillary wall and ECM components	Matrix metalloproteinases (MMPs) 1 and 2
3	EC migration towards the angiogenic stimulus via expansion into the ECM	MMPs, Urokinase and integrins
4	EC re-organization into stable tubules with a central lumen	Angiopoietin 1
5	Formation of a vascular network via interconnection of the newly formed tubules	PDGF

Co-culture of endothelial cells and MSCs can enhance and stabilize endothelial microvascular networks and improve cell functions which to a large extent due to the secretion of pro-angiogenic factors by MSCs stimulating endothelial cells (Kolbe, et al. 2011). These effects can be reproduced using MSC-CM both *in vitro* and *in vivo*. MSC-CM contains a variety of bioactive factors that stimulate angiogenesis, favoring tissue repair. Several bioactive factors have been identified to play key roles in MSC-mediated angiogenesis, including vascular endothelial growth factor (VEGF), fibroblast growth factor basic (FGF-b), platelet-derived growth factor (PDGF), and angiopoietin (Figure 2-10).

VEGF contributes to angiogenesis and restores blood perfusion to injured tissue through a variety of functions, which involve pro-angiogenic activity, inducing migration of cells from macrophage lineage and endothelial cells, and vascular permeability activity (Shibuya 2011) (Ruhrberg 2003) (Gerhardt 2008) (Ferrara 2001) (Hoeben, et al. 2004).

The addition of anti-VEGF-A neutralizing antibodies has been shown to inhibit endothelial cell migration and tubular-like structure formation stimulated by MSC-CM. FGF-b is another key factor that plays various roles in the initiation of angiogenesis through stimulating endothelial cell proliferation, migration, and differentiation (Cross and Claesson-Welsh 2001) (Khurana and Simons 2003) (Chung and Shum-Tim 2012) (Guan, Stankus and Wagner 2007). PDGF plays roles in vascular maturation and remodeling, as it targets mural cells and recruits them to vascular structure (Abramsson, Lindblom and Betsholtz 2003) (Xue, et al. 2012) (Heldin, Hellberg and Östman 2010). In addition, it has been demonstrated that PDGF exhibits a direct antiangiogenic effect on endothelial cells by inhibiting angiogenic response to FGF-b (De Marchis, et al. 2002). Angiopoietins are very important to the stabilization and destabilization processes of vascular networks. Two different angiopoietins, angiopoietin-1 and angiopoietin-2, have been proven to exert opposing effects in angiogenesis. Angiopoietin-1 helps to maintain a stable state of blood vessels by mediating several effects such as improving endothelial cells survival, tightening endothelial cell junctions, and promoting endothelial-mural cell interaction (Sakurai and Kudo 2011) (J. Folkman 2007). Angiopoietin-2 competes with angiopoietin-1 by binding to the same receptor during vascular remodeling, while its exact effects depend on the presence or absence of VEGF (Pitera, et al. 2004) (J. Folkman 2007).

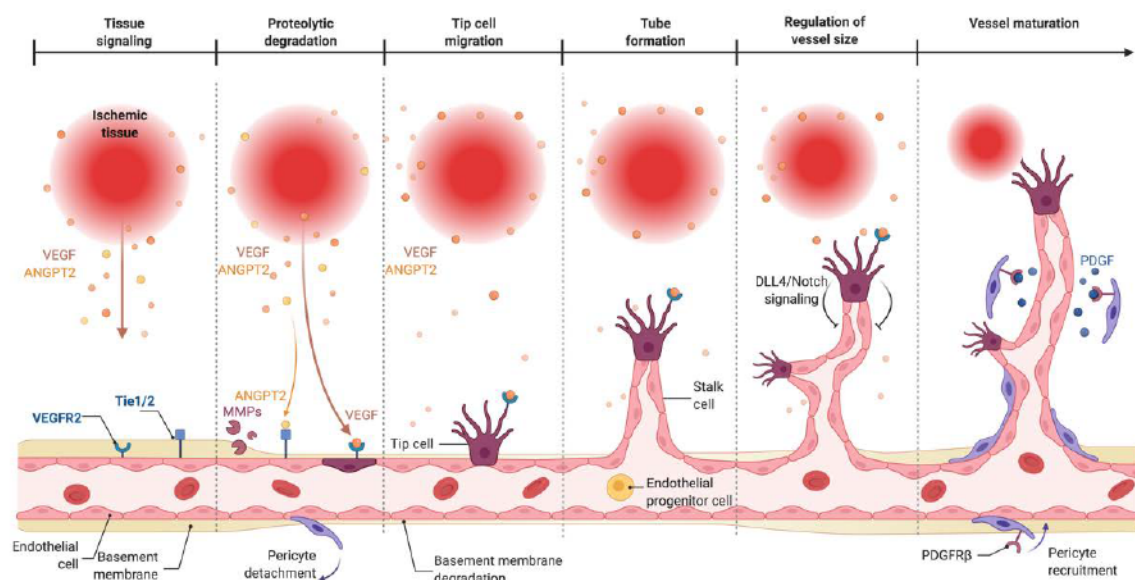


Figure 2-10 Schematic illustration of notable growth factors released from ischemic tissue which promote angiogenesis. Adapted from (Brudno, et al. 2013). (Created with biorender.com).

Numerous other factors are secreted by MSCs, such as substantial amounts of stromal cell-derived factors 1 (SDF-1), which plays an important role in recruiting endothelial progenitor cells to home to sites of injury and accelerate repair (Deshane, et al. 2007) (Askari, et al. 2003) (Yamaguchi, et al. 2003), and in improving the survival of endothelial cells making up the inner lining of vascular vessels (Zou, et al. 2012) In fact, SDF-1-overexpressed MSCs promoted higher density of newly formed blood vessels, thus accelerate wound healing, when compared to normal MSC (Nakamura, et al. 2013). Moreover, these bioactive factors signaling are not static but exhibit dynamic and context-specific secretory signatures (Kshitiz, Ellison, et al. 2019), which support dynamic angiogenic processes (Figure 2-10) are tightly regulated in a spatial and temporal manner (Mastrullo, et al. 2020) (Bentley and Chakravartula 2017) (Carmeliet 2005) (Ferrara and Kerbel 2005) (Carmeliet and Jain 2011).

3 MSCs Metabolism

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The functionality (and ultimately therapeutic efficacy) of MSC-containing construct is a joint action of the MSCs and their microenvironment. Biochemical stimuli (e.g., cytokines, extracellular matrix proteins, etc.) and biophysical cues (such as stiffness and topography of the substrate material scaffold, mechanical stresses and strains exerted on cells, etc.) have been shown to regulate MSC-mediated TE. Advances in several fields (such as cellular and molecular biology, TE, biochemistry, etc.) provided evidence that a regenerative response requires cellular metabolism to provide necessary energy.

3.1 A Brief Overview of Glucose Metabolism in MSCs

Like most mammalian cells, MSCs produce energy by varying the ratios of anaerobic glycolysis and oxidative phosphorylation. Cultured human MSCs (hMSCs) are highly glycolytic, though in the presence of sufficient oxygen, by the enzymatic conversion of glucose to lactate, which contrasts with resting somatic body cells that favor oxidative phosphorylation (OXPHOS) (Salazar-Noratto, et al. 2019) (Nuschke, et al. 2016). This highly glycolytic phenotype provides not only rapid Adenosine triphosphate (ATP) generation, but also carbon precursors biomass generation to support cellular growth. Indeed, glucose can contribute to the amino acid biosynthesis and

nucleotide biosynthesis via glycolysis and its interlinked side pathways, including pentose phosphate pathway (PPP), hexosamine biosynthesis pathway (HBP), and the serine-synthesis pathway (Figure 3-1) (Lunt and Vander Heiden 2011) (Koopman, Ly and Ryall 2014).

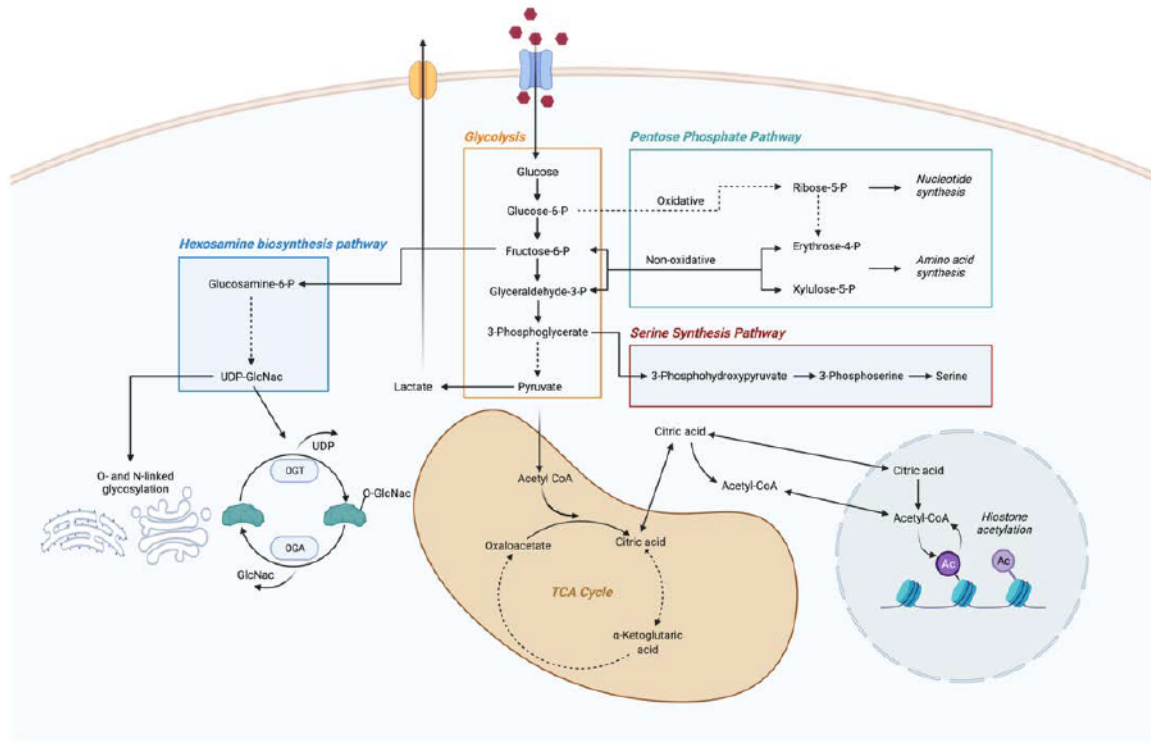


Figure 3-1 Overview of the glucose metabolic pathways in MSC. Glucose is metabolized through glycolysis to generate various metabolites that serve as substrate for multiple metabolic pathways. The pyruvate generated from glycolysis can be either converted to lactate and excreted from the MSC, or transported into the mitochondria and oxidated to acetyl-CoA to feed the TCA cycle and drive OXPHOS when sufficient oxygen is present. Acetyl-CoA can also be transported to the nucleus in which it serves as acetyl donor for histone acetylation. The pentose phosphate pathway and serine synthesis pathway branch from glycolysis and contribute to nucleotide, amino acid and lipid biosynthesis. Hexosamine biosynthesis pathway is another branch of glycolysis that contributes to UDP-GlcNac production, which is a key intermediate for post-translation modification of serine and threonine residues. (Created with biorender.com)

3.1.1 The Glucose Uptake from the Extracellular Milieu

Glucose is a small hydrophilic molecule that circulates freely in the blood but cannot cross the cell's plasma membrane because of its hydrophobicity; instead, it is uptaken through endolysosomal pathways or imported through facilitative diffusion by ATP-independent cell surface nutrient transporters referred to as Glucose Transporters (GLUT1 to 14). Glucose transporters GLUT1, GLUT3, and GLUT4 have been identified for glucose uptake in hMSCs (Unek, et al. 2020). Their high affinity for glucose permits its uptake at a high rate under normal physiological conditions. GLUT1, which is ubiquitously expressed, is considered responsible for basal glucose uptake (Scheepers,

Joost and Schurmann 2004). Oxygen tension, nutrient deprivation, Phosphoinositide 3-kinase (PI3K)/AKT pathway regulate GLUT1. In mouse MSCs (mMSCs), GLUT1 is needed for osteoblast differentiation and bone formation through a mechanism involving the regulation of both the master osteogenic transcription factor RUNX2 and the nutrient sensor AMP-activated protein kinase (AMPK) (Wei, et al. 2015) (van Gestel and Carmeliet 2021). Similarly, an increase in GLUT1 expression associated with the increased glycolytic activity of hMSCs and mMSCs has been reported in MSC immunomodulatory properties (Liu, et al. 2019) (Contreras-Lopez, et al. 2020). GLUT4 is an insulin-sensitive glucose transporter. It is sequestered intracellularly in GLUT4 storage vesicles, which undergo exocytosis to the plasma membrane upon fixation of insulin on its receptor and activation of the PI3K/AKT signaling cascade. This translocation of GLUT4 leads to a 10- to 20- fold increase in glucose uptake (Scheepers, Joost and Schurmann 2004). To the best of the author's knowledge, the exact role of GLUT3 and GLUT4 in MSC biology remains to be determined. Last but not least, glucose uptake in mammalian cells, although mainly regulated by growth factors, can be secondarily modulated by intrinsic sensors such as AMPK. Despite the importance of growth factors acting as cell-extrinsic regulators of glucose uptake and usage, there is a current paucity of studies that seek to identify specific growth factors responsible for glucose uptake in MSCs.

3.1.2 The Glucose Signaling Network

In TE applications/clinical scenarios, MSCs are suddenly exposed to an injured or traumatic tissue (such as bone defect, acute myocardial infarction, and skin wound). The transition from the cultured MSC to such a harsh metabolic milieu characterized by low oxygen tension and nutrient depletion requires a metabolic adaptation to face limited substrate availability (Coyle, et al. 2019) (Salazar-Noratto, et al. 2019). Several nutrient sensors such as AMPK and mechanistic target of rapamycin (mTOR) play critical role in integration between changes in local metabolic milieu and MSC biology since they can convert a change in the extracellular environment to innate signals within cells (Ly, Lynch and Ryall 2020). These nutrient sensors do not belong to strictly linear signaling pathways. They have functional interdependencies acting as both antagonists and agonists of each other's activity and collectively determining the response of a given cell to the extracellular signals (Figure 3-2). Briefly, AMPK is of utmost importance in the capacity

of cells to sense nutrient availability and respond to changes. It is activated allosterically by increasing concentrations of Adenosine monophosphate (AMP). Upon activation of its catalytic alpha subunit, AMPK phosphorylates substrates to restore cellular bioenergetic homeostasis. The activation of AMPK directly impacts glucose homeostasis through promoting increased expression and activity of the glucose transporters (Ly, Lynch and Ryall 2020) (Bando, et al. 2005) (Marsin, et al. 2000) (Sakamoto and Holman 2008). Indeed, substantial evidence demonstrates that AMPK is involved in regulating MSC proliferation, differentiation, and immunomodulatory properties (De Meester, et al. 2014) (Hu and Li 2018) (Kim, et al. 2012) (Pantovic, et al. 2013). Another important signaling pathway mTOR integrates metabolic regulation by growth factors and nutrients. mTOR consists of two complexes (mTORC1 and mTORC2) with divergent functions. A salient feature of mTORC1 is that it responds to energy status, cellular oxygen, amino acids and glucose levels. It is inhibited in cells that are energetically stressed or faced a depletion in oxygen or amino acids even in the presence of growth factors. In these conditions, mTORC1 suppression repress anabolic pathways and induces autophagy and lysosomal biogenesis. In contrast, in the presence of ample nutrients and growth factors, mTORC1 activation upregulates glycolysis, protein and lipid synthesis as well as mitochondrial metabolism. Alterations to mTORC1 signaling play important role in survival/adaptation of hMSCs after long-term starvation (Ferro, et al. 2019), osteoblast proliferation and differentiation (Schaub, et al. 2019), and hMSC immunomodulatory properties (Wang, et al. 2017). In contrast to mTORC1, mTORC2 is not sensitive to nutrients but primarily responds to growth factors. Intracellular nutrient sensors not only respond to metabolite levels, but also are regulated by growth factors signaling pathways. For example, mTORC1 is activated by the serine-threonine kinase AKT (an anti-apoptotic factor), while AKT antagonizes activation of AMPK. Interestingly, accumulating evidences demonstrate the activation of AKT does not inhibit cell death, but renders cells more sensitive to metabolic stress instead. In fact, AKT is evolutionarily conserved and regulates glucose metabolism by promoting glycolysis through increasing GLUT1 trafficking to the cell surface, and through HK and PFK activation (Coloff, et al. 2011) (Nogueira, et al. 2008). Some studies indicate the anti-apoptosis capacity of AKT is coupled with glucose metabolism (Coloff, et al. 2011) (Elstrom, et al. 2004) (Gottlob, et al. 2001) (Graham, et al. 2012). Regarding the role of AKT on MSCs, previous studies show that AKT is involved in regulating mMSC survival in ischemic tissues (Zhu, et al.

2014), rat MSC (rMSC) senescence induced by high glucose (Zhang, et al. 2017), and hMSC osteogenesis (Baker, Sohn and Tuan 2015).

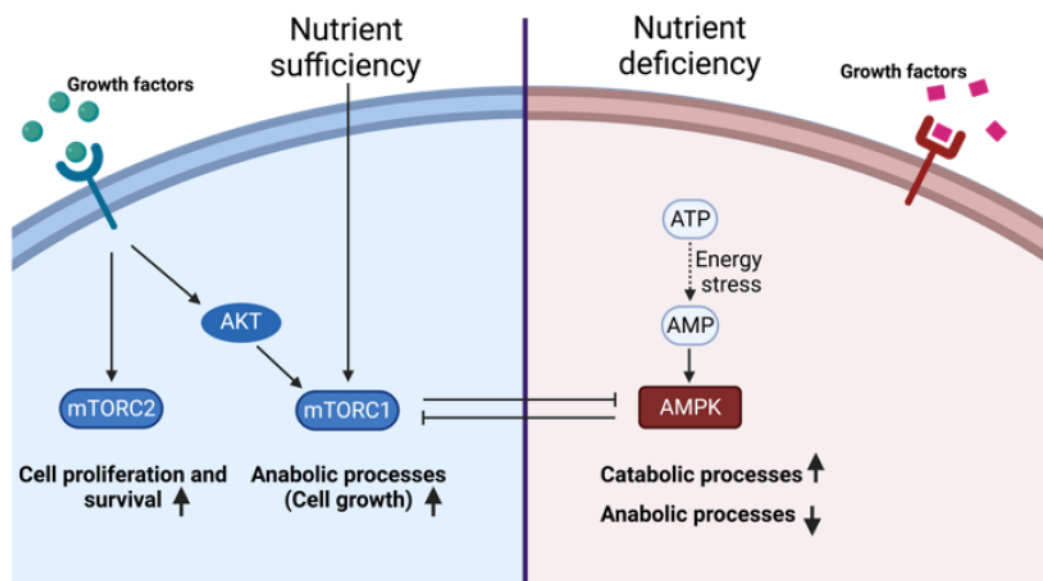


Figure 3-2 **Schematic illustration of the nutrient sensors mTOR/AMPK pathways and growth factor signaling pathway.** A) Under nutrient sufficient condition, mTORC1 is activated. Activation of mTORC1 is also regulated by growth factors signaling pathway via AKT. The activated mTORC1 phosphorylates many downstream targets to increase anabolic processes, thus increases m-TORC1-driven cell growth. In contrast to mTORC1, mTORC2 is primarily activated by growth factors signaling, and controls cell proliferation and survival. B) Under nutrient deficient condition, which induces energy stress, AMPK directly senses the increase of AMP, leading to its activation. Once activated, AMPK participates in a wide scope of pathways and cellular processes, including increase of the catabolic processes and inhibition of the anabolic processes. In addition, the current model shows the interplay between mTOR and AMPK pathways, mTORC1 activation inhibits AMPK while AMPK activation switches off the mTORC1 complex. (Created with biorender.com)

3.1.3 MSCs Exhibit Metabolic Flexibility

Cell metabolism is the sum of all chemical reactions through which energy is created from breaking chemical bonds and used to synthesize basic components. These reactions are directly linked to the activity/ function of cells; in fact, different activities and functions have different energy demands and require synthesis/removal of different molecules. In this regard, plasticity in energy metabolism (or bioenergetics) allows stem cells to match the divergent energy demands of self-renewal and lineage specification (Folmes, et al. 2012) and, in the case of transplanted cells, of cell survival.

Figure 3-3A illustrates the basic bioenergetic reactions that take place in MSCs. Energy generated in cells is stored in energy carrier molecules called ATP, which can be used in future cell chemical reactions. In MSCs, ATP is produced by two pathways: glycolysis and OXPHOS. In glycolysis, glucose is consumed to produce pyruvate and ATP; pyruvate is either converted into lactate or, in the presence of sufficient oxygen, oxidized in mitochondria to acetyl-CoA, which feeds into the tricarboxylic acid (TCA) or,

Krebs, cycle. In OXPHOS, electron by-products of the TCA cycle pass through the electron transport chain (ETC) at the mitochondrial cristae and produce ATP molecules. Although OXPHOS averages a significantly higher ATP production than glycolysis (specifically, 34-36 vs 2 ATP molecules per glucose molecule) (Folmes, et al. 2012) (Lunt and Vander Heiden 2011), studies have consistently shown that MSC survival is not dependent on OXPHOS but rather on glycolysis. Under standard culture conditions, treatment of BM-rMSCs with 2,4-dinitrophenol (DNP), a mitochondrial uncoupler that inhibits OXPHOS showed no increase in cell death up to 72 hours post-DNP treatment (Mylotte, et al. 2008), whereas inhibition of glycolysis by 2-deoxyglucose (2-DG) induced a significant decrease in cell viability as early as 24 hours after treatment in BM-rMSCs (Mylotte, et al. 2008) and BM-hMSCs (Moya, et al. 2018). Moreover, Moya et al (Moya, et al. 2018) showed that, in near-anoxia (0.1% pO₂), disrupting the TCA cycle using Antimycin A or Malonate (TCA cycle inhibitors) does not further impair the MSC metabolism nor does it significantly increase the cell mortality rate. Altogether, these findings confirm that only glycolysis is crucial for cell survival.

Beyond survival, glycolysis is also a hallmark of “stemness” and the preferred pathway for stem cell self-renewal/proliferation. Compared with OXPHOS, glycolysis is less efficient in generating energy; however, inefficient ATP production is a problem only when resources are scarce, which is not the case for proliferating mammalian cells *in vivo* (Heiden, Cantley and Thompson 2009). In fact, proliferating cells have important metabolic requirements that extend beyond ATP, and aerobic glycolysis meets both the bioenergetics and biosynthetic demands of producing new cells (Lunt and Vander Heiden 2011)(Heiden, Cantley and Thompson 2009). Aerobic glycolysis is able to fuel the anabolic intermediates needed for the synthesis of amino acids and nucleotides, via the pentose phosphate pathway (Figure 3-3A) (Lunt and Vander Heiden 2011)(Heiden, Cantley and Thompson 2009) (Shyh-Chang and Ng 2017). Aerobic glycolysis is defined as the conversion of glucose to lactate even in the presence of sufficient oxygen to support glucose catabolism *via* the TCA cycle with OXPHOS (Lunt and Vander Heiden 2011). Vander Heiden et al (Heiden, Cantley and Thompson 2009) approximated that, following glycolysis in proliferating cells, only 5% to 10% of the pyruvate is diverted to mitochondria, whereas 85% is converted to lactate, essentially diminishing the contributions from the TCA and OXPHOS pathways during cell proliferation (known as the Warburg Effect).

Similarly, when MSCs begin to differentiate, they switch their metabolic activity depending on the differentiation pathway they undertake. For example, BM-hMSCs cultured under chondrogenic conditions prefer glycolysis, as demonstrated by their reduced oxygen consumption and OXPHOS activity (Pattappa, et al. 2011). In contrast, BM-hMSCs undergoing osteogenic differentiation exhibit a decrease in glycolytic enzymes but increases in respiratory enzymes, oxygen consumption, and mitochondrial biogenesis; these results suggest a bioenergetics switch from glycolysis to OXPHOS (Chen, et al. 2008). hMSCs experience a similar switch to promote adipogenic differentiation (Tormos, et al. 2011). Because these experiments have been conducted *in vitro*, however, differentiation protocols may play a role in the observed metabolic changes.

Metabolic flexibility allows MSCs to perform their functions; however, it should be noted that metabolic pathways are highly sensitive to cellular and environmental cues, such as oxygen levels, availability of substrates, and pH levels. In order to develop better cell-based therapies and improve MSC survival and their therapeutic potential, scientists should consider the microenvironment in which the cells are transplanted and its potential effect on cellular metabolic activities that ultimately affect cell survival and function.

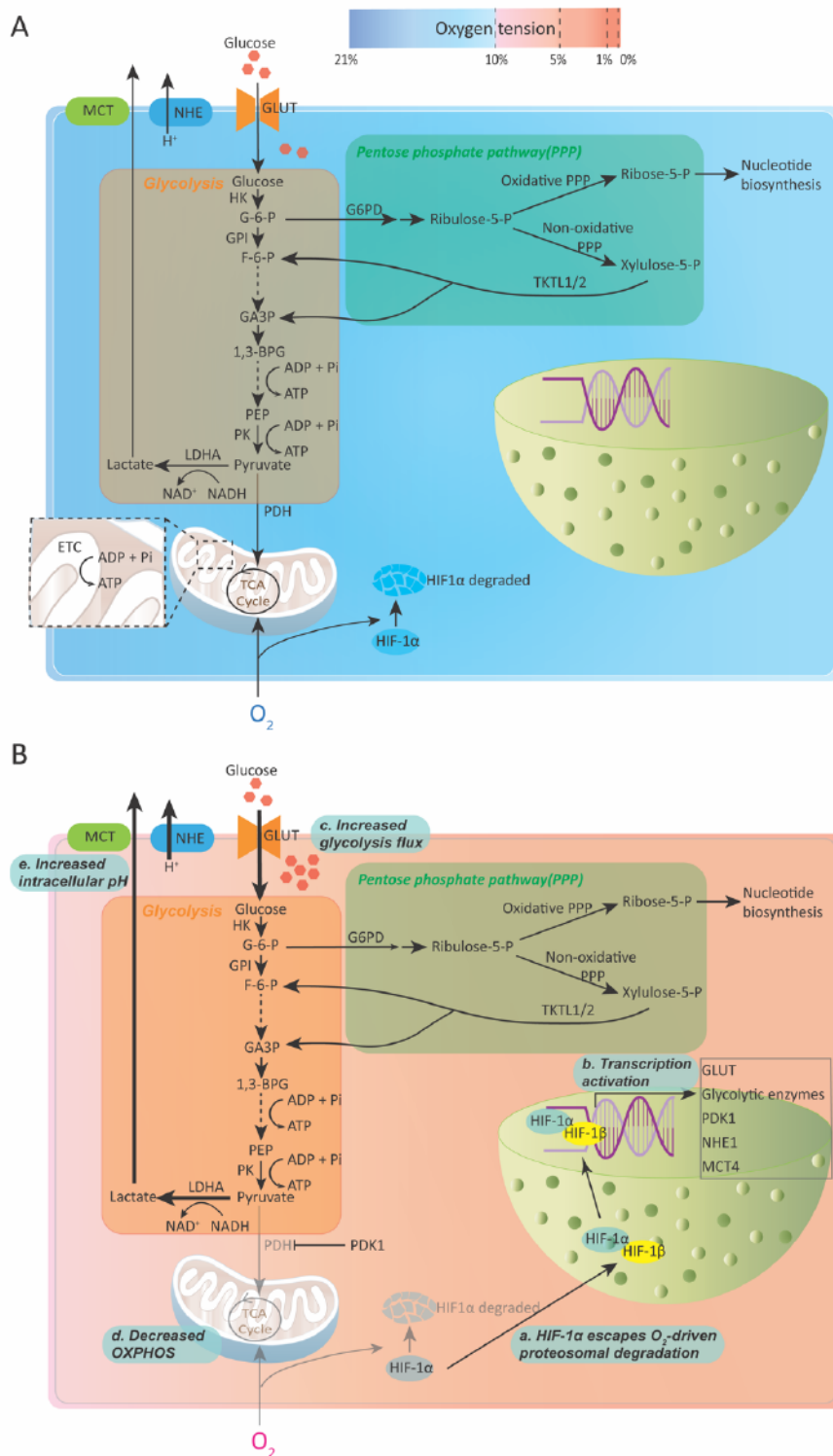


Figure 3-3 Schematic diagrams illustrate metabolism in MSCs under different oxygen tension levels. A, Under “sufficient” oxygen, glucose molecules are broken down in glycolysis for the production of ATP molecules. By-products of glycolysis and other chemical components enter the tricarboxylic acid (TCA) cycle in mitochondria. In OXPHOS, electron by-products of the TCA cycle pass through the electron transport chain (ETC) at the mitochondrial cristae and produce more ATP molecules. Moreover, HIF-1 α is degraded under “sufficient” oxygen-level conditions by proteasomes. **B,** In a near-anoxia environment ($pO_2 < 0.1\%$), however, (a) HIF-1 α escapes oxygen-driven proteosomal degradation and translocates to the nucleus, where it dimerizes with HIF-1 β . Accumulation of HIF-1 α/β activates (b) the transcription of genes that increase expression of glucose transporters and glycolytic enzymes, which (c) increase glycolytic flux; and (d) decrease activity and eventually block the OXPHOS pathway. Increased activity of the glycolytic pathway yields a buildup of lactate, which (e) increases intracellular pH.

3.2 Parameters Affect MSC Metabolism

3.2.1 Effects of Different Oxygen Tension Levels

It is well established that oxygen tension has a direct effect on cellular energy metabolism and, thus, affects cell survival and their therapeutic potential. For this reason, it is important to understand the oxygen tension levels that cells will experience upon transplantation and their subsequent effects on cell metabolism. Oxygen tension varies greatly depending on the targeted tissue, as well as the state (healthy vs injured) of said tissue. Oxygen tension was shown to fall from 2.6% to 3.3% to 0.9% pO₂ within the first 5 minutes of an arterial occlusion in rodent hearts (Khan, et al. 2015) and to reach near-anoxia (0.2% pO₂) after 30 minutes of ischemia (Zhao, et al. 2005). In joint diseases, oxygenation levels of the synovial fluid are lower in inflammatory cases (Ng, et al. 2010) (Treuhaft and McCarty 1971). In situ measurements in the intervertebral disc, however, reported a minimum of 0.3% to 0.7% pO₂ in the disc center in both healthy canine (Holm, et al. 1981) (Holm, Selstam and Nachemson 1982) and degenerated human disks (Bartels, et al. 1998). In general, oxygen tension in various parts of the mammalian body is considerably lower *in vivo* than the atmospheric oxygen tension (21%) and ranges from 12% in the blood to as low as 1% in the deep zone of cartilage regions (Das, et al. 2010). Under standard cell culture conditions, MSCs are kept at 21% pO₂, which is considered “normoxia” but is not physiologically relevant (physioxia). Upon implantation, cells are affected by their new microenvironment, which triggers metabolic changes and signaling pathways. Overall, lack of oxygen triggers the “switch” from high-energy yielding processes (specifically, glycolysis combined with OXPHOS) to the low-energy yielding process of glycolysis (Figure 1B) (Das, et al. 2010). In environments with pO₂ between 1% and 3%, BM-hMSCs (Rosová, et al. 2008) and BM-rMSCs (Peterson, et al. 2011) further activate the Akt signaling pathway by phosphorylation. Akt facilitates metabolic reprogramming toward glycolysis and maintains cell proliferation when the oxygen supply is restricted (Chae, et al. 2016). This signaling pathway plays a key role in the stabilization of the transcription factor hypoxia-inducible factor (HIF)-1 (Das, et al. 2010). In the presence of “sufficient” oxygen levels, the oxygen-regulated HIF-1 α subunit is degraded by proteasomes (Palomäki, et al. 2013) (Simon and Keith 2008). Under hypoxic conditions, however, HIF-1 α escapes oxygen-driven proteasomal degradation. Moya et al (Moya, et al. 2018) reported that exposure of BM-hMSCs to near-anoxia (specifically, 0.1% pO₂) for 3 days resulted in significantly higher HIF-1 α expression and

bioactivity compared with results obtained when BM-hMSCs were maintained at 1%, 5%, and 21% pO₂. Stabilized HIF-1 α translocates to the cell nucleus, where it dimerizes with HIF-1 β (Figure 1B). The HIF-1 α/β dimers activate the transcription of genes encoding glycolytic enzymes and glucose transporters (Saraswati, et al. 2015) (Zhu, et al. 2014). These upregulated glycolytic enzymes aid in shunting energy metabolism from OXPHOS to glycolysis alone and in boosting glycolytic activity (Figure 1B) (Ladurner 2006) (Lavrentieva, et al. 2010). Increased expression of glucose transporters further increases the influx of glucose molecules and overall glycolytic activity in cells. It is worth noting that small differences in oxygen tension (e.g., 1% vs 0.1%) result in a cascade of metabolic and signaling pathways in MSCs. For this reason, it is essential that studies report the level of oxygen tension as accurately as possible. Traditionally, control of pericellular oxygen tension is achieved by fixing the oxygen tension in the gas phase above the medium under which cells are cultured (Mamchaoui and Saumon 2000) (Pettersen, et al. 2005) (Wolff, Fandrey and Jelkmann 1993). Pericellular tension, however, also depends on the number of cells present and their metabolic rate as well as on the diffusion distance from the medium surface to the bottom of the cell culture plasticware where cells are attached (Pettersen, et al. 2005). Pettersen et al (Pettersen, et al. 2005) showed that after 7 days under standard cell culture conditions (21% pO₂) of human T47D cells, the pO₂ was 17.63% at the top of the culture medium and only 7.51% at the bottom of the plasticware surface where the cells were cultured. In order to better simulate the *in vivo* milieu of the transplantation site *in vitro*, researchers should study the oxygen tension level that more closely resembles that of the targeted site *in vivo*.

3.2.2 Effects of Energy Substrate Availability

Historically, the lack of oxygen had been regarded as the primary cause of cell death in an ischemic environment. Recent research, however, provided evidence that the lack of nutrients, notably glucose, is the main culprit of cell death post-implantation. Published reports by many groups have shown that BM-hMSCs (Moya, et al. 2018) (Nuschke, et al. 2016) (Deschepper, et al. 2013), BM-rMSCs (Mylotte, et al. 2008), and BM- sheep MSCs (Deschepper, Oudina, et al. 2011) are able to survive in a hypoxic to near-anoxic environment *in vitro*, given that glucose is available. Glycolysis, which is inherently anaerobic, is the only production path for ATP in cases where little-to-no oxygen is present, which renders glucose the most important energy substrate for MSC survival.

Moya et al (Moya, et al. 2018) confirmed experimentally that BM-hMSCs cultured under 0.1% pO₂ are unable to use exogenous glutamine, serine, or pyruvate to produce ATP. The MSC mortality rate under each of the aforementioned conditions was approximately 93% by day 14 of cell culture *in vitro* (Moya, et al. 2018). In contrast, BM- hMSCs supplied with glucose remained up to 60% viable under near-anoxia by day 14 of cell culture (Moya, et al. 2018). Nevertheless, it should be noted that the high sensitivity of MSC survival to glucose deprivation cannot be explained solely by the loss of ATP (Mylotte, et al. 2008). It is now known that, beyond ATP depletion, inhibition of glycolysis due to glucose deprivation also leads to loss of essential prosurvival signals such as Akt (Mylotte, et al. 2008) (Rathmell, et al. 2003). Furthermore, even in the presence of oxygen, glucose deprivation or starvation, activates metabolic stresses, which may induce cell death (Altman and Rathmell 2012) (Deldicque, Hespel and Francaux 2012) (Ikesugi, et al. 2006) (Schröder and Kaufman 2005). In this scenario, autophagy is activated as a protective mechanism to mitigate cell stress-induced damage and provide nutrients for short-term survival (Figure 3-4) (Altman and Rathmell 2012). In the face of limited or no access to nutrients, cells activate their nutrient-sensing mechanisms to switch from an anabolic program to a catabolic one (Figure 3-4). By decreasing their anabolic activities, cells diminish their metabolic demand, and by activating catabolic pathways, they cannibalize themselves in order to increase their metabolic supply. Cells accomplish this switch through two major cell signaling mechanisms: (i) inactivation of the mTOR pathway, a master regulator of protein translation and proliferation, and (ii) activation of the AMPK. These mutually antagonistic signaling pathways reduce energy-intensive processes (such as protein and lipid synthesis) and induce autophagy and lysosomal biogenesis in order to recover metabolites and energy from existing macromolecules and thus ensure cell survival (Hindupur, González and Hall 2015) (Valcourt, et al. 2012). A study by Nuschke et al (Nuschke, et al. 2016) reported that MSC autophagy was highly sensitive to glucose concentrations. BM-hMSCs that were transferred from a hyperglycemic (4.5 g/L) to a physiological level of glucose medium (1 g/L), thereby lowering their glucose supply, accumulated higher levels of LC3-II (an autophagy marker) as a function of time. In contrast, various oxygen tensions (specifically, 21%, 4%, and 1% pO₂) did not have an effect on the accumulation, or lack, of autophagosomes (Nuschke A 2016). Autophagy is a temporary response to the shortage of glucose, and cell death may still occur if the problem is not addressed in a timely manner (Altman and Rathmell 2012). Therefore, regardless of the level of oxygen

tension, the lack of glucose may lead to cell death. To the best of our knowledge, there are no reported *in vivo* measurements of glucose concentrations in the heart, bone, or articular cartilage in the literature. Because glucose is paramount for the survival of MSCs, knowledge of this parameter is needed and should be used by scientists when designing delivery vehicles for MSCs for TE and regenerative medicine applications.

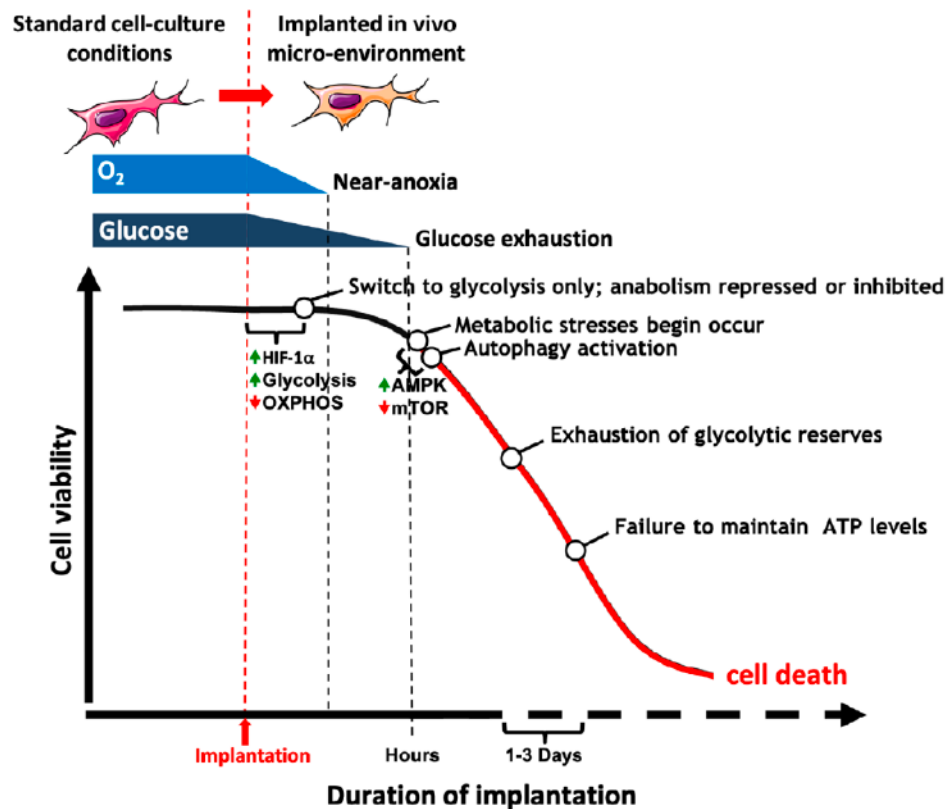


Figure 3-4 Schematic illustrates the envisioned timeline of bioenergetic metabolic activity of cells upon implantation. Before implantation, mesenchymal stem cells (MSCs) are generally cultured under standard conditions of 21% pO_2 and with “sufficient” glucose. Upon implantation in an ischemic site, MSCs experience insufficient levels of oxygen tension, which triggers a switch to glycolysis only and represses, eventually inhibiting, anabolic activities. Upon glucose exhaustion, MSCs begin to experience metabolic stresses, which are, in part, counteracted by the activation of autophagy. Autophagy, or self-catabolism, reduces sources of stress and provides nutrients during times of nutrient withdrawal. Eventually, the glycolytic reserves are exhausted, and autophagy is unable to either provide MSCs additional cell nutrients or mitigate cell stress. Ultimately, cell death occurs when cells do not meet their minimal bioenergetic demands. The time points indicated are according to literature reports (30,65-67) and may vary according to different culture conditions, such as cell density and mass transport parameters. This figure was modified from the graphical abstract from (Moya, et al. 2018)

3.2.3 Effects of the Mechanical Microenvironment

Recent scientific studies provided evidence that the mechanical environment is an integral component of mammalian cell and tissue milieu and should also be taken into consideration regarding cell metabolism. For example, dynamic compression was shown

to increase ATP and lactate production, as well as glucose consumption of pig intervertebral disc cells (Fernando, et al. 2011) (Salvatierra, et al. 2011), and to enhance the glycolytic energy flux in human chondrocytes (Zignego, Hilmer and June 2015). Mechanical stress was also shown to facilitate the transport of glucose into rat podocytes by increasing the number of glucose transporters on the surface of these cells, thus altering energy production and metabolism (Lewko, et al. 2005). Although MSCs are highly mechanosensitive, as shown in many studies regarding stem cell fate in response to mechanical stimulation (Peng, et al. 2017) (Yavropoulou and Yovos 2016) (Yeatts, Choquette and Fisher 2013), there are no literature reports correlating MSC mechanosensitivity and metabolism.

3.2.4 Effects of pH

In addition to the effects on oxygen and nutrient levels, the lack of vascular network results in the accumulation of lactic acid, which acidifies the local environment (Figure 3-3B). Although less detrimental than glucose shortage, lower intracellular and extracellular pH levels inhibit MSC metabolism, proliferation, and osteogenic differentiation (Chano, et al. 2016) (Chen, Zhou and Tan 2009) (Fliefel, et al. 2016) (Kohn, et al. 2002) (Massa, et al. 2017) (Meleshina, et al. 2018) (Monfoulet, et al. 2014). Acidifying the extracellular medium of rabbit MSCs to pH 6.8-7.0 resulted in large decreases in the glucose consumption rate and the lactate production rate (48.3% and 95.1%, respectively), compared with standard cell culture conditions at pH >7.4 (Chen, Zhou and Tan 2009). This finding suggests a reduction in glycolysis, which may be a feedback system to avoid further acidification of the extracellular space that would ultimately lead to an excessive acidification of the cytosol and cell death (Chano, et al. 2016) (Chen, Zhou and Tan 2009). Moreover, Massa et al (Massa, et al. 2017) showed that extracellular acidosis (specifically, pH 6.5) induces the quiescence state in BM-hMSCs, which may partially explain the observed reduction in cell proliferation and differentiation potential under acidosis. In conclusion, the effects of oxygen tension levels, glucose supply, mechanical stress, and pH levels so far reported in the literature regarding MSC metabolism highlight and reiterate the importance of these conditions in the microenvironment that MSCs encounter upon transplantation *in vivo*. Unfortunately, there is limited pertinent information in the literature about the *in vivo* conditions at different transplantation sites and their impact on cell metabolism. Furthermore, research

on MSC metabolism has been largely restricted to standard cell culture practices, which are not analogous to either the transplantation milieu or other *in vivo* scenarios. For all the aforementioned reasons, there is a crucial need to bridge this knowledge gap in order to elucidate the *in vivo* processes of MSC metabolism and to improve MSC survival and, ultimately, MSC-based therapeutics.

4 The Role of Glucose Metabolism in MSCs-Mediated TE

Paragraph 4.1 to 4.4 are from the literature review article entitled ‘The critical role of glucose metabolism in mesenchymal stem cell mediated tissue engineering’ (as first author) that will be submitted to journal Tissue Engineering Part B – Review.

4.1 The Role of Glucose Metabolism in MSC Expansion *in vitro*

A large-scale expansion of MSCs is typically carried out for clinical use. MSCs regardless of the source from which they are isolated are rare (less than one MSC per 1000 aspirated nucleated cells) (Murphy, Moncivais and Caplan 2013) and clinical applications of MSCs require large numbers of cells. For example, approximately 1-20x10⁶ MSCs/cm³ construct are used for TERM applications in bone and/or cartilage regeneration (Labibzadeh, et al. 2016) (Al-Najar, et al. 2017) (Emadedin, et al. 2017) (Gjerde, et al. 2018) (Dilogo, et al. 2019) (Rojewski, et al. 2019). This cellular expansion is carried out either in conventional 2D cultures or in bioreactor-based culture systems that closely monitor and tightly control the principal culture parameters (e.g., pH, nutrient supply, and dissolved oxygen).

Glucose metabolism is central to MSC expansion. It acts as an energy source for ATP generation and a major source of carbon for lipid, nucleotides, and non-essential amino acids, which are required for the biosynthesis of macromolecules to generate daughter cells. The contribution of glycolysis to ATP generation in proliferating MSCs varied from 67% (Pattappa, et al. 2011) to 97% (Fillmore, et al. 2015). To match their metabolic needs, proliferating MSCs have to switch from a glycolytic metabolism (in quiescent MSCs) to a mixed metabolism of OXPHOS and glycolysis (in proliferating MSCs). This metabolic remodeling is reflected by the change in the yield of lactate from glucose ($Y_{lac/glc}$) during MSC expansion which decreased from 2 mol/mol (suggesting anaerobic glycolysis) at day 0 to 3 to 1 mol/mol (indicating a combination of anaerobic glycolysis and OXPHOS) at later time points of MSC expansion (Schop, et al. 2008).

Thorough knowledge of the factors that influence MSC glucose requirements (measured as the cell-specific consumption rate of glucose (qGlc)) is required for achieving smooth continuous *in vitro* MSCs expansion. The qGlc depends on the biochemical and biophysical milieu parameters and the species from which the MSCs are isolated. Firstly, the qGlc in proliferating MSCs is affected by basal culture media choice (Chen, et al. 2015). For instance, qGlc of hMSCs expanded in α MEM-based medium ($6.6 \pm 1.2 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) was lower than in DMEM medium ($19.1 \pm 1.1 \mu\text{mol}/10\text{E}6 \text{ cells/day}$). Secondly, the qGlc in proliferating MSCs is influenced by oxygen tension. Thus, hMSCs expanded at 0,1% oxygen tension consume more glucose than hMSCs expanded at 20% oxygen tension (2 versus 1.5 mM per well per day) (Mischen, et al. 2008). Along the same line, qGlc of hMSCs expanded at 2% oxygen tension ($14\text{-}17 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) was consistently higher than the one of hMSCs expanded at 20% oxygen tension ($12 - 14 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) from the beginning of the culture to day 7 but became similar after that (Dos Santos, et al. 2010). Surprisingly, culturing hMSCs at 2% oxygen tension resulted in lower $Y_{\text{lac/glc}}$ than culturing them at 20% oxygen tension ($2.0\text{-}/+0.1$ versus $2.4\text{-}/+0.5 \text{ mol/mol}$) (Dos Santos, et al. 2010). Thirdly, the qGlc in proliferating MSCs is also correlated with the growth rate. Goat MSCs that grow at a high rate during the exponential phase consume more glucose per cell than goat MSCs that grow at a lower rate during the stationary growth phase (D. Schop, et al. 2009). These changes in qGlc are probably accompanied by a metabolic shift from anaerobic glycolysis to OXPHOS, as suggested by the progressive decrease of qGlc over time. Fourthly, qGlc of hMSCs expanded in a microcarrier-based bioreactor was slightly lower than in conventional planar culture (4.3 ± 0.7 versus $6.6 \pm 1.2 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) (D. Schop, et al. 2008), suggesting the metabolic difference between 3D and 2D cell culture. Fifthly, qGlc by proliferating MSCs also depends on the species from which the MSCs are isolated. Thus, metabolic analysis revealed that hMSCs during the exponential cell growth phase under a 20% oxygen tension exhibited a higher qGlc ($9.2 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) than rat ($5.9 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) and goat ($2.6 \mu\text{mol}/10\text{E}6 \text{ cells/day}$) MSCs (D. Schop, et al. 2009) (Dos Santos, et al. 2010).

An additional important factor when addressing the *in vitro* MSC expansion is the upper glucose concentration threshold impacting MSC proliferation negatively. This factor depends on the duration of MSC exposure to glucose. Thus, glucose concentrations of up to 25 mM did not influence hMSC proliferation in short-term-culture (4 days) whereas glucose concentrations of 25 mM resulted in a slight but significant decrease in

proliferation compared to 5,6 mM glucose in long-term culture (4 weeks) (Li, et al. 2007). Similarly, another study reported that hMSC cultured at 25 mM glucose exhibited a time-dependent (7, 14, 21, and 28 days) decrease in proliferation evidenced by reduction in cumulative population doubling and BrdU incorporation (Chang, Hsu and Wu 2015). Caution should, however, be exercised in interpreting these data as 4 week-exposure of hMSCs to 40 mM glucose concentrations did not have any effect on MSC proliferation (Li, et al. 2007). Moreover, the glucose concentration threshold is species-dependent. Thus, in contrast to hMSCs, mouse (Wang, et al. 2013) and rat (Chen, et al. 2017) MSCs proliferate optimally at 25 mM glucose. Finally, in addition to a direct cytotoxic- or hyperosmolarity-mediated impact of glucose on MSC proliferation, glucose may impact cell proliferation through lactate release. The growth-inhibiting concentration of lactate is species-dependent (*i.e.*, 35.4, 16 and 28.4 mM for hMSCs, rat- and goat-MSCs, respectively) (D. Schop, et al. 2009).

4.2 The Role of Glucose Metabolism in MSC Viability Post-Implantation

Substantial evidence demonstrates that the massive MSC death post-implantation is a major roadblock for unraveling the full potential of MSC-based therapy (Becquart, et al. 2012) (Logeart-Avramoglou, et al. 2010) (Manassero, Paquet, et al. 2016) (Dégano, et al. 2008) (Haider and Ashraf 2008) (Tögel, et al. 2008). Excellent review articles have been published describing the different factors involved in MSC death post-implantation, such as nutrient deprivation and hypoxia, increased cytokine production in the wound milieu, enhanced reactive oxygen species and oxidative stress (Becquart, et al. 2012). Here, we will focus on aspects related to the metabolic needs of MSCs.

Post-implantation MSCs face an ischemic environment characterized by low oxygen tension and either low concentration or lack of nutrients (Becquart, et al. 2012). Historically, oxygen has been considered the main culprit in this process of cell death for three main reasons: (i) it is a poorly diffusible molecule; (ii) the literature is replete with statements suggesting its prominent role in the death of MSCs (Chen, et al. 2018) (Das, et al. 2010) (Potier, et al. 2007); (iii) oxygen tension in wounds and tissue constructs devoid of a pre-existing vascular network is low (respectively 0,2% and 0,1%) (Deschepper, Oudina, et al. 2011) (Moya, Paquet, et al. 2018). However, recent research provided a shift of paradigm and demonstrated that the lack of glucose (but not oxygen) is responsible for this massive MSC death post-implantation (Moya, et al. 2018)

(Deschepper, Oudina, et al. 2011) (Deschepper, et al. 2013) (Nuschke, et al. 2016) (Mylotte, et al. 2008).

In vitro studies that delineate the pivotal role of glucose metabolism on MSC survival were carried out under near-anoxia ($pO_2 \leq 0,2\%$) to reflect the *in vivo* milieu experienced by implanted hMSCs (Moya, et al. 2018). In this near anoxia model, glucose deprivation induced massive death of sheep MSCs whereas glucose supply resulted in the maintenance of sheep MSC viability until the end of the experiment (for up to day 12 of culture). These results were confirmed with hMSCs over a period of up to 21 days *in vitro* (Moya, et al. 2018) (Deschepper, et al. 2013). The use of glutamine or serine as alternative energy substrates to glucose resulted in massive hMSC death in near anoxia as soon as day 3 underscoring the need of glucose for hMSCs to survive. Interestingly, in the presence of pyruvate, more than 60% hMSCs remained viable at day 7, but cell viability drastically decreased at day 14 (Moya, et al. 2018). This critical role of glucose was further confirmed by the gene expression profiles of metabolic enzymes (Moya, et al. 2018) which revealed for the first time that glycolysis (not pentose phosphate, TCA cycle, or glycogenolysis pathways) is the only energy-related pathway involved in hMSC survival under near-anoxia (Moya, et al. 2018). These results were further confirmed by addition of inhibitors of glycolysis (either 2DG or NaOx) which induced a rapid and drastic decrease in cell viability. these *in vitro* studies also allow to determine that hMSCs have minimal glycolytic reserves. In fact, hMSCs loaded into fibrin hydrogels and implanted subcutaneously exhibited a rapid decrease (66%) of their glycolytic reserves during the first 24 hours post-implantation. This was paralleled by a rapid decrease in their ATP content. Specifically, 40% of the ATP stock was lost within 24 hours, and 63% within 3 days; no more ATP was detected at day 7 post-implantation (Moya, et al. 2018). These results are also consistent with the observations that hypoxia or serum deprivation preconditioning of MSCs induced metabolic adaptation by either decreased glucose consumption or increased glucose stock, possibly improving cell retention *in vivo*, since glucose was available to MSCs for longer term (Beegle, et al. 2015) (Zhu, et al. 2014).

It is noteworthy that, apart from ATP depletion, absence/decrease of glycolysis as a result of glucose deprivation also induces the loss of crucial anti-apoptotic signals such as AKT (Salazar-Noratto, et al. 2019) (Rathmell, et al. 2003) (Majewski, et al. 2004). Moreover, decreased glucose metabolism due to glucose deprivation represses the generation of many biosynthetic precursor molecules, including nucleic acids, fatty acids,

and acetyl-CoA for acetylation (Wellen, et al. 2009); as well as inhibits proper glycosylation and protein folding in the endoplasmic reticulum (Kaufman, et al. 2010). There is also evidence that glucose deprivation as a metabolic stress activates the cellular process autophagy as a protective mechanism to mitigate damage and provide nutrients for short-term survival (Bernales, McDonald and Walter 2006) (Altman and Rathmell 2012).

Altogether, these findings challenge the current paradigm that the massive cell death observed upon implantation is ascribed to oxygen depletion *per se*. They provide clear evidence that MSCs can withstand exposure to severe, continuous near anoxia, providing that a glucose supply is available to fuel ATP production via glycolysis and meet the high energy demands of MSCs (Figure 4-1). This is best illustrated by the four-to-five-fold increase in the number of viable hMSCs in constructs enriched with glucose compared to that of glucose-free constructs, 14 days after implantation in an ectopic mouse model (Moya, et al. 2018). Taken together, these data establish that the presence of glucose in engineered constructs is an essential and missing piece to the puzzle of MSC survival post-implantation and prompt for the development of glucose-enriched scaffolds to enable MSCs to cross the bioenergetic “valley of death” between the time of implantation and the arrival of the new blood vessels.

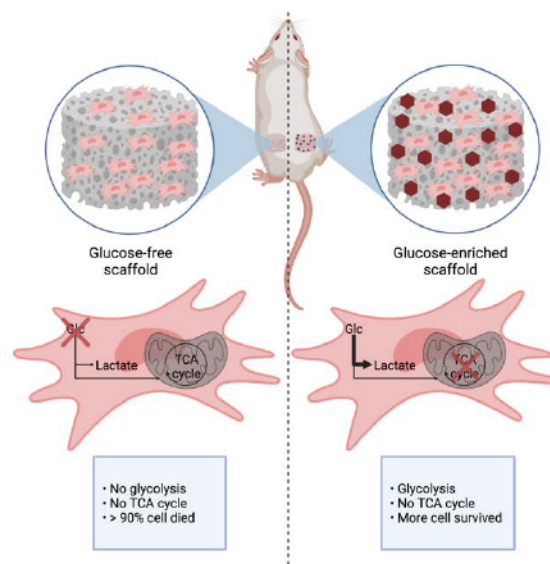


Figure 4-1 Schematic illustration of positive effects of glucose on MSC survival post-implantation. MSCs seeded into glucose-free scaffolds encounter the switch off of glycolysis and TCA cycle leading to massive and rapid cell death, while MSCs seeded into glucose-enriched scaffolds enhances cell survival relying on glycolysis. (Created with biorender.com)

4.3 The Role of Glucose Metabolism in MSC Differentiation

Differentiation is a highly regulated sequence of events that are tightly linked to the cell metabolic activity. MSC metabolism is highly sensitive to extracellular cues, and both oxygen tension and glucose availability play large roles in differentiation (Salazar-Noratto, et al. 2019). We will hereby focus on the impact of glucose on MSC differentiation towards osteogenic and chondrogenic pathways. MSCs undergo either endochondral ossification or intramembranous ossification, and their metabolic activity manages and controls the differentiation pathway as well as the quality of the end-product (differentiated tissue) in terms of amount of mineral deposition.

4.3.1 Endochondral Ossification

Endochondral ossification occurs in three major steps: first, MSCs differentiate into chondrocytes and form a cartilaginous template, which later mineralizes as chondrocytes mature to hypertrophy; next, the cartilaginous template is replaced by *de novo* bone formed by invading osteoprogenitor cells from the newly formed vasculature. The quality of newly-formed bone depends on the quality of the differentiated chondrocytes at the tissue regeneration site (Poulet, et al. 2016) (Zhao, et al. 2013). MSCs undergoing chondrogenic differentiation *in vitro* using the “pellet culture method” significantly reduce their oxygen consumption after 24 hours in culture and shift towards a predominantly glycolytic metabolism (Pattappa, et al. 2011) (Chen, et al. 2008) (Wang, et al. 2005). During chondrogenesis, the MSC energy metabolism primarily (89%) depends on glucose metabolism for the total ATP production (Pattappa, et al. 2011). This change in bioenergetics is not surprising since it is similar to that of chondrocytes, which have a high level of glycolysis with minimal oxygen consumption in 3D *in vitro* cultures (Pattappa, et al. 2011) (Rajpurohit, et al. 1996) (Heywood and Lee 2008). In the past, this phenomenon was associated with an adaptation to the 3D pellet culture conditions rather than with chondrogenesis itself (Pattappa, et al. 2011); however, recent research demonstrated that specific chondrogenic signals modulate bioenergetics towards glycolysis and away from oxidative phosphorylation. For example, treatment of murine primary articular chondrocytes with transforming growth factor (TGF)- β 1 realigned the ATP production dependency on glycolysis (and away from mitochondrial respiration), and upregulated several key glycolytic genes, including Glut1, Hexokinase II (HKII),

Pyruvate Kinase M2 (PKM2) and Lactate deshydrogenase A (LDHA) (Wang, Shen and O'Keefe 2009). Moreover, genetic deletion of the glucose transporter Glut1 severely impairs chondrocyte proliferation and hypertrophy *in vivo* (Lee, Abel and Long 2018). These observations provide evidence of the tightly knit relationship between chondrogenesis and glucose metabolism.

Because MSCs undergoing chondrogenic differentiation *in vitro* and *in vivo* predominantly rely on glucose metabolism for their bioenergetics, glucose availability significantly affects the cell differentiation process. In the presence of high levels of glucose (from 20.4 mM to 25 mM), chondrogenic differentiating MSCs and mature chondrocytes increase their glucose intake and improve chondrogenesis, as quantified by the quality of the extracellular matrix (Heywood, Bader and Lee 2006) (Zhong, et al. 2016). In addition to serving as an energy substrate, glucose also plays a key role in cell functions pertinent to the synthesis of chondrogenic extracellular matrix components such as a precursor to GAGs, glycoproteins, and glycolipids (Wang, Shen and O'Keefe 2009) (Mobasheri, et al. 2006) (Mobasheri, Glucose: An energy currency and structural precursor in articular cartilage and bone with emerging roles as an extracellular signaling molecule and metabolic regulator 2012). Chondrogenic differentiating MSCs exhibited almost a 2.5-fold increase in GAG production when cultured in medium supplemented with 25.2 mM than with 5.6 mM glucose (Zhong, et al. 2016). Similarly, chondrocytes cultured in medium supplemented with 20.4 mM (versus 5.5 mM) glucose yielded a 73% increase in GAG production (Heywood, Bader and Lee 2006).

4.3.2 Intramembranous Ossification

Intramembranous ossification is the direct differentiation of MSCs to osteoblasts, the bone-forming cells. Unlike chondrogenesis, MSCs undergoing osteogenesis *in vitro* increase, or at the very least maintain, their oxygen consumption and suppress glycolysis (Pattappa, et al. 2011) (D. Schop, et al. 2008). Evidence for the decreased glycolytic activity are (1) the observed progressive reduction in lactate production during MSC osteogenesis, compared with that obtained from non-differentiating MSCs (Pattappa, et al. 2011) (Chen, et al. 2008), and (2) the decreased expression of glycolytic enzymes, such as glucophosphate isomerase (GPI) and phosphofructokinase (PFK), in osteogenic MSC cultures compared with non-differentiating, expanding MSCs (Chen, et al. 2008).

Even when glycolysis is suppressed during MSC osteogenesis, glucose metabolism still plays a key role in the differentiation process of these cells. For example, Wnt signaling is a major mechanism involved in bone formation, and it does so in part through stimulating glucose metabolism in osteoblast lineage cells. *In vitro* and *in vivo*, Wnt7b depends on the glucose transporter Glut1 and on glucose consumption to promote bone formation (Chen, et al. 2019). Runt-related transcription factor 2 (Runx2), a master transcription factor in regulating osteogenic differentiation of MSCs, is required for the induction of major bone matrix genes in immature osteoblasts (Komori 2010). One recent study demonstrated that the feedforward regulation between Runx2 and Glut1 facilitates the initiation of osteoblast differentiation (Wei, et al. 2015) (Chen, et al. 2016). Nevertheless, high glucose (from 15.5 mM to 35 mM) may have a negative impact on the osteogenic differentiation of MSCs, by suppressing mineralization (Wang, et al. 2013) (W. Wang, et al. 2010) and, instead, promoting adipogenesis (W. Wang, et al. 2010). In fact, MSCs cultured under high (25 mM) glucose osteogenic conditions have downregulated expressions of BMP2 and Runx2, resulting in suboptimal extracellular matrix formation (Wang, et al. 2013) (Chen, et al. 2017). There is recent evidence that demonstrates that MSCs cannot efficiently downregulate glucose transporters, and thus glucose intake, in the excess of glucose. Normoglycemic (5 mM) and hyperglycemic (25 mM) culture of human placental amnion-derived MSCs did not change the expression of Glut1, Glut3 and Glut4 protein 24 and 48 hours post-culture (Unek, et al. 2020). For all the aforementioned reasons, changes in exogenous glucose may significantly affect MSC metabolism and, thus, cell differentiation.

4.4 The Role of Glucose Metabolism in MSC-Mediated Paracrine Functions

In addition to their capability to differentiate into constituent cells of several tissues, MSCs also possess a regenerative capacity through the secretion of biologically active molecules, collectively referred to as the “secretome”. This secretome, which contains growth factors, cytokines, lipid mediators, cell-adhesion chemical compounds, exosome, microvesicles, etc., plays critical roles in various biological processes such as pro-angiogenesis, anti-apoptosis, anti-fibrosis, immunomodulation, etc. (L., et al. 2019). Cues from the extracellular microenvironment including soluble factors and physical factors (such as ECM composition, geometry of scaffold, and oxygen tension) are of critical importance to direct MSC-mediated paracrine activity (G. D. Kusuma, et al. 2017).

Interestingly, some differences in the metabolite dynamics (Caseiro, et al. 2019) (particularly on glucose metabolism), accompanying different secretome profile, have been observed when conditioned MSCs derived from the umbilical cord stroma and from the dental pulp were used. We will hereby focus on the impact of glucose on MSC-mediated angiogenesis and immunomodulation.

4.4.1 MSC mediated-angiogenic properties

Conditioned media harvested from MSCs improved the healing of ischemic tissue via the secretion of bioactive, pro-angiogenic growth factors, including VEGF (Potapova, et al. 2007) (Burlacu, et al. 2013) (Bhang, et al. 2004) (Kuljanin, et al. 2017) (Yang, et al. 2018), FGF-b (Caseiro, et al. 2019) (Potapova, et al. 2007) (Bhang, et al. 2004) (Kuljanin, et al. 2017) (Paquet, et al. 2015), HGF (Caseiro, et al. 2019) (Bhang, et al. 2004) (Paquet, et al. 2015), PDGF-BB (Caseiro, et al. 2019), and monocyte chemoattractant protein 1 (MCP-1) (Caseiro, et al. 2019) (Paquet, et al. 2015).

Recent studies by our research team have demonstrated that glucose plays a critical role in MSC-mediated angiogenic paracrine functions. Conditioned media harvested from hMSCs cultured in the presence of either 5.6 or 33 mM glucose under near-anoxia (0.1% pO₂) conditions for 3 days increased human umbilical vein endothelial cell (HUVEC) migration for up to 36 hours when compared to results obtained using supernatant conditioned media collected from hMSCs cultured without glucose (Luo, et al. 2020). The increased HUVEC migration was corroborated by increased amounts of several pro-angiogenic factors, specifically, Angiogenin, VEGF-A, VEGF-C, Angiopoietin-1, Endostatin, and CCL2 (Luo et al., 2020 ORS). Importantly, implanted hMSC-containing hydrogels loaded with glucose exhibited an increase in the volume of newly-formed blood vessels when compared to hMSC-containing hydrogels without glucose, respectively. The volume of newly-formed blood vessels in cell-containing hydrogels without glucose and in cell-free hydrogels loaded with glucose were similar and minimal, indicating that the angiogenesis is MSC-driven and this effect is minimal in the absence of glucose (Luo, et al. 2020). Furthermore, delivery glucose to MSCs via hydrolysis of starch (a polymer of glucose) significantly increased MSC-mediated angiogenesis, including more HUVECs migration and tubular structure formation *in vitro* and higher volume of newly-formed blood vessels *in vivo* (Denoëud, Paquet, et al. 2019).

To date, the underlying mechanism(s) regarding how glucose regulates MSC-mediated angiogenesis remains partially elusive. In fact, glucose regulates HIF-1 (one of the major regulators of MSC-mediated angiogenesis) expression and/or stability in several cell types including primary dermal fibroblast (Catrina, et al. 2004), endothelial cell (Catrina, et al. 2004) (Kikuchi-Taura, et al. 2020), primary cortical neurons (Guo, et al. 2008), and cancer cells (Vordermark, et al. 2005) (Nishimoto, et al. 2014) (Kwon and Lee 2005) (Liu, et al. 2013). Deschepper et al. reported that glucose increased HIF-1 α expression and bioactivity in hMSCs cultured under near-anoxia (0.1% pO₂) (Deschepper, et al. 2013). Such regulation of HIF-1 α is a plausible molecular mechanism for explaining the role of glucose on MSC-mediated angiogenesis. Recently, glucose transfer via gap junction-mediated cell-cell interaction has been reported as an alternative mechanism in angiogenesis (Kikuchi-Taura, et al. 2020) (Kikuchi-Taura, Okinaka and Saino, et al. 2021). MSCs mainly uptake glucose from the extracellular milieu, while a recent study observed the uptaken glucose in HUVEC was transferred to mMSCs via gap junction-mediated cell-cell interaction (Kikuchi-Taura, Okinaka and Saino, et al. 2021). A similar study also observed the uptaken glucose in bone marrow mononuclear cells was transferred to HUVECs via gap junction-mediated cell-cell interaction with an increase of HIF-1 α activation and an increase in VEGF uptake leading to an increased angiogenesis at ischemic tissue location (Kikuchi-Taura, Okinaka and Takeuchi, et al. 2020). Collectively, these data prompt for further investigations to determine whether the transferred glucose between HUVEC and MSCs regulates MSC-mediated angiogenesis through HIF-1 α activation. In addition, many pertinent questions (such as whether glucose regulates MSC-mediated angiogenesis directly or indirectly through improved MSC survival followed by increased release of pro-angiogenic secretome) remain unanswered

4.4.2 MSC mediated-immunomodulatory properties

The immunomodulatory properties of transplanted MSCs in response to stimuli from the recipient bed are crucial in providing anti-inflammatory signals favoring tissue regeneration. These MSC-driven signal cascades favor macrophage polarization from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype, inhibit maturation and activation of antigen-presenting dendritic cells, reduce proliferation of B cells, inhibit proliferation of conventional T cell, and induce expansion of regulatory T cell (M.

Krampera 2011) (Bowles, et al. 2020). These immunomodulatory capabilities of transplanted MSCs are not constitutively expressed but depend on pro-inflammatory stimuli in a process known as ‘licensing’. In general, MSC licensing is induced through stimulation with either one or combined cytokines such as IL-1 β , Tumor necrosis factor (TNF)- α , and IFN- γ . Licensed MSCs then acquire the immunomodulatory properties by releasing a range of soluble chemical compounds such as IDO, PGE2, NO, IL-10, and programmed death ligand-1 (PD-L1).

Many recent studies have shown that glucose plays a crucial role in the immunomodulatory properties of MSCs in at least two ways. Firstly, a bioenergetic switch towards enhanced aerobic glycolysis occurs during the process of MSC licensing (Liu, et al. 2019) (Böttcher, et al. 2016) (Jitschin, et al. 2019) (Strauch, et al. 2020). IFN- γ individually or combining TNF- α licensed hMSCs displayed increased aerobic glycolysis evidenced by increased surface expression of GLUT1, increased mRNA expression of several key glycolytic genes (such as enolase 1 (ENO1), pyruvate dehydrogenase kinase (PDK) 3, and HK2), higher glucose consumption, and higher extracellular acidification rate (ECAR) (Liu, et al. 2019) (Jitschin, et al. 2019). A gene-set enrichment analysis suggested that the IDO expression was correlated with glucose and pyruvate metabolism (Jitschin, et al. 2019). Additional studies established that glucose metabolism via glycolysis, and not pyruvate metabolism, is responsible for IDO production and the immunosuppressive properties of hMSCs (Liu, et al. 2019). More specifically, when IFN- γ licensed hMSCs were cultured in media containing various concentration of glucose and pyruvate, the addition of 2 mM sodium pyruvate alone did not increase IDO activity (compared to pertinent results obtained with glucose-free and pyruvate-free media). In contrast, addition of glucose at either 1, 2, or 20 mM, in the absence of pyruvate, significantly ($p < 0.05$) increased the IDO activity (Liu, et al. 2019). Moreover, an inhibition of glycolysis via 2-DG treatment impacted IDO activity, IDO protein and IDO mRNA levels, resulting in the inhibition of T cell proliferation. Similarly, IFN- β licensed MSCs exhibit an induction of their immunomodulatory properties via the long-lasting mTOR-associated promotion of glucose metabolism (Vigo, et al. 2019). Furthermore, compared to mMSC conditioned media containing 11.2 mM glucose, the mMSC conditioned media containing high 25.2 mM glucose concentration increased inhibition of T cell proliferation by boosting mMSC glycolysis (Vigo, et al. 2019). This observation indicates extracellular glucose concentration affects MSC metabolic reprogramming, thus MSC immunomodulatory properties. Secondly, protein glycosylation, including O-linked

N-acetylglucosamine (O-GlcNAcylation) and N-glycosylation, controls licensed MSC immunomodulatory properties. For example, exposing IFN- γ and TNF- α licensed hMSCs to 2-DG significantly ($p < 0.05$) reduced IFN- γ and TNF- α licensing-mediated induction of PD-L1 transcription, and abolished the upregulation of PD-L1 cell surface expression and release (Strauch, et al. 2020). The disruption of N-glycosylation of PD-L1, rather than glycolysis-inhibitory effects, were responsible for the 2-DG treatment as it prevented PD-L1 transportation to the human MSC surface and its subsequent release. Similarly, O-GlcNAcylation modification of STAT1, which depended on the glycolytic flux, controlled IDO1 mRNA and protein expression in licensed hMSCs (Jitschin, et al. 2019). The authors showed that IFN- γ and TNF- α licensing hMSC upregulated overall O-GlcNAcylation modification of signal transducer and activator of transcription (STAT)1, and blocking the glycolytic flux with 2-DG reduced O-GlcNAcylation of STAT1 and IDO1 levels together with the MSCs' T- cell suppressive capacity. These reductions of O-GlcNAcylation of STAT1 and IDO1 occurred when genetic silencing or pharmacologically blocking of O-GlcNAc transferase (OGT) (Jitschin, et al. 2019). In contrast, supplying hMSCs with GlcNAc, which increases O-GlcNAc protein modifications by enhancing the HBP flux, significantly rescued IDO1 upregulation during inhibition of glycolysis (Jitschin, et al. 2019).

Thus, upon pro-inflammatory cytokine licensing, glucose plays a crucial role in the MSC immunomodulatory properties due not only to a bioenergetic slanting towards increased aerobic glycolysis but also to important modification of glycosylation for production of immunomodulatory chemical compounds.

5 Endoplasmic Reticulum Stress and MSCs

The growing interest in the role of glucose metabolism in MSC survival and function has placed the focus on mitochondria, the cellular organelles in which cells generate energy through OXPHOS. Mitochondria are extremely dynamic organelles that communicate with other organelles, particularly the endoplasmic reticulum (ER), lipid droplets, and lysosomes. The issue is that the ER is another essential organelle that has received far less attention, despite the fact that the hostile ischemia milieu experienced by MSCs is a well-established stressor that induces ER stress in MSCs.

5.1 ER Stress and Unfolded Protein Response

The ER is an intracellular organelle where secreted proteins are synthesized and folded before they are processed through the secretory pathway. Only properly folded proteins are translocated to the Golgi apparatus, then are secreted into extracellular space via secretory vesicles. To prevent the potentially toxic accumulation of improperly folded proteins, the ER has a dynamic capacity to fold and process incoming proteins (Moore and Hollien 2012). The biosynthesis and folding of proteins in the ER represent an intensive energy-consumption process. Therefore, several cellular stressors that compromise the energetic status of cells, such as nutrient or oxygen deprivation, reactive oxygen species accumulation, or ER luminal Ca^{2+} depletion, can give rise to the accumulation of improper folding proteins in the lumen of the ER, and therefore induce ER stress. Cells cope with ER stress via initiating a series of adaptive mechanisms that together are termed as unfolded protein response (UPR). In mammals, the UPR is initiated by three main ER transmembrane sensors: the protein kinase RNA-like ER kinase (PERK) axis (Figure 5-1A), inositol-requiring enzyme-1 α (IRE1 α) (Figure 5-1B), and the activating transcription factor 6 (ATF6) (Figure 5-1C). These three master regulators sense and interpret protein folding status in the ER and transmit this information across the ER membrane to regulate downstream effectors (Ghosh, et al. 2010). Activation of the UPR helps to restore protein-folding homeostasis by affecting many aspects of the secretory pathway. In contrast, UPR signaling results in cell death if cell damage is sufficiently severe (Hetz, Chevet and Harding 2013) (Tabas and Ron 2011). Molecular insight into these three UPR pathways has been reviewed extensively elsewhere and will be briefly outlined below.

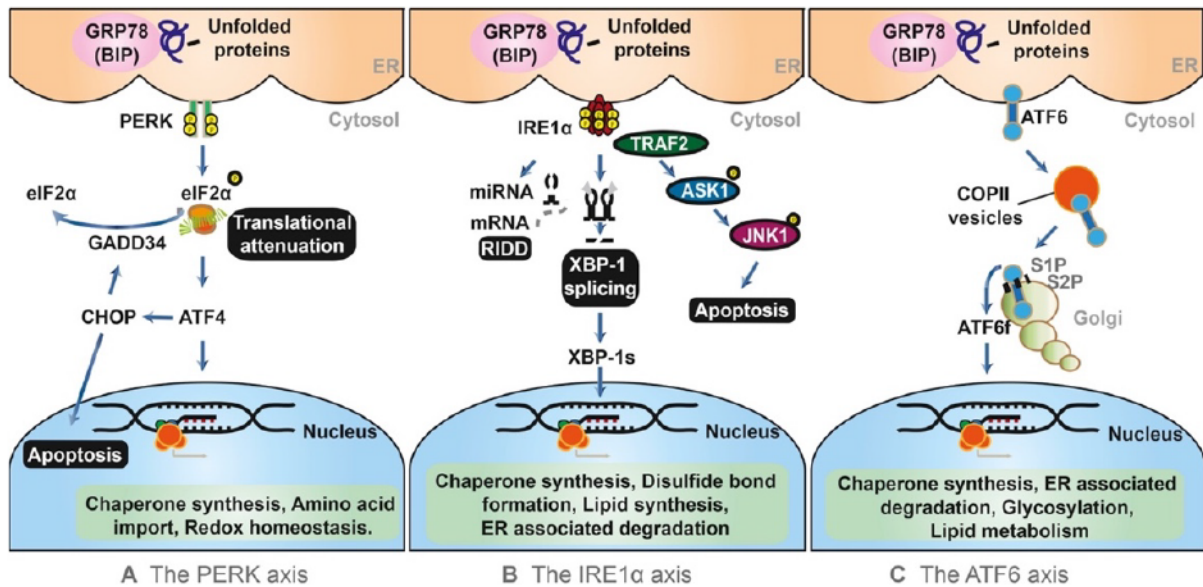


Figure 5-1 Pathways of the unfolded protein response. Image from (Binet, François and Sapieha, Przemyslaw, 2015)

5.1.1 UPR Signaling through PERK

Activation of PERK involves its dimerization and trans-autophosphorylation. Activated PERK inhibits general protein translation through the phosphorylation of eukaryotic translation initiator factor-2 (e-IF2 α). This transient inhibition of protein synthesis rapidly reduces the amount of proteins entering the ER, thus is important to promote cell survival (Harding, et al. 2000). Concomitantly, phosphorylation of eIF2 α initiates the translation of mRNAs harboring short open reading frames in their 5'-untranslated region, such as activating transcription factors 4 (ATF4). ATF4 regulates the expression of genes that encode proteins involved in redox homeostasis, amino acid metabolism, and ER chaperones and foldases (Harding, Zhang, et al. 2003) (Lange, et al. 2008) (Hetz, Chevet and Harding 2013). ATF4 also controls the expression of important genes involved in apoptosis, such as the transcription factor C/EBP-homologous protein (CHOP) and growth arrest and DNA damage-inducible 34 (GADD34). In addition, GADD34 participates in a feedback loop to dephosphorylate eIF2 α to restore protein synthesis through interacting with protein phosphatase 1C (PP1C) (Novoa, et al. 2001) (Hetz, Zhang and Kaufman 2020) (Han, et al. 2013).

5.1.2 UPR Signaling through IRE1 α

The activation of IRE1 α is similar to PERK, which also involves dimerization, oligomerization, and trans-autophosphorylation to activate its RNase domain (Zhou, et al.

2006). The activated IRE1 α excises a small 26-nucleotide intron from the mRNA that encodes the transcription factor X-box binding protein 1 (XBP1), which shifts the coding reading frame (Yoshida, et al. 2001) (Calton, et al. 2002) (Shen, et al. 2001). This leads to the expression of a more stable and active XBP1 which is known as XBP1s for the spliced form. XBP1s transactivates many target genes related to ER-associated protein degradation, protein folding, protein translocation to the ER, and protein secretion (Acosta-Alvear, et al. 2007) (Lee, Iwakoshi and Glimcher 2003). Activated IRE1 α also participates in a process known as regulated IRE1 – dependent decay (RIDD), in which IRE1 α cleaves a subset of ER-associated mRNAs or premature microRNAs, leading to their degradation (Hollien and Weissman 2006). Thus, RIDD lowers mRNA abundance and hence protein folding burden in the ER. This additional output of IRE1 has an important biological function in the control of inflammation, apoptosis, and glucose metabolism (Maurel, et al. 2014) (Hetz and Papa 2018). In addition, the IRE1 α cytosolic domain interacts with adaptor proteins, such as tumor necrosis factor receptor-associated factor2 (TRAF2), to undergo crosstalk with inflammatory response pathways (Urano, et al. 2000) (Hu, et al. 2006) (Binet and Sapieha 2015).

5.1.3 UPR Signaling through ATF6

ATF6 is a type II ER transmembrane protein that contains a basic leucine zipper (bZIP) transcription factor on its cytosolic domain (Hetz and Papa 2018). ER stress triggers dissociation of glucose-regulated protein 78 (GRP78) from ATF6. This leads to ATF6 translocates to the Golgi apparatus where it is cleaved by site-1 protease (S1P) and S2P protease to release its cytosolic domain, ATF6f (Haze, et al. 1999) (Asada, et al. 2011) (Hetz, Chevet and Harding 2013). ATF6f is a bZIP transcription factor that is relocated to the nucleus to regulate the expression of select UPR target genes (Yamamoto, et al. 2007) (Lee, et al. 2002) (Ye, et al. 2000). Interestingly, ATF6f exhibits crosstalk with XBP1s to regulate transcription of genes that encodes ER chaperones and enzymes promoting ER protein translocation, folding, maturation, secretion, and degradation of misfolded proteins (Yamamoto, et al. 2007) (Hassler, et al. 2015) (Wu, et al. 2007) (Shoulders, et al. 2013). Moreover, ATF6f and XBP1s promote ER and Golgi apparatus biogenesis to enhance the secretory capacity of cells upon ER stress (Bommiasamy, et al. 2009) (Sriburi, et al. 2004) (Shaffer, et al. 2004) (Hetz, Zhang and Kaufman 2020). Overall, the UPR signaling pathways operate as a complex network that restores ER

proteostasis and preserves cell function under ER stress by adjusting the ER folding capacity in a dynamic manner.

5.2 ER Stress-Mediated MSCs Apoptosis

A prolonged ER stress induced by an ischemic environment has been proposed to affect MSC survival post-implantation. It has been confirmed by *in vitro* studies that BM-MSCs cultured under conditions of oxygen, glucose, and serum deprivation exhibited higher expression of ER stress markers, such as p-PERK, GRP78, ATF4, and CHOP, as well as higher apoptotic rate. In the same study, silencing CHOP expression significantly reduced the apoptotic rate of MSCs. In addition, oxidative stress, another feature of the ischemic environment, has also been demonstrated to promote MSC death by inducing ER stress.

Ample evidence indicates that UPR signaling integrates information about the duration and the intensity of the stress stimuli to restore protein-folding homeostasis or induce cell death. Hence, when the capacity of UPR fails to restore ER proteostasis, UPR signaling continues to persist and ultimately induces cell death (Shore, Papa and Oakes 2011). PERK and IRE1 α have been proved to upregulate a distinct set of pro-apoptotic genes that contribute to cell death if ER stress cannot be resolved. For example, PERK hyperactivation can upregulate the expression of CHOP transcription factor, which promotes cell death by inhibiting anti-apoptotic genes but stimulating pro-apoptotic genes (Urrea, et al. 2013) (Puthalakath, et al. 2007). In addition, GADD34 is a downstream target of CHOP, which resumes protein synthesis in stressed cells, thus sensitize cells to cell death (Marciniak, et al. 2004). Similarly, when under chronic ER stress, hyperactivation of IRE1 α contributes to cell death through two distinct mechanisms: 1) activation of apoptosis signal-regulating kinase 1 (ASK1) (Kim, et al. 2009) and JUN N-terminal kinase (JNK)1 (Urano, et al. 2000) by interacting with TRAF2, and 2) sustained RIDD causing massive decay of hundreds of ER-localized mRNAs to depletes ER cargo and protein-folding components (Hollien and Weissman 2006) (Han, et al. 2009) (Hetz and Papa 2018). Moreover, the differential kinetics of activation and attenuation of stress sensor proteins has been proposed as one of the mechanisms underlying the shift of UPR signaling from adaptive to pro-apoptotic responses (Hetz, Chevet and Harding 2013). For example, the IRE1 α is switched off in cells upon chronic ER stress, whereas the PERK pathway is sustained conditionally (Hetz, Chevet and Harding 2013) (Lin, et al. 2007)

(Lin, Li and Zhang, et al. 2009). This leads to ablate the protective effects of XBP1s expression and promote the expression of ATF4 and CHOP/GADD34 (Hetz, Chevet and Harding 2013).

5.3 ER Stress and Angiogenesis

In recent years, there has been an increasing amount of literature indicating that the highly evolutionarily conserved pathways of UPR, which are either triggered secondary to perturbations of ER or independently, can regulate vascular growth (Karali, et al. 2014) (Lenna, Han and Trojanowska 2014) (Maamoun, et al. 2019) (Paridaens, et al. 2014).

Several studies have revealed a role of UPR signaling in vascular development using genetic loss-of-function studies of various stress sensors. For example, IRE1a-inactivation embryos exhibit widespread and severe developmental deficiencies and generally die after 12.5 days of gestation. This embryonic death thought to occur through reduced expression of placental VEGF and severe dysfunction of the labyrinth in the placenta, a highly developed tissue of blood vessels (Zhang, et al. 2005) (Iwawaki, et al. 2009). In the same vein, XBP-1-deficient mice display stalled retinal vascularization and impaired the angiogenesis triggered by ischemia. (Zeng, et al. 2013). Further studies indicate that transcription factors from all three branches of UPR, such as ATF4, sXBP-1, and ATF6f, have consensus sites on VEGF promoter and have been shown to bind and upregulate its expression (Ghosh, Lipson, et al. 2010). In addition to VEGF, UPR signaling also contribute to regulate other factors modulating angiogenesis, such as IL-8, FGF-b, angiogenin, MMP2, and MMP9 (Gargalovic, et al. 2006) (Li, et al. 2003) (Pereira, et al. 2010), thus UPR can contribute to angiogenesis by increasing synthesis of pro-angiogenic factors.

However, it is important to bear in mind that the UPR induces cell death under chronic, severe ER stress, thus negatively affecting angiogenesis. Under chronic, severe ER stress, sustained hyperactivation of UPR induces cell death via increasing expression of CHOP/GADD34 or/and RIDD activity (Gómora-García, et al. 2021) (Marciniak, Yun, et al. 2004) (Oyadomari and Mori 2004) (Tabas and Ron 2011). Similarity, ER stress can also drive an anti-angiogenic response associated to the expression of CHOP and RIDD activity. For example, postischemic neovascularization is increased in CHOP-deficient mice with surgically induced hind-limb ischemia, suggesting that under chronic and/or severe ER stress, CHOP drives an anti-angiogenic response (Loiuard, et al. 2012). In

addition, mild ischemic retinal neurons produce netrin-1 to promote angiogenesis, but severe ischemic neurons trigger sustained activation of both PERK and IRE1 α pathways, and thereby IRE1 α pathway impair proper angiogenesis of ischemic retina through the RIDD activity degrading netrin-1 (Binet, Mawambo, et al. 2013). In view of all that has been mentioned so far, ER stress pathway modulate angiogenesis by either promoting vascular growth under mild stress conditions or inhibiting angiogenesis under prolonged and/or severe conditions (Figure 5-2). Therefore, this study offers some important insights into whether and how glucose regulates ER stress of hMSCs under ischemic conditions.

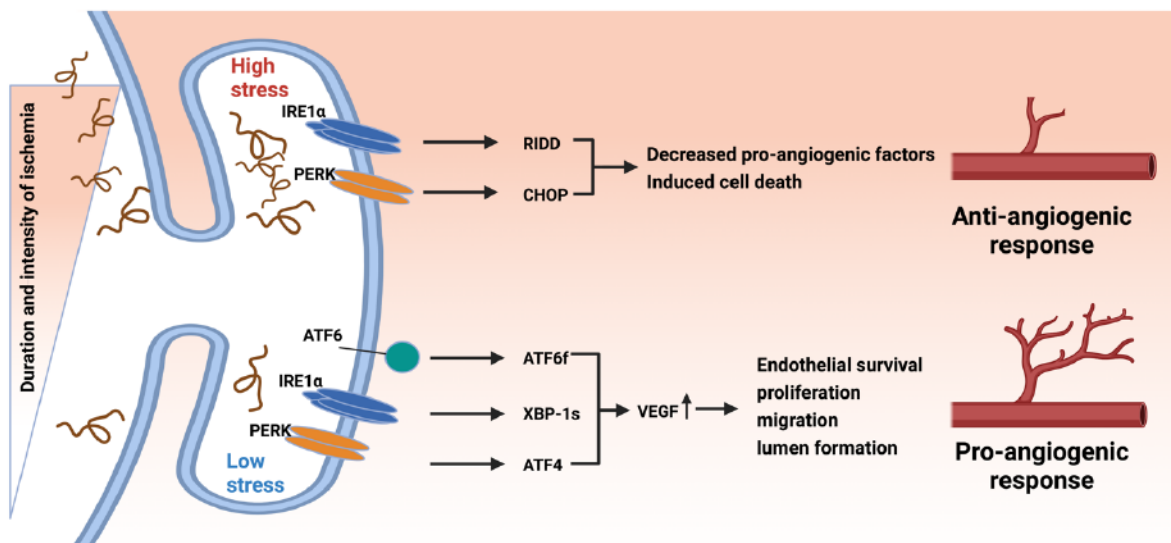


Figure 5-2 ER stress and angiogenesis. (Created with biorender.com)

6 Strategies to Improve MSC Functionalities

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In TE and regenerative medicine applications, strategies to mitigate cell death take precedence since increased cell survival is hypothesized to enhance the effectiveness of cell-based therapies. It is imperative that cells maintain at least a minimum energy status in order to carry out life-sustaining metabolic reactions and, therefore, survive. In order to maintain the necessary energy status in the compromised metabolic milieu of transplanted sites, scientists have pursued two main strategies: (i) providing cells with the substrates necessary in order to maintain their bioenergetics levels and (ii) modulating the metabolism in MSCs to promote cell adjustment to their new environment.

6.1 Providing MSCs with Nutrients and Oxygen

In the design of cell-containing scaffolds for TE, the scaffold architecture should be optimized to enable the mass transport of oxygen and vital nutrients necessary for the energy requirements of the transplanted cells. Because upon implantation, cell-containing scaffolds and the surrounding tissue are avascular, there is no convective mass transport. Consequently, the grafted cells rely mainly on the availability of nutrients, whose diffusion coefficients into the 3D material scaffolds are generally relatively low. In this context, the structural features of the scaffold are critical for optimal mass transport conditions (Karande, Ong and Agrawal 2004). Pore size, geometry, orientation, interconnectivity and branching, and channels may all affect diffusion. Because tissue regeneration and engineering specifications vary with specific tissue requirements, there is no consensus regarding the optimal structural parameters of material scaffolds. Recently, new technologies, such as additive manufacturing, or 3D printing, enable production of scaffolds with customizable macroscopic porous networks that were not possible before.

Nevertheless, focusing on designing scaffolds that improve mass transport of nutrients may not be the ideal or, at least, a sufficient approach considering that MSC-containing scaffolds are often implanted in ischemic environments and, thus, require extracellular nutrients to promote a regenerative response to the injured tissue. Consequently, research should refocus its effort to rationally engineer a new generation of cell-containing scaffolds that provide nutrients tailored to the needs of transplanted cells. Given that glucose (and not oxygen) is essential to ensure MSC survival via glycolysis, (Mylotte, et al. 2008) (Moya, et al. 2018) (Deschepper, et al. 2013) recent strategies have focused on delivering this chemical compound to cells. Reports by our research group have provided evidence that glucose-loaded scaffolds result in an increased expression of HIF-1 α by BM-hMSCs and in a fivefold enhancement of BM-hMSC survival rate post-implantation in an ectopic mouse model at day 14 (Deschepper, et al. 2013) In addition to short-term survival, it is essential that engineered scaffolds deliver the amount of glucose necessary to meet at least the minimum MSC energy requirements until newly formed vessels reach a density within the scaffold sufficient to provide cells with the needed nutrients. The goal is to aid transplanted cells survive until vascularization takes place. After all, in order to contribute to tissue regeneration via cell proliferation and

differentiation, cells cannot be in either a nutrient- or an oxygen-deprived environment; their bioenergetics should not operate at the minimum level. To this end, glucose delivery is a challenging endeavor because this molecule, which is uncharged and highly water-soluble, is rapidly released from scaffolds. Furthermore, increasing the concentration of glucose within scaffolds is not a viable strategy since glucose at high concentrations can disturb the osmotic pressure and cause cell lysis (Sharifi, et al. 2009). These challenges motivated our research group to develop an enzyme-controlled glucose-delivery hydrogel that uses starch, similar to that present in plants, to store large amounts of glucose while reducing the associated osmotic pressure (M. Deschepper 2016) (see full details below). In this novel system, the starch, which is osmotically inactive, is enzymatically converted to glucose by amyloglucosidase (AMG). Other research groups have proposed oxygen delivery systems, including oxygen-carrier scaffolds and oxygen-producing scaffolds (Cook, et al. 2015) (Lee, et al. 2015) (Newland, et al. 2018). The rationale of these studies was that oxygen is required to ensure MSC survival. However, based on recent data, which provide strong evidence that MSCs rely on glycolysis to survive in near-anoxia (Mylotte, et al. 2008) (Moya, et al. 2018) (Deschepper, Oudina, et al. 2011), the authors of this review propose another hypothesis; specifically, that supplying oxygen to MSCs enables flexibility in energetic substrate choice because oxygen-dependent energetic pathways become functional under these conditions. An additional positive effect of delivering oxygen to transplanted MSCs is that oxygen tension is a critical regulator in maintaining the stemness of MSCs and in determining their differentiation fate (review reference (Mohyeldin, Garzón-Muvdi and Quiñones-Hinojosa 2010)).

Although oxygen delivery systems have been largely explored, there are few studies concerning MSCs directly. Namely, Newland et al (Newland, et al. 2018) showed that coculturing oxygen-producing microspheres with hMSCs at near-anoxia (0.1% pO₂), with 0.05 mg/mL of glucose (contained in the fetal bovine serum), maintained cell viability up to day 4 *in vitro*. In a similar strategy, oxygen-loaded microspheres were embedded within polyprolactone constructs (termed oxygen microtanks) in order to enhance their oxygen delivery capability. *In vitro* coculture of oxygen microtanks with adipose-derived hMSCs at 0% pO₂ also maintained cell viability up to day 4, then drastically dropping off to approximately 20% viability at day 6 (Lee, et al. 2015). *In vivo*, the use of synthetic oxygen-carrier-enriched hydrogels for delivering murine mesenchymal stem-like C3H10T1/2 cells resulted in increased cell survival in an ectopic mouse model at day 3 but not at day 7 (Kimelman-Bleich, et al. 2009). In this study, delivery of oxygen did not

increase blood vessel volume density, number, or thickness (Kimelman-Bleich, et al. 2009). A possible explanation for this outcome is that delivery of oxygen may have temporarily “mised” transplanted cells, so that they did not reorient their paracrine functions appropriately toward an angiogenic response. Consistent with this hypothesis, MC3T3-E1 cells cultured on oxygen-releasing hollow particles exhibited a significant downregulation of both HIF-1 α and VEGF when compared with cells cultured on hollow particles alone at 1% pO₂ (Lee, et al. 2015). In contrast, conditioned medium from hMSCs exposed to near-anoxia led to a twofold increase in chemotaxis of human umbilical vein endothelial cells and to a threefold increase in the formation of vascular structures when compared with the results obtained using conditioned medium from hMSCs cultured at either 21% or 5% pO₂ (Paquet, et al. 2015). Taken together, these results provide evidence that, although delivering oxygen may enhance cell survival, it may also hinder angiogenesis and, thus, affect regeneration in some tissues. Nevertheless, further research is needed to provide mechanistic insights into the relationship among oxygen delivery, MSC survival, and angiogenesis, and to establish the importance of oxygen delivery as a strategy to improve cell survival post-transplantation. Oxygen delivery systems must be reassessed using MSCs, because different cell types require distinctly different energetic and biosynthetic pathways to support their specific functional needs. Last but not least, the field has become increasingly invested in developing vascularization techniques in order to enable in situ nutrient supply and cell metabolic waste removal by convective transport. Unfortunately, induced vessel ingrowth into the cell-containing scaffolds is often too slow to provide adequate, timely nutrient transport and availability to the cells in the core of 3D transplanted constructs. To overcome this limitation, newly developed strategies include material scaffold design, inclusion of pro-angiogenic components (such as angiogenic growth factors or seeded endothelial cells), and *in vitro* pre-vascularization. For more comprehensive information about these developments, the reader is referred to the following reviews (Novosel, Kleinhans and Kluger 2011) (Rouwkema and Khademhosseini 2016) (Rouwkema, Rivron and van Blitterswijk 2008).

6.2 Modulating MSC Metabolism

Keeping in mind that transplanted MSCs encounter environments lacking nutrients and other chemical requirements for their bioenergetics needs, several research groups

have turned to modulating MSC metabolism in order to lower their bioenergetics-related demand. For example, a recent strategy has consisted in driving MSCs to a reduced metabolic state before their implantation in animal models (Moya, Larochette, et al. 2017). To this end, cells are induced to quiescence (by serum deprivation), which is a state that stem cells physiologically adopt in their biological niche in order to preserve their key functions. This quiescence preconditioning allowed BM-hMSCs to withstand exposure to total glucose depletion under continuous near-anoxia (0.1% pO₂) for up to 14 days (Moya, Larochette, et al. 2017) and under hypoxia (2% pO₂) for up to 75 days *in vitro* (Ferro, et al. 2019). *In vivo*, quiescence preconditioning significantly improved BM-hMSC viability when ectopically implanted in cell-containing constructs for up to 7 days (Moya, Larochette, et al. 2017). More importantly, upon *in vitro* reperfusion, preconditioned BM-hMSCs maintained both their proliferation and secretory functions, as well as their trilineage differentiation potential (Moya, Larochette, et al. 2017) (Ferro, et al. 2019). From a metabolic perspective, quiescent BM-hMSCs exhibited reduced ATP-consuming anabolic functions, such as nucleotide and protein syntheses, but maintained their intracellular ATP and protein contents (Moya, Larochette, et al. 2017), suggesting that quiescence may have redirected the energy metabolism of BM-hMSCs toward essential “housekeeping” functions (Boutillier 2001). Furthermore, quiescence preconditioning stimulated autophagic activity in BM-MSCs, as measured by the sustained inhibition of mTOR throughout the early (3-day) period of ischemia exposure *in vitro* (Moya, Larochette, et al. 2017). Overall, our research group hypothesizes that, through reprogramming toward a reduced metabolic state, quiescence preconditioning provides a protective adaptation of hMSCs against abrupt transition to the deleterious ischemic environment.

Strategies have also been developed by other research groups to precondition cells prior to transplantation to a hostile *in vivo* environment, thus enhancing their survival and functional performance. Because MSC-based therapies are delivered to ischemic/hypoxic injured sites, it was suggested that a hypoxic treatment could precondition the cells to adapt better to the ischemic environment. In fact, increasing numbers of literature reports have shown that hypoxic preconditioning of MSCs, under various oxygen concentrations and periods of time, enhances *in vitro* cell viability and *in vivo* cell engraftment (Beegle, et al. 2015) (Chen, et al. 2017) (Chen, et al. 2017) (Ho, et al. 2018) (Hu, et al. 2008) (Luo, et al. 2019) (Peck, et al. 2019) (Pourjafar, et al. 2017) (Sart, Ma and Li 2014) (Wang, et al. 2018) (Wei, et al. 2012). For example, Beegle et al demonstrated that hypoxia-

preconditioned BM-hMSCs had an approximately twofold increase in survival at 6 days under serum deprivation and hypoxia (1% pO₂) as compared with control BM-hMSCs (Beegle, et al. 2015). Preconditioning cells for 96 hours at 1% pO₂ reduced glucose consumption and lactate secretion in MSCs (Beegle, et al. 2015) and regulated their glycogen metabolism through PI3K/AKT and HIF-1/glycogen synthase kinase-3 β -mediated pathways, thus producing glycogen-based energy prestorage (Zhu, et al. 2014). Furthermore, Stegen et al (Stegen, et al. 2016) reported that genetic or pharmacological HIF-1 α stabilization (which occurs under hypoxia) in skeletal progenitor cells prior to implantation improved survival of these cells by adapting glutamine and glycogen metabolism to preserve redox and metabolic energy balance, resulting in enhanced bone repair. Apart from ischemia, the site of injured tissue is usually associated with oxidative stress, inflammation, and acute immune response (Sart, Ma and Li 2014); therefore, other preconditioning and treatment strategies such as exposure to oxidative stress and heat shock treatment have been explored for MSCs (Moloney, et al. 2012) (Zhang, et al. 2012). Although no direct survival studies have been carried out, results showed an enhancement of the therapeutic potential of such preconditioned MSCs (Zhang, et al. 2012). For a deeper discussion of these preconditioning strategies, the readers are referred to the following reviews (Sart, Ma and Li 2014) (Haider and Ashraf 2008).

6.3 Glucose Delivery Strategy: An Enzyme-Controlled Glucose-Delivery Hydrogel to Fuel MSCs Metabolism Post-implantation

Hydrogels are three-dimensional networks composed of hydrophilic polymers cross-linked by forming covalent bonds, capable of absorbing a significant amount of liquid, thus allowing the diffusion and attachment of molecules within their network. Due to their biocompatibility, biodegradability, and the similarity of their physical properties to natural tissue, they are frequently used in TE for a wide variety of scenarios, including wound healing, cartilage/bone regeneration, and the sustained release of drugs (Catelas, et al. 2006) (Eyrich, et al. 2007). Therefore, an enzyme-controlled glucose delivery hydrogel can serve as a source of glucose for MSCs to maintain their survival and functionalities within the post-implantation ischemic environment. Specifically, this novel glucose-delivery system is composed of the following products: fibrinogen, thrombin, aprotinin, wheat starch, and AMG.

The principle of this hydrogel is that starch is hydrolyzed by AMG, allowing the production of glucose to feed the MSCs seeded within the hydrogel (*Figure 6-1*). This innovative device, described as a nutritive hydrogel, has been the subject of a European patent application concerning the glucose delivery system (EP14306700, CNRS, 2014). The proof of concept of the starch/AMG nutrient hydrogel in improving MSCs survival and functionalities post-implantation will be demonstrated within this thesis project.

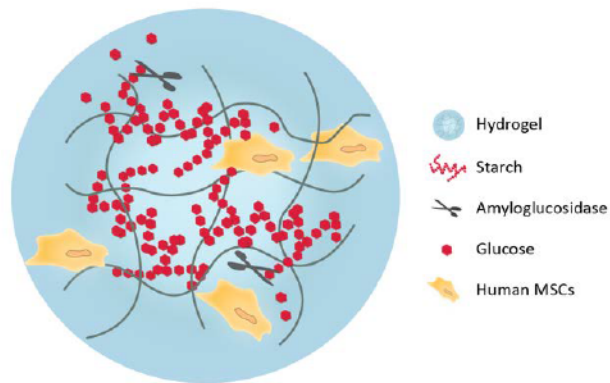


Figure 6-1 Schematic illustration the principle of the nutritive hydrogel.

Aims of the Thesis

Current evidence clearly suggests that MSC-based therapy has an enormous potential in TE applications. The therapeutic potential of MSCs largely results from their survival and cell engraftment, as well as their ability to secrete various bioactive factors post-implantation to improve tissue repair. However, rapid and massive MSC death post-implantation has been observed, which makes the therapeutic potential of MSCs fall short of their initial promise and hype. The hostile ischemic microenvironment, mainly characterized by oxygen and nutrient deprivation, is responsible for the rapid and massive MSC death upon implantation. Recent studies from our lab (B3OA), as well as from other teams, established that the absence of glucose (but not the lack of oxygen) is the culprit for this MSC death. Exogenous glucose supply was proven to maintain *in vitro* survival of hMSCs under near-anoxia (0.1% pO₂) for 21 days and *in vivo* survival in an ectopic model for 14 days post-implantation.

The overall aim of this thesis was to increase our understanding of the role of glucose on MSCs survival and functionalities, as well as of how the glucose delivery approach in TE could be applied to enhance MSCs survival and functionalities upon transplantation and ultimately improve their therapeutic efficacy. The specific aims of the thesis included are stated below.

Specific aims:

- to investigate whether various glucose concentration affects hMSC survival under near anoxia (Article I)
- to evaluate the role of glucose on hMSC-mediated angiogenesis *in vitro* and *in vivo* (Article I)
- to analyze the role of glucose on ER stress of hMSCs under near anoxia (Article I)
- to develop a novel glucose delivery system composed of fibrin hydrogel, starch (a polymer of glucose), and amyloglucosidase (AMG, an enzyme that releases glucose from starch) (Article II)
- to characterize the glucose production of the novel starch/AMG hydrogel (Article II)

- to assess the effect of starch/AMG hydrogel on hMSCs survival and their mediated angiogenesis both *in vitro* and *in vivo* (Article II)

RESULTS

This thesis is based on the work presented in article I and II to develop a novel glucose delivery system for hMSCs in TE application.

7 Article I: Glucose Promotes Transplanted Human Mesenchymal Stem Cell Survival and Paracrine Effects by Regulating Endoplasmic Reticulum Stress

My contribution for this article: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing

7.1 Introduction

The rapid and massive MSCs death post-implantation considerably reduces their therapeutic potential in TE applications, such as large bone defects, myocardial infarction, and many others. The limiting factor for MSC survival within the ischemic microenvironment is not the absence of oxygen but glucose. The supply of exogenous glucose can maintain MSCs survival both *in vitro* near-anoxia conditions and *in vivo* ectopic model because glucose appears as an essential nutrient for MSCs to produce energy under near-anoxia. Thus, it is rational to assume that the increased MSC persistence *in vivo* may benefit their paracrine functions, which are considered essential in tissue repair. The paracrine effects, particularly the pro-angiogenic functions, of hMSCs are relevant aspects of MSC-based therapies' success. Specifically, the release from hMSCs of bioactive factors plays a critical role in MSC-mediated angiogenesis; in addition, cell-secreted bioactive factor(s) depend on active nascent protein biosynthesis within the endoplasmic reticulum (ER) of endothelial cells. Therefore, it is reasonable to hypothesize that nascent protein synthesis plays a role in MSC-mediated angiogenesis in TE.

In the article, we initially reproduce the pro-survival effect of glucose on hMSC under near-anoxia. Then we evaluate the nascent protein biosynthesis of hMSCs in the absence or presence of glucose under near-anoxia and the effects of glucose on hMSC-mediated angiogenesis *in vitro* under near-anoxia and *in vivo* in an ectopic transplantation mouse model. Last but not least, since hypoxia/anoxia and nutrient deprivation can induce

ER stress, which inhibits nascent protein biosynthesis via unfolded protein response; thus, we also investigate whether and how glucose regulates ER stress of hMSC under near-anoxia.

Glucose promotes transplanted human mesenchymal stem cell survival and paracrine effects by regulating endoplasmic reticulum stress

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Key words: Mesenchymal Stem Cells, Tissue Engineering, Glucose, Cell Survival, Ischemia, Angiogenesis, Endoplasmic Reticulum Stress.

ABSTRACT

Mesenchymal stem cells (MSCs) are promising candidates for tissue engineering applications. Nonetheless, after transplantation, MSCs were exposed to an ischemic milieu characterized primarily by oxygen and nutrient deprivation, resulting in rapid and massive MSC death, a significant impediment to their fully therapeutic potential. Our previous research established that the observed MSC death is caused by a lack of glucose, not a lack of oxygen. However, the effect of glucose and the underlying mechanism on the paracrine effects of human MSCs (hMSCs), particularly hMSCs-mediated angiogenesis, are not yet clear. We demonstrated in this study that supernatant conditioned media (CM) obtained from hMSCs cultured in the presence of glucose (1 and 5 g/L) exhibited significantly enhanced proangiogenic properties when compared to CM obtained from hMSCs cultured in the absence of glucose. This finding is corroborated by the significantly increased amount of proangiogenic factors released. Most importantly, in addition to preserving hMSC viability, a hMSC-containing hydrogel loaded with glucose promoted the formation of new blood vessels when subcutaneously implanted. Mechanistically, it was discovered that when hMSCs are cultured in the presence of glucose, endoplasmic reticulum (ER) stress, which is induced by ischemic insults, is mitigated. These findings contribute to our understanding of the possible mechanism through which glucose regulates MSC-mediated angiogenesis and pave the way for the development of glucose-delivery scaffolds to further increase the therapeutic potential of MSC-mediated tissue engineering.

Significance Statement

A major roadblock for MSC-based therapy is the poor survival of grafted MSCs at the injured sites. The survival of grafted MSCs upon implantation has been found to be improved by glucose supply. In the current study, we further demonstrated that glucose promotes hMSC-driven vascular growth in an ischemic environment. Additionally, one possible mechanism for glucose's beneficial effects seems to be that glucose promotes vascular growth by mitigating ER stress in hMSCs. These findings contribute to the understanding of glucose's impact and underlying mechanism on hMSC-driven vascular growth, paving the way for the development of a glucose-delivery system to optimize MSC-based therapeutic efficiency.

INTRODUCTION

Mesenchymal stem cells, also known as multipotent stromal cells (MSCs), are a promising source of cells for a variety of tissue engineering and regenerative medicine applications due to their capacity to differentiate into a variety of mesodermal tissues^[1-6] and to induce a regenerative milieu via the secretion of bioactive chemicals that promote both angiogenesis and tissue regeneration as well as inhibit apoptosis, fibrosis, and inflammation^[7-13]. However, rapid and massive cell death within the injured tissue following transplantation *in vivo*^[14-27], caused by disruption of nutrient and oxygen supplies, has been a major obstacle to MSC use in therapeutic applications; as a result, MSCs have fallen short of the initial promise and hype. While both oxygen and nutrients are required for cell survival and functions associated with new tissue formation, published reports by our lab and other teams have established that the absence of glucose, but not oxygen, is fatal to human MSCs (hMSCs) because glucose acts as a pro-survival molecule for hMSCs following transplantation^[28-32].

The paracrine effects of hMSCs, particularly their pro-angiogenic properties, are also critical to the success of MSC-based therapies^[33-38]. Specifically, the release of bioactive factors from hMSCs is required for MSC-mediated angiogenesis; additionally, cell-secreted bioactive factor(s) are dependent on active nascent protein biosynthesis within the endoplasmic reticulum (ER) of endothelial cells^[39-41]. It is reasonable to hypothesize that nascent protein biosynthesis contributes to MSC-mediated angiogenesis. Both hypoxia/anoxia and nutrient deprivation, to the author's knowledge, can induce ER stress, a condition that inhibits nascent protein biosynthesis via PERK-mediated eIF2 phosphorylation^[42-45]. Additionally, ER stress may be a mechanism by which cells in injured or pathological tissue regulate blood supply, as is the case with ischemic heart disease, cerebral ischemia, and ischemic retinopathies^[41,46-55].

However, a comprehensive understanding of the mechanisms by which glucose affects hMSC-mediated angiogenesis via ER stress regulation remains elusive. For this reason, we hypothesized that nascent protein biosynthesis contributes to MSC-mediated angiogenesis and examined the effects of glucose on hMSC survival and their-mediated angiogenesis *in vitro* ischemic model and *in vivo* using an ectopic transplantation mouse model in order to determine newly-formed blood vessels, and then investigated whether and how glucose regulates ER stress via PERK-mediated pathway. The present paper

provides evidence that glucose promotes hMSCs survival under near anoxia conditions. Viable hMSCs exert their beneficial effects in this condition by releasing bioactive chemicals. Indeed, our findings establish for the first time that glucose enhances hMSC-mediated angiogenesis by selectively regulating the secretion of ER-related bioactive chemical factors.

MATERIALS AND METHODS

Cell isolation and culture

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow using a procedure adapted from a literature report^[56]; this bone marrow was obtained as discarded tissue during routine orthopedic surgery from 4 donors with the respective informed consent according to Lariboisiere Hospital (Paris, France) regulations. The isolated hMSCs were expanded in alpha minimum essential medium (α -MEM; PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Les Mureaux, France) and 1% penicillin/streptomycin (PAA Laboratories GmbH) under standard culture conditions, specifically, a humidified environment of 5% CO₂/95% air at 37 °C. When the cultures were 80-90% confluent, the cells were rinsed twice with Dulbecco's phosphate-buffered saline (DPBS), treated with trypsin-EDTA and reseeded at a density of 10,000 cells/cm². Passage up to number 5 hMSCs from the four donors were pooled in an equal ratio and were used in the experiments.

Near-anoxia cell culture condition

Near-anoxia cell culture condition was achieved using a well-characterized, finely-controlled, proOx-C-chamber system (C-Chamber, C-374, Biospherix, New-York, USA). The oxygen tension was maintained at 0.1% using a residual gas mixture composed of 5% CO₂ and balance nitrogen. The temperature of the system was maintained at 37°C for the duration of the experiments.

For experiments under near-anoxia (0.1% oxygen), the hMSCs in α -MEM with 10% FBS and 1% penicillin/streptomycin were seeded into individual wells of tissue culture plates at a density of 12,500 cells/cm² well surface area and cultured under standard culture conditions. When 60-70% confluence was reached, the cells were rinsed twice with DPBS and cultured in fresh α -MEM without FBS, in either the absence or presence

of D-glucose at 1 g/L or 5 g/L and exposed to near-anoxia conditions (0.1% oxygen) for the time periods specified in the ***IN VITRO EXPERIMENTS*** section of this manuscript. In order to sustain constant oxygen tension, the hMSC cultures were maintained undisturbed without supernatant media changes until the end of the respective experiments.

IN VITRO EXPERIMENTS

Assessment of hMSC viability

The hMSC viability was assessed at day 0, 1, 2, 3, 5, 8, 11, and 14 of cell culture under near-anoxia (0.1% oxygen) conditions. Briefly, at the prescribed time points, hMSCs were collected and stained with propidium iodide (PI; at a final concentration of 1 µg/mL) and Hoechst 33258 (HE; at a final concentration of 1 µg/mL); cell viability was assessed using a Attune flow cytometer (LifeTechnologies, Saint Aubin, France). HE⁺ and PI⁻ cells, but HE⁺ and PI⁺ were considered as ‘viable’ and ‘dead’ cells, respectively. Data were analyzed using Attune Software (LifeTechnologies). The results were expressed as the ratio of viable cell number at each prescribed time point versus the respective viable cell number at day 0 of culture.

Nascent protein biosynthesis detection

Nascent protein biosynthesis was assessed using the Click-iT™ HPG Alexa Fluor™ 488 protein synthesis assay kit according to the manufacturer’s instructions. Briefly, after days 1 and 3 of cell culture under near-anoxia (0.1% oxygen), the supernatant media were collected and stored for further analysis (as specified section), while the hMSCs were rinsed with DPBS twice and then treated with methionine-free medium in either the absence or presence of the methionine analog L-homopropargylglycine (HPG; final concentration 50 µM) for 1 hour. At that time point, the hMSCs were rinsed with DPBS and fixed using 4% paraformaldehyde for 15 min, followed by permeabilization using a saponin buffer for 20 min. Next, the hMSCs were treated with the Alexa Fluor™ 488 detection reagents for the Click-iT reaction in a cocktail buffer at room temperature for 30 min according the manufacture’s protocol. After the Click-iT reaction, the hMSCs were analyzed using a Attune flow cytometer to determine the incorporation of HPG.

Determination of the ADP/ATP ratio

The ADP/ATP ratio of hMSCs was assessed at day 0, 1, 2, 3, 5, 8, 11, and 14 post exposure to near-anoxia (0.1% oxygen) conditions using the ADP/ATP ratio assay kit (ALX-850-248-KI01, Enzo) according to the manufacturer's instructions. Briefly, the hMSCs were lysed using nucleotide releasing buffer and gentle shaking at room temperature for 5 minutes. At that time, the supernatant media were collected and stored at -80°C until ADP/ATP analysis. The ATP and ADP levels in the hMSCs were determined by measuring luminescence in either the absence or presence of ADP-converting-enzyme using a luminometer (Tecan). The respective ADP/ATP ratios were calculated using a formula provided by manufacturer.

Assessment of the chemotactic effect of conditioned media (CM)

The chemotactic potential of CM from hMSCs after exposure to near-anoxia conditions (0.1% oxygen) in either the absence or presence of glucose at either 1 g/L or 5 g/L for 3 days was determined using the Incucyte[®] Live Cell Analysis System (Sartorius). Briefly, 200 µL of each respective CM mixed with endothelial basal media 2 (EBM2; containing 0.25% FBS) in a ratio of 1:1 were added to the appropriate cell-containing wells of the reservoir plate, and 1,400 HUVECs (suspended in EBM2 media containing 0.25% FBS) were seeded into each insert. Subsequently, the ClearView Cell Migration plates were placed into the IncuCyte[®] system and scanned up to 72 hours; the system setting used were: objective:10X; channel selection: phase contrast; scan type: chemotaxis; and scan interval: every 2 hours. The data regarding migrated HUVECs were analyzed using the IncuCyte[®] Chemotaxis Software^[57].

Assessment of released bioactive mediators

Release of chemotactic and proangiogenic bioactive mediators from hMSCs after exposure to near-anoxia (0.1% oxygen) conditions in either the absence or presence of glucose at 1 g/L or 5 g/L for 3 days were determined using Luminex technology (Millipore, Billerica, USA). The levels of 20 mediators, specifically, the chemotactic growth factors IL-8, CCL2, CCL4, CCL5, MMP9, Chemerin, MIF, CXCL2, CXCL5, CXCL10, CXCL16 and proangiogenic growth factors VEGF-A, VEGF-C, VEGF-D, ANGPT-1, ANGPT-2, ANG, FGF-BASIC, Endostatin, and PDGF-BB released from the hMSCs were determined using a Milliplex MAP kit (Millipore) and following the manufacturer's instructions. The presence and concentration of each of the

aforementioned bioactive mediators were evaluated using the MasterPlex QT 1.0 system (MiraiBio, Alameda, USA) and analyzed using the Luminex-100 software version 1.7 (Luminex, Austin, USA).

Quantitative Real-Time Polymerase Chain Reaction

mRNA expression of PERK, eIF2 α , CHOP, and GRP78 of hMSC was assessed at day 1, 2, and 3 post near-anoxia (0.1% oxygen) exposure. Briefly, total RNA was extracted using the NucleoSpin[®] RNA method (Macherey-nagel, Germany); the quantity and purity of RNA was determined using a NanoDrop[™] 1000 Spectrophotometer (Labtech France, Palaiseau, France). Complementary DNA (cDNA) synthesis was performed using 0.5 μ g of RNA using the first-strand synthesis kit of SuperScript[™] II Reverse Transcriptase (Invitrogen) with a iCycler thermal cycler RT-PCR Detection System. Quantitative real-time PCR was performed using the TaqMan[™] Gene Expression Assays Protocol (human PERK: Hs00169345_m1; human eIF2 α : Hs00187953_m1; human CHOP: Hs00358796_g1; human GRP78: Hs00607129_gH; and human 18S rRNA: Hs99999901_s1; Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using MyiQ[™] Single-Color software (Bio-Rad, Hercules, CA, USA). Relative levels of gene expression were analyzed using the $2^{-\Delta\text{CT}}$ method and 18S as an endogenous control.

Western Blotting

The phosphorylation levels of eIF2 α and protein expression of eIF2 α , CHOP, and ATF4 of hMSC was assessed at day 1, 2, and 3 post near-anoxia (0.1% oxygen) exposure. Briefly, hMSC lysates were prepared using lysis buffer containing protease inhibitor and phosphatase inhibitors. After centrifugation, the protein concentration in each supernatant was determined using the Bio-Rad protein assay system. Heat-denatured proteins were electrophoresed on 12 % SDS-PAGE, followed by transfer onto nitrocellulose membranes. These membranes were blocked using 5 % bovine serum albumin at room temperature for 30 min and incubated overnight at 4 °C with primary antibodies against p-eIF2 α (1:1000), total eIF2 α (1:1000), ATF4 (1:1000), CHOP (1:1000), and Actin (1:1000; Cell Signal Tech), at 4°C overnight and then incubated with either horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:3000; Jackson ImmunoResearch) as the secondary antibody at room temperature for 2 h. Bound

antibodies were detected using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK) and visualized using the IVIS Lumina Bioluminescent imaging system (Xenogen, Caliper Life Science, Tremblay-en-France, France). The results were analyzed using Image J software.

***IN VIVO* EXPERIMENTS**

Fibrin hydrogel preparation

The hMSCs proangiogenic potential was assessed using 125 μ L fibrin hydrogels loaded with 1,250,000 hMSCs in either the absence or presence of various (0, 1, 5, 10, and 20 g/L) glucose concentrations, and was encapsulated at the core of a silicone cylinder (4-mm internal diameter; 6-mm external diameter; 16-mm height; Silicone DIA; Weber Métaux, France). Fibrin hydrogels loaded with 20 g/L glucose without hMSCs were used as glucose control. The 3-mm height from the edges of both the top and the bottom of each silicone cylinder were filled with 1 mg/mL fibrin, without aprotinin, in order to maintain the hydrogels at the center of silicone cylinder.

Animals

Ten-week-old, female, nude mice (Janvier, Le Genest-Saint-Isle, France) were used in an ectopic implantation model to assess the proangiogenic potential. The surgery protocol was approved by the Ethics Committee on Animal Research at the Lariboisiere/Villemin (Paris, France; number S158 / 2014-09-07). All procedures regarding housing, handling, surgery, and recovery of the animals after surgery were conducted in compliance with the guidelines of the new European Directive 2010/63/EU regarding protection of the animals used for scientific purposes.

Animal anesthesia

The procedure was performed as described previously^[58]. Briefly, each mouse was given a dose (0.1 mg/kg weight) of buprenorphine (Buprecare[®]; Axience, Pautin, France) by subcutaneously injection. Subsequent anesthesia was induced, and maintained, using intraperitoneal administration of 100 mg/kg weight ketamine (Ketamine1000[®]; Virbac, France) and 8 mg/kg animal weight xylazine (Rompun[®] 2%; Bayer HealthCare, Berlin, Germany).

Ectopic implantation

Briefly, the skin of each mouse was treated with povidone-iodine (Betadine[®]; Vetoquinol). Two symmetrical incisions on both sides of the spine were made and subcutaneous pouches were created. Next, hydrogels of interest to the present study were randomly chosen and carefully inserted into each pouch. Skin closure was accomplished using 4.0 polyglactin sutures (Ethicon; Issy-les-Moulineaux, France) with an interrupted suture pattern.

Assessment of hMSCs proangiogenic potential

Formation of new blood vessels which had invaded the hydrogel post 21 days of implantation was visualized after each mouse was injected with a Microfil[®] compound (Flowtech; Carver, MA, USA). Briefly, after deep anesthesia (as described in the **Animal anesthesia** section), the skin from the thorax and the rib cage of each mouse was incised to access the heart. The left ventricle was catheterized using a 20G canula (BD Venflon; Beckson Dickinson Infusion, Sweden); the right atrium was cut as an output of contained blood, which was removed through perfusion from the left ventricle using DPBS containing 100 UI/mL heparin using a pump (at 6 mL/min flow rate). After DPBS-containing, heparin-perfusion, each mouse was manually perfused with 14 mL Microfil[®] (prepared according to the manufacturer's instructions using 6.3 mL of Microfil[®], 7 mL of the specific diluent, and 0.7 mL of the specific curing agent) using an intracardiac canula. The Microfil[®] perfused mice were stored at 4 °C overnight to allow polymerization of the Microfil[®]; at that time, the silicone cylinders were explanted and fixed using 4% paraformaldehyde overnight. Each sample was analyzed according to the techniques described in the **Micro-CT imaging, reconstructions and data computation** section.

Micro-CT imaging, reconstructions and data computation

Explanted silicone cylinder samples were imaged *in vitro* using a Skyscan1172 CT-scanner (45 kV; 100 mA; 85 ms; 0.3-degree rotation step; without filter; and 10 µm pixel sizes). The acquired images were then reconstructed as a stack of slices using Nrecon software. The volumes of interest (VOI; 2 per samples) were set as cylinders overlapping the internal diameter of the silicone tube (4-mm internal diameter), between the 1st-mm and the 3rd-mm height from both the top and the bottom of each silicone cylinder edge. The neo-vessel volume was expressed as the amount of binarized object-volume

measured within the designated volume of interest exhibiting threshold gray values between 110-255 on the CTAn software.

Statistical analyses

Each of the *in vitro* experiments was conducted in triplicate and repeated at least three separate occasions. Each of the *in vivo* experiments was conducted using at least 6 samples for each experimental group. Numerical data were expressed as means \pm standard deviation (SD). Statistical analyses were performed using commercially available software (GraphPad 6.0 Software, California Corporation, USA). Data were analyzed statistically using analysis of variance and the Bonferroni post-test, and the Mann-Whitney test for parametric and non-parametric tests, respectively. For all analyses, the confidence interval was set at 95% and the significant level at $p < 0.05$.

RESULTS

Glucose sustained hMSCs survival in near anoxia conditions

Given that hMSCs are exposed to ischemia (i.e., low oxygen tension and nutrient deprivation) during implantation, we cultured hMSCs in near-anoxia ($pO_2 = 0.1\%$) and confirmed the effect of glucose on their survival. hMSCs cultured in the absence of glucose experienced rapid decrease in their survival with less than 10% hMSCs remaining viable after day 4 (*Figure 7-1 A*). In contrast, hMSCs cultured in the presence of either 1 or 5 g/L glucose underwent an adaptation period between days 1 and 3, and then remained viable at the level reached on day 3 for up to day 14 (*Figure 7-1 A*).

Based on the fact that decreased ATP levels and increased ADP levels indicate apoptotic or necrotic cells, a high ADP/ATP ratio indicates dying cells, whereas a low ADP/ATP ratio indicates viable cells. The results of this point of the study indicated that hMSCs cultured in the presence of either 1 or 5 g/L glucose for 14 days had a low ADP/ATP ratio, whereas when hMSCs were cultured in the absence of glucose, a sharp increase in ADP/ATP ratio was observed starting on day 1 and lasting up to 4 days

(*Figure 7-1B*). Due to undetectable levels of ATP, this ratio was not monitored at subsequent time points.

Taken together, these results confirmed that glucose at 1 or 5 g/L maintained hMSC survival under near-anoxia; in contrast, glucose depletion resulted in hMSC death.

Glucose increased human umbilical vein endothelial cell (HUVEC) migration, tubular structure formation and selectively increased bioactive factors released in near anoxia conditions

Because the paracrine effects of hMSC, particularly their pro-chemotactic and pro-angiogenic functions, are critical for the success of hMSC-based therapies, the release of bioactive chemical factors is critical for the paracrine functions of hMSC. The migrate curves of HUVECs were analyzed using the Incucyte[®] Live Cell Analysis System in order to determine the chemotactic potential of supernatant conditioned media (CM) collected on day 3. As shown in *Figure 7-2A*, supernatant CM from hMSCs cultured with either 1 or 5 g/L glucose under near-anoxia for 3 days promoted significant ($p < 0.05$) increases in HUVEC migration for up to 36 hours when compared to supernatant CM from hMSCs cultured in the absence of glucose. The migration of HUVECs induced by the supernatant CM from hMSC cultured with either 1 g/L or 5 g/L glucose was comparable (*Figure 7-2A*). To determine the angiogenic potential of the same supernatant CM, the formation of tubular structures was evaluated *in vitro* using the Matrigel assay. Light microscopy was used to visualize the formation of tubular structures (*Figure 7-2B*). The quantification of the total length of tubular structures revealed that the supernatant CM obtained when hMSCs were cultured in the presence of glucose (1 or 5 g/L) under near-anoxia induced significantly ($p < 0.05$) more tubular structures than the CM obtained when hMSCs were cultured in the absence of glucose under near-anoxia (*Figure 7-2C*). Additionally, the formation of tubular structures induced by the supernatant CM obtained from hMSCs cultured with 1 g/L or 5 g/L glucose was comparable (*Figure 7-2C*).

When the angiogenic and chemotactic growth factor profiles of supernatant CMs were analyzed using a protein array that quantifies 24 bioactive factors using Luminex technology, it was discovered that hMSCs cultured in the presence of glucose at a concentration of 1 g/L released significantly ($p < 0.05$) more Angiogenin, VEGF-A, VEGF-C, Angiopoietin-1, Endostatin, and CCL2 compared to hMSCs cultured in the absence of glucose (*Figure 7-2D*). In contrast, hMSCs cultured in the presence of 1 g/L

glucose released significantly ($p < 0.05$) less amounts of FGF-basic and MIF when compared to hMSCs cultured in the absence of glucose (Figure 7-2D). Last but not least, hMSCs cultured in the absence (0 g/L) or the presence of glucose at 1, and 5 g/L released comparable amounts of angiopoietin-2, PDGF-BB, EGF, MMP-9, VEGF-D, IL-8, CXCL10, MIP-1b, Eotaxin, Chemerin, CXCL5, CXCL16, RANTES, MDC, CXCL2, IP-10, Midkine and Lymphotactin (Data not shown).

Overall, these data provided evidence that glucose promoted HUVEC migration, tubular structures formation, and regulated selective secretion of bioactive chemical factors.

Glucose increased hMSC-mediated angiogenesis *in vivo*

The effects of glucose on hMSC-mediated angiogenesis were investigated *in vivo* using an ectopic transplantation mouse model. Glucose is a small, uncharged and highly diffusible molecule whose release from the implant is rapid. In order to compensate for this rapid glucose release and achieve prolonged presence of glucose in the hMSC-containing hydrogels, we envisioned to load hydrogels with the highest amount of glucose not cytotoxic to hMSCs. To this aim, increased dose of glucose was tested for glucose toxicity by exposing hMSCs at glucose concentration ranging from 1 to 80 g/L in near anoxia conditions for 3 and 8 days. Because the hMSCs remained viable in the presence of glucose concentrations up to 20 g/L (Figure 7-5), cell-containing hydrogels loaded with either 0, 1, 5, 10, or 20 g/L glucose were prepared. Cell-free hydrogels but loaded with 20 g/L glucose were included as controls. All these hydrogels were implanted ectopically in nude mice, and the volume of newly-formed blood vessels was quantified 21 days post implantation. A dichotomous tree, i.e., a well-defined hierarchal blood vessel branching network, developed within the region of interest surrounding cell-containing hydrogels loaded with glucose (Figure 7-3A). In contrast, this hierarchal branching pattern was disrupted showing only a few large-diameter blood vessels branching into small-diameter blood vessels within the region of interest surrounding cell-containing hydrogels without glucose and cell-free hydrogels loaded with 20 g/L glucose (Figure 7-3A). Most importantly, the implanted cell-containing hydrogels loaded with either 5, 10, and 20 g/L glucose exhibited a 2.4-, 2.8-, and 2.4-fold increase ($p < 0.05$) in the volume of newly-formed blood vessels in the region of interest when compared to cell-containing hydrogels without glucose, respectively (Figure 7-3B). The volume of newly-formed blood vessels in cell-containing hydrogels without glucose and

in cell-free hydrogels loaded with 20 g/L glucose was similar (Figure 7-3B). To ensure the newly-formed vessels are vascular structures, an immunohistological analysis was performed and found that CD31 positive endothelial cells infiltrated the implants and were abundantly present in vessels (Figure 7-3C).

Taken together, these data demonstrate that glucose increased hMSC-mediated angiogenesis post-implantation.

Glucose increased hMSC survival and paracrine effects by regulating hMSC ER stress

Since nascent protein biosynthesis plays a role in MSC-mediated angiogenesis, the effect of glucose on hMSCs nascent protein biosynthesis was determined. hMSCs cultured in the absence of glucose exhibited significantly ($p < 0.05$) lower nascent protein biosynthesis at day 1 (Figure 7-4A). When compared to hMSC cultured in the presence of either 1 or 5 g/L glucose, there was no nascent protein biosynthesis at day 3 (Figure 7-4A). This result provided evidence that these hMSCs encountered ER stress because nascent protein biosynthesis inhibition is a hallmark of ER stress.

Since both hypoxia/anoxia and nutrient deprivation experienced by hMSCs upon transplantation can induce ER stress, cells cope with ER stress by initiating a series of adaptive mechanisms that together are termed “unfolding protein response (UPR)”. In this respect, PERK-mediated eIF2 α phosphorylation, preferential translation of ATF4 mRNA and induction of CHOP is one of the mechanism during UPR (Figure 7-4B). For this reason, we investigated whether glucose-regulated UPR via the PERK-mediated pathway regulated the ER stress in our study. hMSCs cultured in the absence of glucose exhibited significantly ($p < 0.05$) lower mRNA expression of PERK at day 3 and of eIF2 α at days 2 and 3, and significantly ($p < 0.05$) higher mRNA expression of CHOP and GRP78 at all times tested when compared to hMSCs cultured in the presence of glucose (Figure 7-4C). hMSCs cultured in the absence of glucose exhibited increased level of phosphorylated eIF2 α when compared with hMSCs cultured in the presence of glucose at day 1 and 2 (Figure 7-4C). This increase in phosphorylated eIF2 α , however was drastically decreased when hMSCs were cultured in the absence of glucose at day 3. hMSCs cultured in the absence of glucose exhibited higher expression of CHOP when compared to hMSCs cultured in the presence of glucose (Figure 7-4D), however, ATF4,

another downstream targets of eIF2 α phosphorylation was not detectable (Data not shown).

Taken together, these data provided evidence that glucose regulated UPR, thus relieving hMSC ER stress in near anoxia conditions; this information provides a plausible explanation for the beneficial effects of glucose on hMSC survival and paracrine functions.

DISCUSSION

Rapid and massive MSC death following disruption of oxygen and nutrient supply during transplantation places a cap on MSC-based tissue engineering therapies. Previously, we and others demonstrated that glucose is critical for MSC survival in an ischemic microenvironment post-implantation^[28–32]. Apart from enhancing MSC survival, the following functionalities are critical for MSC-based therapies in tissue engineering. For example, revascularization of injured tissue by hMSCs is a critical biological event during tissue repair. Thus, the purpose of this study was to determine the effect of glucose and the underlying mechanism on hMSC-mediated angiogenesis in an ischemic microenvironment post-implantation.

Angiogenesis generates new vascular structures from pre-existing blood vessels, aiming to recover nutrient transfer, oxygen supply, and metabolic waste removal. Angiogenesis is a multistep process involving the activation and survival of endothelial cells (ECs), basement membrane degradation, EC proliferation and migration, tube formation, elongation, and remodeling, as well as maturation. MSCs have been identified as being in close proximity to newly formed blood vessels but not integrated into the vasculature structure^[59]. This finding indicated that MSC promote angiogenesis via a paracrine effect rather than directly differentiating and integrating into the vasculature structure. The effect of glucose on hMSC-mediated angiogenesis was investigated in this study by measuring HUVEC migration and tubular structure formation using supernatant CM obtained from hMSCs cultured in near-anoxia for three days. We found that CM derived from hMSCs cultured with glucose (1 or 5 g/L) significantly ($p < 0.05$) increased HUVEC migration for up to 36 hours and significantly increased tubular structure formation (total length of tubular structure) when compared to CM derived from hMSCs cultured in the absence of glucose (*Figure 7-2*). The findings observed in this

study mirror our previous studies that have examined the effect of oxygen tension on hMSC paracrine functions, demonstrating that hMSCs cultured with 5 g/L glucose without serum under near-anoxia for up to 14 consecutive days exhibit both pro-chemotactic and pro-angiogenic response^[60]. Surprisingly, no differences were found in HUVEC migration and the total length of tubular structure induced by the supernatant CM from hMSCs cultured in the presence of glucose at either 1 or 5 g/L (Figure 7-2). This finding suggests that a wide range of glucose concentrations may be used in applications involving hMSC-mediated angiogenesis. However, it is critical to keep in mind that when supraphysiological glucose concentrations (e.g., 5 g/L) are used to prepare supernatant CM for tissue engineering, it has been demonstrated that supraphysiological glucose concentrations have a detrimental effect on other cellular behaviors. Indeed, other studies have demonstrated that increasing extracellular glucose concentrations (up to 20 mM) increases endothelial cell permeability dose-dependently^[61] and a high glucose concentration (20-25 mM) impairs endothelial progenitor cell proliferation^[62,63], migration and tube formation functions^[63].

Along with improved hMSC survival *in vivo* following implantation, the current study found that implanted hMSC-containing hydrogels loaded with glucose increased the volume of newly formed blood vessels when compared to hMSC-containing hydrogels without glucose (Figure 7-3). By contrast, the volume of newly formed blood vessels in cell-containing hydrogels devoid of glucose was comparable to that in cell-free hydrogels loaded with glucose. Last but not least, a histological analysis was performed using CD31 antibody staining to determine the presence of ECs. CD31, also known as PECAM-1 (platelet endothelial cell adhesion molecule 1), is a transmembrane glycoprotein. It is an endothelial intercellular junction constituent. It is required for the adhesion cascade between ECs during angiogenesis, which qualifies it as an EC marker^[64]. Histological sections revealed that CD31 positive endothelial cells were abundantly present in vessels and infiltrated the implants (Figure 7-3). These findings suggest that hMSCs are responsible for forming new blood vessels and that exogenous glucose promotes vascular growth.

Given that MSC paracrine functions are mediated by the secretion of a variety of bioactive mediators, we investigated the release of chemotactic and angiogenic bioactive mediators into the supernatant CM. The current study discovered that when hMSCs were cultured with glucose, they released significantly ($p < 0.05$) more Angiogenin, VEGF-A, VEGF-C, Angiopoietin-1, Endostatin, and CCL2 than when they were cultured without

glucose (*Figure 7-2*). According to the current findings, previous research has established that the secretion levels of four major factors (angiogenin, CCL2, VEGF, and IL-8) may serve as effective biomarkers for predicting MSC revascularization efficacy^[65].

Moreover, VEGF and CCL2 are two of four bioactive mediators released by hMSCs (MCP-1, IL-8, VEGF, and RANTES) that have been identified as critical factors in hMSC-mediated angiogenesis under near-anoxia^[60]. These results indicated that exogenous glucose supply of hMSCs cultured in near-anoxia enhanced MSC-mediated angiogenesis by increasing the release of pro-angiogenic factors into the culture media. However, one unexpected finding was that the amount of FGF-b, which is known to promote angiogenesis synergistically with VEGF, is significantly ($p < 0.05$) higher in the CM collected from hMSCs cultured in the absence of glucose compared to hMSCs cultured with glucose (*Figure 7-2*). This could be because the result was obtained from a single time point, whereas the secretome profile is dynamic^[66]. Another possibility is that MSCs contain a significant reservoir of active FGF-b, which is passively released into culture media upon cell death or even sub-lethal injury, rather than being secreted actively^[67]. Thus, it is possible that increased MSC death in the absence of glucose resulted in a robust release of active FGF-b from the intracellular depot into the culture media, resulting in observed HUVEC migration and tubular structure formation. The released FGF-b from MSCs upon cell death may propose the emerging concept of dying stem cell hypothesis as a novel mechanism for MSC therapy, though most researchers focus on the role of apoptotic extracellular vesicles on immunomodulation. The current study did not examine the effect of glucose on the immunomodulatory properties of MSCs, another critical factor in the success of MSC-mediated tissue engineering. Additional research will be required to demonstrate the beneficial effects of glucose.

According to a review of the literature, there is a strong correlation between ER stress and angiogenesis. The majority of pro-angiogenic bioactive mediators are secreted proteins that require active nascent protein biosynthesis and proper protein folding within the ER, and transport from the ER to the Golgi apparatus (GA) and then to the plasma membrane via secretory vesicles or secretory granules^[68]. The biosynthesis and folding of proteins in the ER are high-energy processes. Thus, several cellular stressors that impair cells' energetic status, such as nutrient or oxygen deprivation, can accumulate misfolded proteins in the ER lumen, triggering the UPR. The UPR is induced by three major transmembrane stress sensors: PERK, IRE1, and ATF6. The UPR maintains a balance between cell survival and death, and thus can result in either a pro- or anti-angiogenic

response. Indeed, ER stress may be a mechanism through which cells in injured tissue adjust blood supply. Under conditions of mild ER stress, all three arms of the UPR can promote angiogenesis by increasing pro-angiogenic factor synthesis. By contrast, under prolonged, severe ER stress, the UPR may switch from a pro-angiogenic to an angiostatic vascular growth program. The current study discovered that when hMSCs were cultured in the absence of glucose under near-anoxia, they exhibited significantly ($p < 0.05$) lower nascent protein biosynthesis at day 1 than when they were cultured in the presence of glucose (*Figure 7-4*). The current findings appear to be consistent with previous research indicating that glucose availability promotes protein synthesis in a variety of cell types and tissues^[69-73]. Additionally, our findings suggest that hMSCs cultured in the absence of glucose under near-anoxia experienced a higher level of ER stress, as protein biosynthesis arrest is a hallmark of ER stress. It is somewhat surprising that, on day 3, no nascent protein biosynthesis was observed in hMSC cultured in the absence of glucose under near-anoxia. A possible explanation for the abrogation of nascent protein biosynthesis is the exhaustion of glycolytic reserves and ATP content in hMSCs cultured in the absence of glucose under near-anoxia for 1 and 3 days, respectively^[30].

Another significant finding was that hMSCs cultured in the absence of glucose under near-anoxia expressed significantly ($p < 0.05$) more CHOP and GRP78 mRNA and a significantly higher level of phosphorylated eIF2 and CHOP protein expression than hMSCs cultured in the presence of glucose (*Figure 7-4*). It has been demonstrated that increasing CHOP expression not only causes cell death but also induces an anti-angiogenic response^[74]. In this regard, the increased expression of CHOP may account for the significant hMSC death and relative lack of angiogenic potential observed in the current study. However, because the current study focused exclusively on the role of glucose in PERK pathways, additional research into glucose regulation in the other two major UPR branches (IRE1 and ATF6) is undoubtedly required. Specifically, previous research has demonstrated that severe ischemic neurons activate the IRE1 pathway indefinitely, impairing proper angiogenesis in the ischemic retina via the RIDD activity degrading pro-angiogenic factors^[46]. Additionally, it would be interesting to assess the effect of ER stress inducers and/or inhibitors on hMSC-mediated angiogenesis, providing solid and direct evidence.

CONCLUSION

The purpose of this study was to determine the effect of glucose and the underlying mechanism on hMSC paracrine functions, particularly angiogenesis mediated by hMSC. The results of this study demonstrate that exogenous glucose both *in vitro* and *in vivo* significantly promotes hMSC-mediated angiogenesis via increased secretion of pro-angiogenic factors (Angiogenin, VEGF-A, VEGF-C, Angiopoietin-1, Endostatin, and CCL2). Another significant finding is that glucose's beneficial effect on hMSC-mediated angiogenesis may be due to the ER stress experienced by hMSCs in an ischemic microenvironment. The findings provide the first insight into the mechanism underlying glucose's beneficial effects on hMSC-mediated angiogenesis post-implantation and pave the way for the development of glucose-delivery scaffolds to augment the therapeutic potential of MSC-mediated tissue engineering.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors state no potential conflicts of interest.

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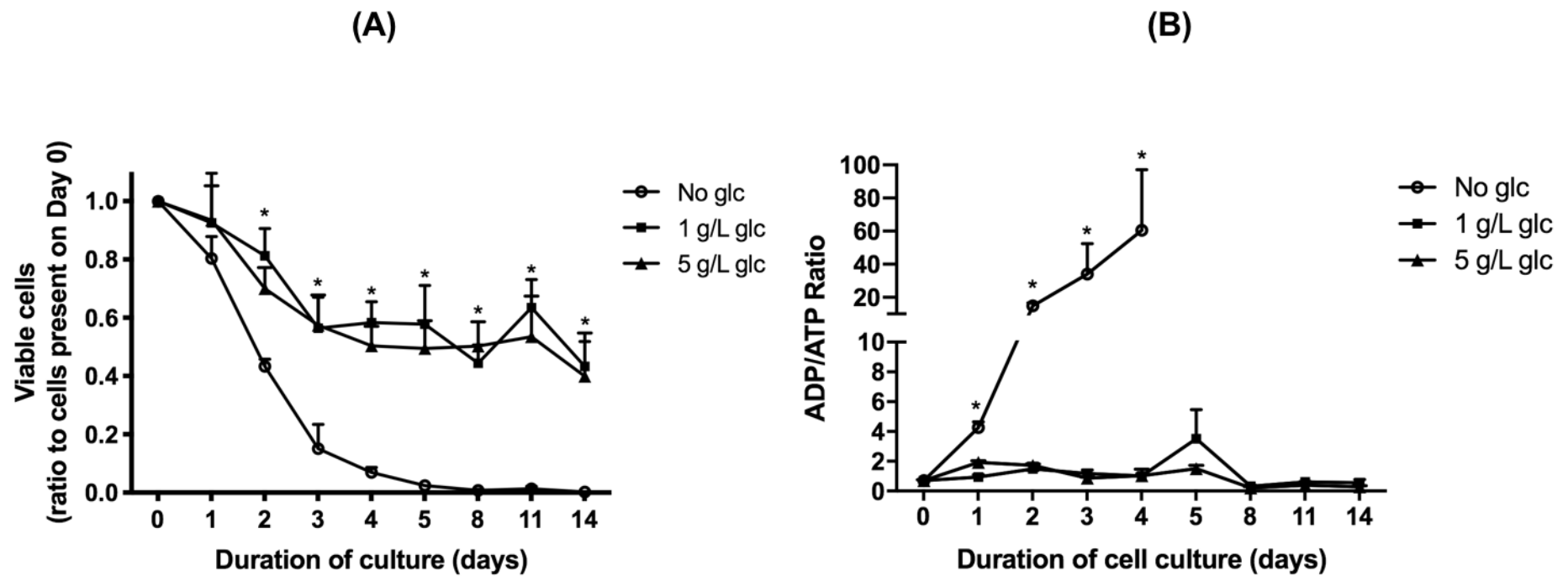


Figure 7-1 **Glucose sustained hMSCs survival in near anoxia conditions** (A) Time course of the viability of hMSCs cultured either in the absence or presence of either 1 g/L or 5 g/L glucose under near anoxia (0.1% oxygen) conditions. (B) Time course of the ADP/ATP ratio of hMSCs cultured in either the absence or presence of 1 g/L or 5 g/L glucose under near anoxia (0.1% oxygen) conditions. Data are mean \pm SD. * $p < 0.05$ compared to the results obtained from hMSC cultured in the absence of glucose. Figure 1A: $n = 9$; stains: Hoescht 33342 and Propidium Iodide. Figure 1B: $n = 6$.

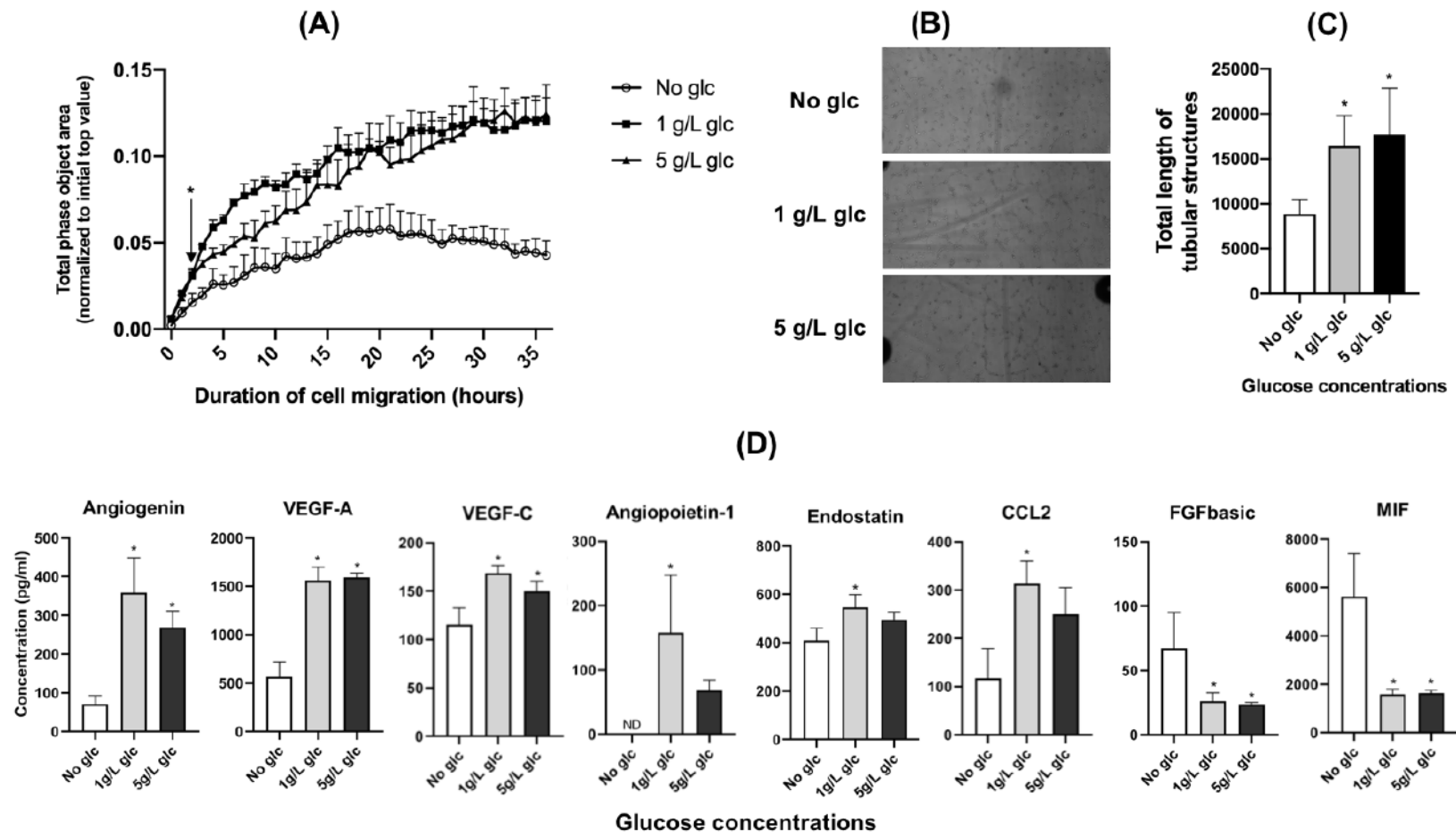


Figure 7-2 Glucose increased human umbilical vein endothelial cell (HUVEC) migration, tubular structure formation and selectively increased bioactive factor release in near anoxia conditions (A) Time course of the migration curve of HUVECs in response to supernatant CM obtained from hMSCs cultured in either the absence or presence of 1 g/L or 5 g/L glucose under near-anoxia for three days. (n = 6). (B, C) Representative light micrographs illustrating the formation of tubular structures (B) and quantitation of tubular structures formed by HUVECs (C) on Matrigel matrices in the presence of supernatant CM obtained from hMSCs cultured in either the absence or presence of 1 g/L or 5 g/L glucose under near-anoxia for three days. (n = 6). Magnification, x10 (n = 6). Release of select bioactive factors (D) from hMSCs cultured in either the absence or presence of 1 g/L or 5 g/L glucose under near anoxia conditions for 3 days (n = 6). Data are mean \pm SD. * p < 0.05 (compared to the results obtained from hMSC cultured in the absence of glucose).

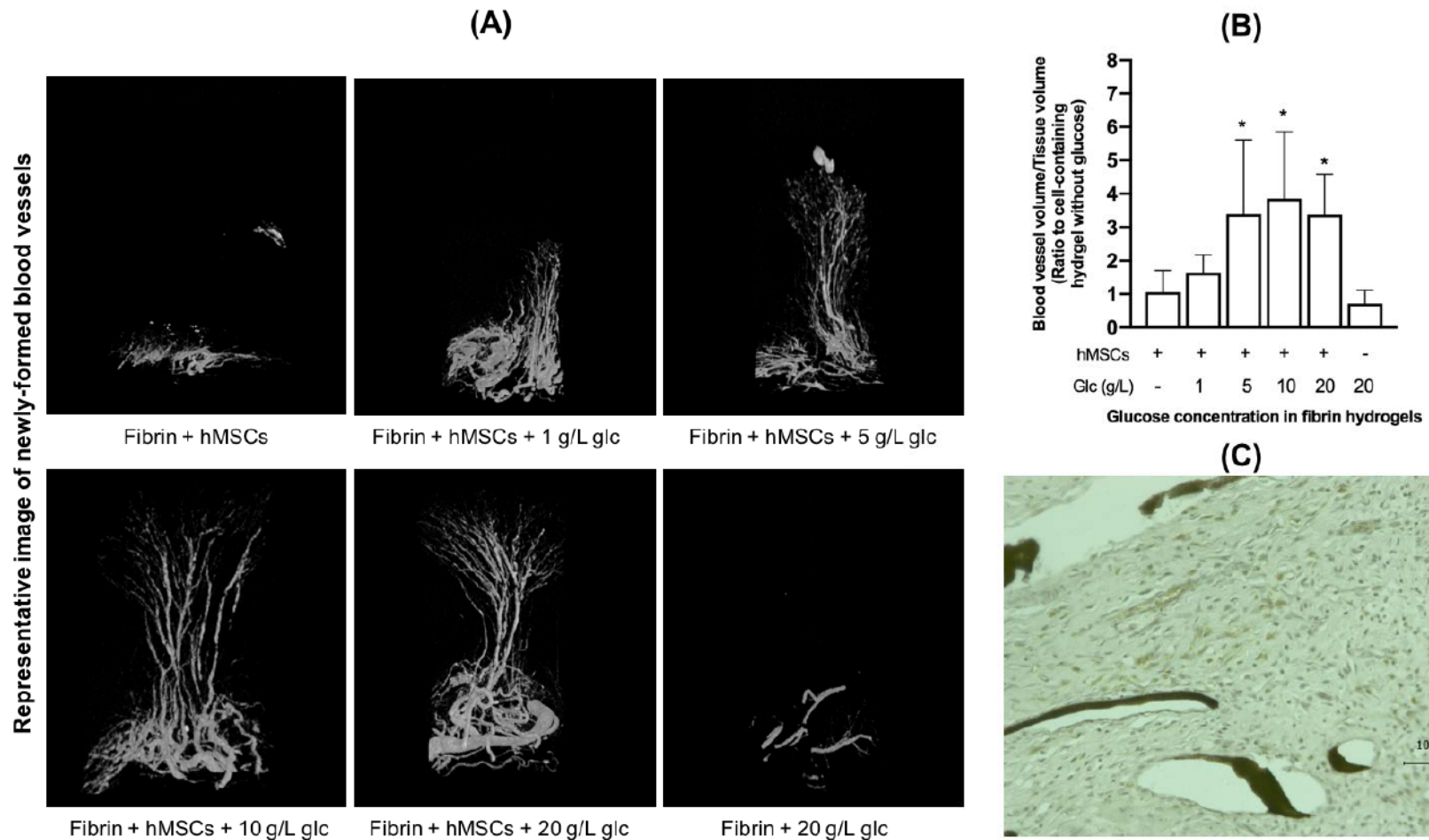


Figure 7-3 Glucose increased hMSC functions pertinent to angiogenesis in vivo. (A, B): Micro-CT vasculature analysis in the vicinity of either hMSC-containing hydrogels loaded with various glucose concentration (0, 1, 5, 10, or 20 g/L) or cell-free hydrogel loaded with 20 g/L glucose after 21 days of ectopic implantation in nude mice. (A). Representative 3D reconstructions of the newly-formed blood vessels into the region of interest defined in the vicinity of either hMSC-containing hydrogels loaded with various glucose concentration (0, 1, 5, 10, or 20 g/L) or cell-free hydrogel loaded with 20 g/L glucose after subcutaneous implantation in nude mice for 21 days. Quantification of the volume of newly-formed blood vessels (B) in the region of interest surrounding cell-containing hydrogels either in the presence of 1, 5, 10, and 20 g/L or absence (0 g/L) glucose and cell-free hydrogels loaded with 20 g/L glucose (n = 6). Data are mean \pm SD. * p < 0.05 (compared to the results obtained from cell-containing hydrogels in the absence of glucose). (C) Histological analysis of the host mouse endothelial cells (CD31 positive staining) that has infiltrated region of interest of the hydrogels after 21 days of ectopic implantation in nude mice. (bar = 100 μ m)

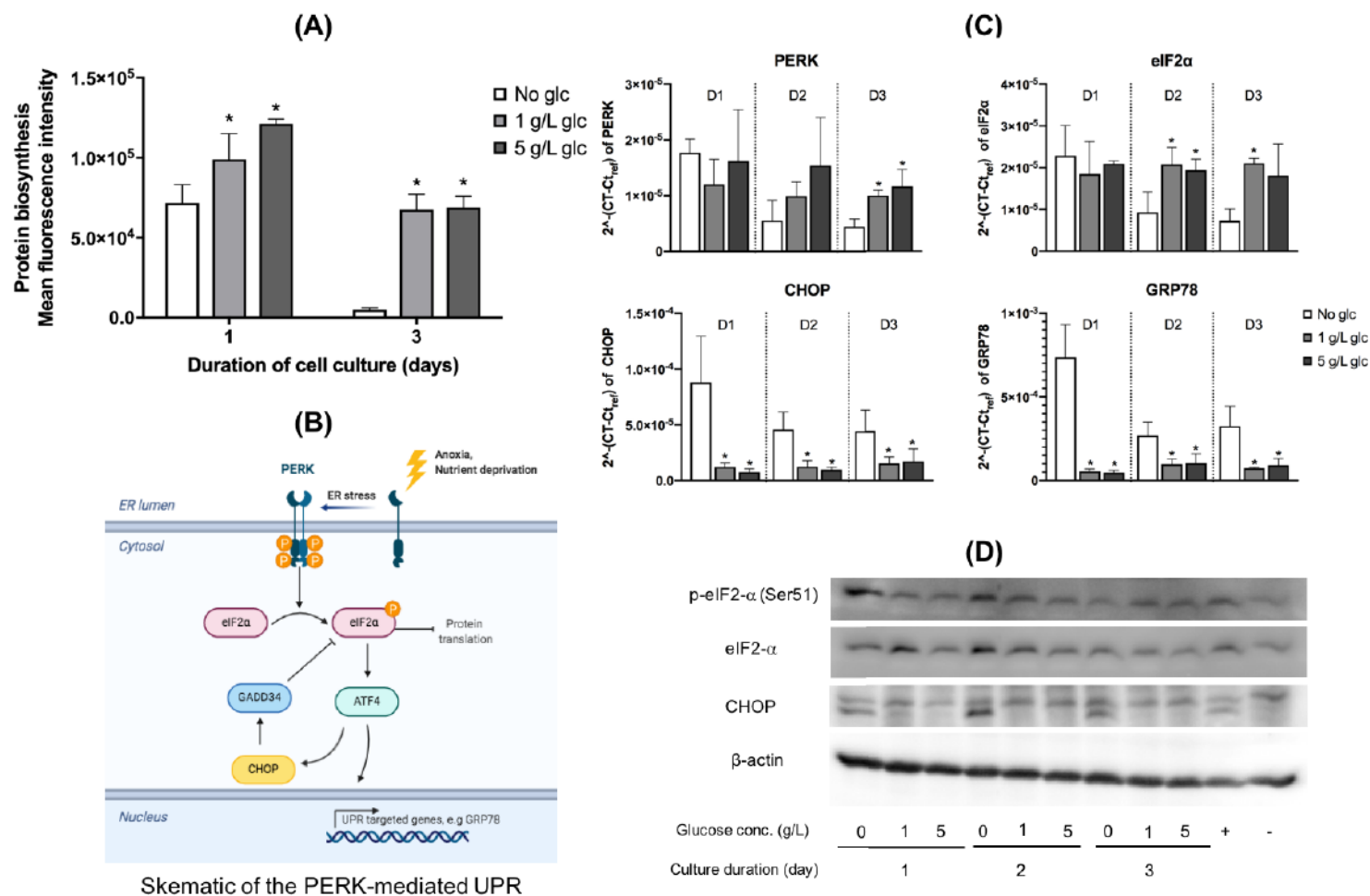


Figure 7-4 Glucose increased hMSC survival and paracrine effects by regulating hMSC ER stress. (A) Nascent protein biosynthesis by hMSCs cultured in either the absence or presence of glucose at 1 g/L or 5 g/L under near anoxia conditions for 1 and 3 days, using homopropargylglycine incorporation and analysed using flow cytometry (n = 6). (B) Schematic of the PERK-mediated eIF2 α phosphorylation pathway. Both anoxia and nutrient deprivation are ER stresses that initiates the UPR and enables dimerization of PERK and its subsequent autophosphorylation. Activation of PERK then phosphorylates eIF2 α , leading to attenuation of global protein translation. Phosphorylated eIF2 α allows selective translation of the ATF4 mRNA. Expression of ATF4 can induce CHOP expression, in turn reversing translational repression by activating GADD34. (C) The mRNA levels of PERK, eIF2 α , CHOP, and GRP78. (D) Phosphorylation of eIF2 α , and protein expression of CHOP in hMSC cultured in either the absence or presence of 1 and 5 g/L glucose under near anoxia at 1, 2, and 3 days (n = 3). Data are mean \pm SD. * p < 0.05 (compared to the results obtained from hMSCs cultured in the absence of glucose).

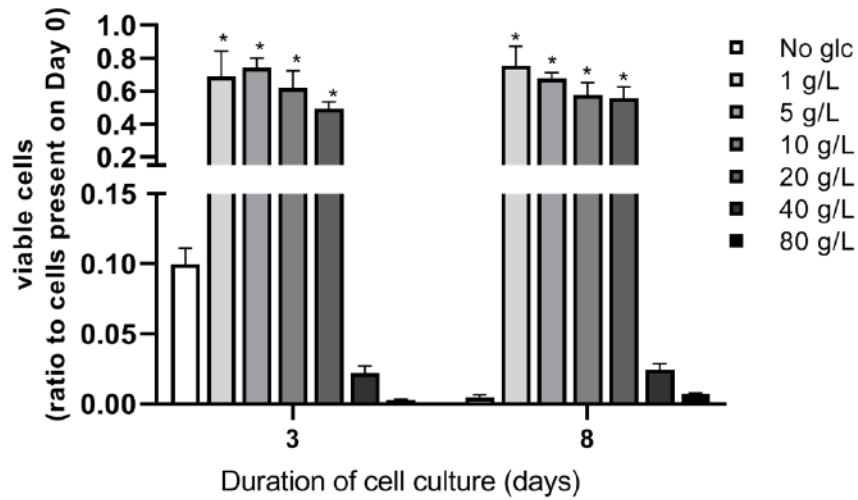


Figure 7-5 **hMSCs remained viable in the presence of high glucose concentration under near anoxia conditions.** Viability of hMSCs cultured in either the absence or presence of glucose in the range of 1 to 80 g/L under near anoxia (0.1% oxygen) conditions at day 3 and 8. Data are presented as mean \pm SD. *: $p < 0.05$ compared to the results obtained from hMSC cultured in the absence of glucose. $n = 3$, stains: Hoescht 33342 and Propidium Iodide.

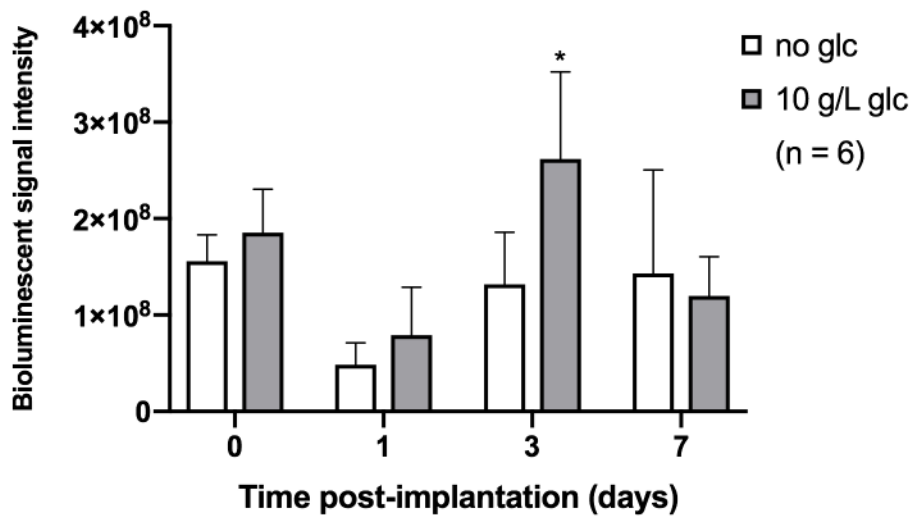


Figure 7-6 **Glucose improved *in vivo* survival of hMSCs after subcutaneous implantation.** Quantification of the bioluminescent signal detected in the hydrogels. Data are mean \pm SD. * $p < 0.05$ (compared to the results obtained from cell-containing hydrogels in the absence of glucose)

8 Article II: Enzyme-Controlled, Nutritive Hydrogel for Mesenchymal Stem Cell Survival and Paracrine Functions

My contribution to this article: assistance in conducting the *in vivo* experiments regarding neovascularization (Figure 8-6 and Table 8-1) and assistance in collecting data.

8.1 Introduction

Previous studies and article I have demonstrated the positive effects of exogenous glucose on MSCs survival and functionalities post-implantation, indicating that a supply of glucose appears to be a key factor for the design and development of strategies to maintain MSCs survival and functionalities after implantation. However, glucose is a small, uncharged molecule without specific affinity to any polymer or matrix; in other words, glucose is highly diffusible and cannot remain within the implant. In addition, adding a supraphysiological concentration of glucose may lead to an increase in osmotic pressure that may induce cell lysis. Thus, a glucose reservoir that continuously delivery glucose to MSCs in a tolerable concentration range to maintain their survival and functionalities is a significant challenge of interest.

To address this problem, we have developed a novel system for the controlled delivery of glucose by an enzymatic reaction. This system consists of starch (a glucose polymer) and AMG (enzyme to release glucose from starch), encapsulated within a fibrin hydrogel. Theoretically, the AMG hydrolyzes the α -1,4 glycosidic linkages of starch to produce glucose that fuel MSC metabolism within the fibrin hydrogel. This system is referred to as a nutritive hydrogel (Starch/AMG hydrogel).

We initially evaluated the amount of glucose produced within nutritive hydrogels (without cells) as a starch and AMG concentration function. In addition to ensuring that the starch/AMG enzymatic reaction releases glucose within these hydrogels, this initial study allowed us to determine a concentration of starch and AMG producing glucose at a concentration within physiological ranges. The nutritive hydrogels were then seeded with hMSCs. MSC survival within these nutritive hydrogels was evaluated, first *in vitro* under near-anoxic conditions (0.1% pO₂) without glucose and serum for 14 days, then *in vivo* after implantation in an ectopic mouse model for 14 days. In order to extend the interest of this system to other fields of TE, the *in vitro* survival of ADSCs and myoblasts

maintained inside the nutrient hydrogels was investigated under near-anoxic conditions for 14 days.

In TE, MSCs are mainly used for their paracrine effect, contributing notably to the migration of host cells towards the injured tissue (chemotactic properties) and the formation of new blood vessels, essential for the transport of oxygen and nutrients (pro-angiogenic properties). Thus, *in vitro*, we evaluated the chemotactic and pro-angiogenic properties of hMSCs within nutrient hydrogels under near-anoxic conditions for 14 days. In addition, the ability of the hMSCs secretome to induce new blood vessel formation under the avascular microenvironment was also evaluated *in vivo*.

ENZYME-CONTROLLED, NUTRITIVE HYDROGEL FOR MESENCHYMAL STEM CELL SURVIVAL AND PARACRINE FUNCTIONS

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ABSTRACT

Mesenchymal stem cells (MSC) are appealing candidates for regenerative medicine applications. Upon implantation, however, cells encounter an avascular microenvironment depleted of oxygen and nutrients that is responsible for their massive death post-transplantation, a major roadblock to successful clinical therapies. To date, various approaches, including development of oxygen-delivery scaffolds, have been proposed by scientists to address this issue. We hereby propose a shift of paradigm for enhancing human MSC (hMSC) survival by designing, developing and testing for the first time an enzyme-controlled, nutritive hydrogel with an inbuilt system of glucose delivery. This novel hydrogel, made of fibrin, starch (a polymer of glucose), and amyloglucosidase (AMG, an enzyme that releases glucose from starch), provides physiological levels of glucose to fuel hMSCs via glycolysis. hMSCs loaded in the novel starch/AMG hydrogels exhibit improved cell viability, and paracrine functions for up to 14 days under near-ischemic conditions. Most importantly, in addition to enhance hMSC viability, these nutritive hydrogels promoted paracrine functions when implanted ectopically in an avascular model.

Keys-words: Tissue engineering, Mesenchymal stem cells, Ischemia, Glucose, Hydrogel, Starch, Amyloglucosidase, Cell survival, Cell paracrine functions.

INTRODUCTION

While there is tremendous excitement regarding the use of mesenchymal stem cells (MSC) for the repair/regeneration of cardiovascular (Martin-Rendon et al., 2008) and osteoarticular tissues (Bruder et al., 1994; 1999; Petite et al., 2000; Yan et al., 2007), it is essential to temper hype with reality. In fact, the initial “proof of concept” of these therapies has not yet been translated into routine clinical practices. A significant roadblock to these therapies is the occurrence of massive MSC death post-implantation (Logeart-Avramoglou et al., 2010; Giannoni et al., 2010; McGinley et al., 2013). Indeed, the lack of functional vascularization in implanted engineered constructs expose grafted MSCs to the rigors of an ischemic milieu, a prime cause responsible for the observed massive cell death post-implantation (Becquart et al., 2012). Progress from just using combinations of cells and material scaffolds to truly integrated and functional tissue constructs requires robust strategies to develop

“niches” for MSCs that are supportive of post-implantation cell survival and their functions pertinent to new tissue formation.

Whereas material scaffolds have been optimized to allow cell attachment, to deliver bioactive chemical compounds, and to expose MSCs to specific mechanical and biological stimuli, these scaffolds rely, at best, on their internal architecture to enable diffusion of vital oxygen and nutrients and, thus, provide energy to the cells. This situation is inadequate when considering that (i) cells are often implanted in an ischemic environment devoid of oxygen and nutrients and (ii) cells crucially require extracellular nutrients to fuel their metabolism and provide regenerative responses.

In an attempt to overcome these issues, we proposed to engineer material scaffolds with an inbuilt system of nutrient supply. Rational engineering of such scaffolds requires thorough understanding of the specific metabolic needs of MSCs exposed to ischemic conditions. To this aim, our research team (Deschepper et al., 2011 and 2013; Moya et al., 2018) and others (Vordermark et al., 2005; Szegezdi et al., 2008; Zhu et al., 2014) explored the respective roles of the two major insults (specifically, glucose deprivation and near-ischemic) associated with ischemia on human MSCs (hMSC) viability and functions. We demonstrated that hMSCs exposed to severe, continuous near-ischemic, but without glucose shortage, remained viable and maintained both their *in vitro* proliferative ability and, most importantly, their functions pertinent to tissue repair *in vivo*. These findings challenged traditional views according to which lack of oxygen *per se* is responsible for the massive death of MSCs observed upon implantation and provided evidence that these cells can withstand exposure to near-ischemic provided that a glucose supply is available. Taken together, these findings underscore the importance of glucose supply to ensure MSC survival post-implantation and justify development of material scaffolds with an inbuilt system for glucose delivery.

Storing and delivering glucose to the grafted MSCs is a challenge because this water-soluble molecule, at high concentrations, can disturb the osmotic pressure and cause cell lysis. To resolve this matter, and taking inspiration from nature, we have hereby developed an enzyme-controlled-glucose-delivery hydrogel that contains starch, the energy storage polysaccharide in plants, in order to store large amounts of

glucose while reducing the associated osmotic pressure. In the present novel system, the starch (which is osmotically inactive) enzymatically delivers glucose molecules by means of embedded amyloglucosidase (AMG). The present study demonstrates for the first time that starch/AMG hydrogels can produce and deliver glucose for *in situ* “fueling” MSCs and, thus, improving their survival and paracrine functions both *in vitro* in near-ischemic conditions and *in vivo* post-implantation.

MATERIALS & METHODS

CELLS AND CELL CULTURE

Culture of Human Mesenchymal Stem Cells (hMSCs)

hMSCs were isolated from bone marrow obtained as discarded tissue during routine surgery from four adult donors at the Lariboisiere Hospital, Paris, France. The tissues of interest were collected with the patient’s informed consent and in agreement with Lariboisiere Hospital regulations. hMSCs were isolated from each donor’s bone marrow using a procedure adapted from a literature report (Gnecchi *et al.*, 2009), characterized by CD marker expression and differentiation potential, and cultured in Alpha Minimum Essential Medium (α -MEM; PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% Fetal Bovine Serum (FBS; PAA Laboratories GmbH, Les Mureaux, France) and 1% antibiotic/antimycotic (atb/atm; v/v, PAA Laboratories GmbH, Cörbe, Germany) under standard cell culture condition, that is, a humidified, 37°C, 5% CO₂, and 95% air environment. When 80-90% confluence was reached, the hMSCs were trypsinized using trypsin-EDTA (Sigma-Aldrich, St Quentin Fallavier, France), and replated at a density of 10×10^3 cells/cm². hMSCs from the four donors were pooled at an equal ratio at passage 1 and were further expanded up to passage 4 before use in experiments.

Transduction of hMSCs

For the *in vivo* study of hMSC survival, the pooled hMSCs (at passage 2) were transduced with a lentiviral vector encoding for the firefly luciferase and the ZSGreen proteins (pRRLsin-MND-Luc-IRES2-ZS Green-WPRE ; TRANSBIOMED, Bordeaux, France) as previously described (Becquart *et al.*, 2012) and further expanded. Flow cytometry analysis of ZSGreen-positive cells provided evidence that 88% of the hMSCs were transduced (data not shown). These cells will be referred to as “Luc-ZSGreen-hMSCs” thereafter.

Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs (Lonza, Walkersville, USA) were cultured in Endothelial Cell Growth Medium (EBM-2; Lonza, Levallois-Perret, France) supplemented with 5% FBS and 1% ATB/ATM, under standard cell culture conditions. When 80-90% confluence was reached, the HUVECs were trypsinized and replated at a density of 15×10^3 cells/cm². Cells at passage 4 were used for the *in vitro* experiments.

HYDROGELS

Preparation of wheat starch solutions

Wheat-derived starch solutions were prepared at either 0.2, 1, 2, or 4% (w/v) concentrations in the 10 mM HEPES buffer (pH 7.4), and mixed with another buffer containing 3 M NaCl, 0.4 M CaCl₂ (pH 7.4) (all compounds from Sigma-Aldrich, Lyon, France). The wheat starch powder was completely dissolved by stirring and heating up the solutions at 92°C for 2 hours.

Preparation of cellularized hydrogels

Four types of cellularized hydrogels were prepared: (i) “Glc-free”: glucose-free hydrogel that represents a negative/empty control hydrogel; (ii) “Glc 5.5 mM”: glucose hydrogel loaded with 5.5 mM glucose that represents a standard control hydrogel; (iii) “Starch”: fibrin hydrogels loaded with starch; and (iv) “Starch/AMG”: fibrin hydrogels loaded with starch and amyloglucosidase (AMG; Sigma-Aldrich, Lyon, France). All types of hydrogel were prepared by mixing 2 solutions (Mix 1 and Mix 2) as described in Table 1. The hMSCs were loaded within the Mix 1 that contained fibrinogen (Merck Chemicals, Fontenay-sous-Bois, France), HEPES buffer and aprotinin (Sigma-Aldrich). Depending of the type of hydrogel prepared, the Mix 2 contained either thrombin (Sigma-Aldrich) and HEPES for the Glc-free hydrogels, or thrombin, HEPES and glucose for the Glc 5.5 mM hydrogels, or thrombin and starch for the Starch hydrogels, or thrombin, starch and AMG for the Starch/AMG hydrogels. 100µl hydrogels were loaded with 10^5 hMSCs for the *in vitro* experiments, while 200µl hydrogels were loaded with 2×10^5 Luc-ZS Green-hMSCs for the *in vivo* assessment of hMSC survival and 125 µl hydrogels were loaded with 1.25×10^5 hMSCs for the *in vivo* assessment of the proangiogenic potential of the hMSC secretome.

Mix 1 was first deposited on a hydrophobic surface (specifically, a polytetrafluoroethylene (PTFE), disk diameter = 5 mm) and Mix 2 was then added and homogenized within the Mix 1. These hydrogels were maintained at 37°C in a humidified atmosphere for 1 hour. After polymerization, each hydrogel was carefully removed from the PTFE surface, placed in individual wells of 12-wells tissue-culture plates, and immersed in 2 mL serum- and glucose-free α -MEM culture medium before use in either the *in vitro* or *in vivo* experiments.

100 μ L Hydrogels ^(a)

Composition	Mix x	Glc free	Glc 5.5 mM	Starch	Starch/AMG	Final Concentration
Fibrinogen (50 mg/mL)	1	36 μ L	36 μ L	36 μ L	36 μ L	18 mg/mL
HEPES (10 mM)	1	5 μ L	5 μ L	5 μ L	5 μ L	0.5 mM
Aprotinin (3000 U/mL)	1	3.7 μ L	3.7 μ L	3.7 μ L	3.7 μ L	112 U/mL
hMSCs number	1	10 ⁵ ^(b)	10 ⁵ ^(b)	10 ⁵ ^(b)	10 ⁵ ^(b)	10 ⁶ cells/mL ^(b)
Thrombin (500 U/mL)	2	3.6 μ L	3.6 μ L	3.6 μ L	3.6 μ L	18.6 U/mL
HEPES (10 mM)	2	46.7 μ L	46.4 μ L	1.7 μ L	NA	5.1 mM
NaCl (3 M) CaCl ₂ (0.4 M)	2	5 μ L	5 μ L	NA	NA	NaCl (0.15 M) CaCl ₂ (0.02 M)
Glc (2,750 mM)	2	NA	0.2 μ L	NA	NA	5.5 mM
Wheat Starch (0.2, 1, 2 or 4% (w/v))	2	NA	NA	50 μ L	50 μ L	0.1, 0.5, 1 or 2% (w/v)
AMG (117 mg/mL)	2	NA	NA	NA	1.7 μ L	1.95 mg/mL

Table 1: Composition of the hydrogels used in the present study. The indicated volumes correspond to a 100 μ L hydrogel sample used for the *in vitro* experiments. ^(a) For 125 μ L hydrogels and 200 μ L hydrogels, each volume was increased 1.25- and 2-fold, respectively. ^(b) 1.25x10⁶ hMSCs were loaded into 125 μ L hydrogels for the *in vivo* assessment of the proangiogenic potential of hMSC secretome while 2x10⁵ Luc-ZS Green-hMSCs were loaded in 200 μ L hydrogels for the *in vivo* assessment of hMSC survival. Glc = Glucose; NA = Not Applicable.

IN VITRO EXPERIMENTS

Hydrogel-contained glucose quantification without hMSCs

The amount of glucose inside the hydrogels was quantified using a glucose electrode biosensor (AniRA-NeuroChem platform, University Lyon I, France) placed at the center of the samples. Briefly, the glucose microelectrode biosensor was composed of a 25 μm diameter platinum wire insulated in a pulled glass capillary with a 100 μm long sensing tip coated with electropolymerized poly-m-phenylenediamine, and with glucose oxidase enzyme immobilized with poly(ethyleneglycol) duglycidyl ether (PEGDE) (Vasilyeva *et al.*, 2011). Glucose oxidation into gluconolactone by the enzyme produced hydrogen peroxide (H_2O_2), which was reoxidized at the platinum electrode, producing an oxidation current which was directly proportional to glucose concentration (based on standard curve). Exogenous glucose released outside the glucose hydrogels and the starch hydrogels loaded with or without AMG was monitored using a biomedical ARCHITECT C8000 (Abbott Technology) robot.

Hydrogels surface topography

The surface topography of the glucose hydrogels and starch hydrogels loaded with or without AMG was assessed using environmental scanning electronic microscopy (Philips eSEM FEG/XL-30.). These samples were treated by liquid nitrogen for 30 seconds just before scanning to make the hydrogel external surface visible by the method used. Images of the hydrogel surface were collected and photographed.

Near-ischemic culture conditions

The “ischemic” environment was simulated *in vitro* by culturing the hydrogel-contained hMSCs under both near-ischemic (0.1% O_2) and nutrient (including both serum and glucose) deprivation. Near-ischemic culture conditions were achieved using a well-characterized, finely-controlled, proOx-C-chamber system (C-Chamber, C-374, Biospherix, New-York, USA). The oxygen concentration in this chamber was maintained at 0.1%, with a residual gas mixture composed of 5% CO_2 and balance nitrogen. The temperature of the system was maintained at 37°C for the duration of the experiments. Environmental nutrient deprivation was achieved using a glucose-free α -MEM (PAN Biotech GmbH).

Quantification of viable and apoptotic cells within hydrogels

The viability of hMSCs maintained inside the hydrogels under near-ischemic conditions was evaluated after 1, 7 and 14 days of culture. At the prescribed times, the

hydrogels were incubated with both 1 µg/mL nucleic acid stain Hoechst 33342 (HE; Sigma-Aldrich) and 1 µg/mL propidium iodide (PI; Sigma-Aldrich) at 37°C for 20 minutes; the hydrogels were then observed and photographed using a Zeiss confocal microscope (LSM 800, Zeiss, Göttingen, Germany). Hydrogels were then digested and hMSCs were detached from hydrogels using trypsin-EDTA for 20 min, then PBS containing Bovin Serum Albumin 2% (BSA, Sigma-Aldrich) was added to stop the chemical action of trypsin. After centrifugation (at 3,500xg for 5 min) the hMSCs were re-suspended in fresh PBS, and analyzed using an Attune flow cytometer (LifeTechnologies, Saint Aubin, France). Cells which had stained both HE positive and PI negative were identified as “viable cells”, whereas cells which had been stained both HE positive and PI positive were identified as “dead cells”. Cell viability was expressed as the number of viable cells at each point tested normalized with the respective viable cell number at day 1 of culture. For each hydrogel tested, cell viability at day 1 of culture was determined to be more than 90% of the 10⁵ hMSCs seeded at day 0.

Proliferative potential of hydrogel-contained hMSC under near-ischemic

After 14 days under near-ischemic, hMSCs were trypsinized from the starch 2%/AMG hydrogels and transferred back into tissue culture-flask (10³ cells/cm²) and standard cell culture conditions (*i.e.*, 21% O₂ and further cultured in α -MEM containing 5.5 mM glucose and 10% FBS) for 10 days. The doubling time of these hydrogel-derived hMSCs was determined and compared to the respective result obtained using naive hMSCs, which had been previously cultured under standard cell culture conditions for 14 days.

Preparation of hMSC conditioned media (CM)

The hydrogel-contained hMSCs were maintained under near-ischemic conditions for 14 days. The supernatant cell culture medium was not changed for the duration of the aforementioned cell culture period. At 7 and 14 days of culture, the supernatant cell culture media were collected, centrifuged (at 700xg for 5 min), aliquoted, and kept at -80°C until further use. These media are designated as conditioned media (CM) thereafter in this paper.

Assessment of the chemotactic effect of conditioned media

The chemo-attractive potential of CM from hydrogel-contained hMSCs maintained under near-ischemic conditions for either 7 or 14 consecutive days was determined using

the Boyden chamber migration assay. Briefly, 600 μ L of each respective CM were added in the bottom well of the Boyden chamber, and 2×10^4 hMSCs were seeded on the top of the 8- μ m-pore-diameter porous membrane (disk diameter = 6.5 mm; Transwell®; VWR International, Fontenay-sous-Bois, France), previously coated with 0.5% gelatin (Sigma-Aldrich) and cultured in glucose- and serum-free media. Serum-free α -MEM and α -MEM supplemented with 10% FBS were added in the bottom well as negative and positive controls, respectively.

After 6 hours maintenance under standard cell culture conditions, the cells on the top of the porous membrane were scrapped in order to remove the hMSCs that had not migrated from the original seeding location. These porous membranes were then fixed using paraformaldehyde 11% (Sigma-Aldrich) at room temperature for 30 minutes and stained using an azure eosin methylene blue 0.4% solution (Giemsa; Sigma-Aldrich) for 3 minutes. The hMSCs that had migrated through the porous membrane were photographed using a Keyence VHX-2000F microscope (Courbevoie, France), and counted using *Image J* free software (National Institute of Health, Bethesda, USA).

Assessment of released bioactive mediators

The presence of chemotactic and proangiogenic mediators in CM were quantified using Luminex technology (Millipore, Billerica, USA) following the manufacturer's instructions. The levels of 16 mediators, specifically, IL-8, CCL2, MMP9, Chemerin, MIF, CXCL2, CXCL5, CXCL10 (known as chemotactic growth factors) and VEGF-A, VEGF-C, VEGF-D, ANGPT-1, ANGPT-2, ANG, FGF-BASIC, Endostatin (known as proangiogenic growth factors) were evaluated using the MasterPlex QT 1.0 system (MiraiBio, Alameda, USA) and analyzed using Luminex-100 software version 1.7 (Luminex, Austin, USA).

IN VIVO EXPERIMENTS

Animals

Ten-week-old female nude mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France). All procedures regarding housing, handling, surgery, and recovery of the animals after surgery were conducted in compliance with the guidelines of the new European Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The surgery protocol used had received approval by the Ethics Committee on

Animal Research at the Lariboisiere/Villemin (Paris, France; number S85 / 2014-07-30; number S158/2018-09-07).

Ectopic implantation

Before each surgical procedure, a dose of buprenorphine (Buprecare®; 0.1 mg/kg animal weight; Axience, Pautin, France) was administrated subcutaneously in each mouse and the skin was prepared for surgery using application of povidone-iodine (Betadine®, Vetoquinol, Paris, France). Anesthesia was induced by intraperitoneal administration of ketamine (Ketamine1000®; 100 mg/kg animal weight; Virbac, France) and xylazine (Rompun® 2%; 8 mg/kg animal weight; Bayer HealthCare, Berlin, Germany). Flowing oxygen was delivered using a specific mask for each animal throughout the surgical procedure. Either two (for the assessment of cell proangiogenic potential) or four (for the assessment of cell viability) symmetrical incisions (each 7.5 mm in length) on both sides of the spine were made on the back of each mouse, and subcutaneous pouches were created. Hydrogels were then carefully and randomly inserted into each pouch. Skin closure was accomplished with an interrupted suture pattern using 4.0 polyglactin 910 sutures (Ethicon, Issy-les-Moulineaux, France). Throughout the post-operative period, the mice were monitored on a daily basis by trained animal-care personnel. Food and water were available ad libitum to the animals.

Assessment of cell viability

At prescribed time points during the 14 days of implantations (*i.e.*, on days 1, 7 and 14) the viability of the Luc-ZS Green-hMSCs contained within the implanted hydrogels was monitored non-invasively using a bioluminescence imaging system (Ivis, Lumina II, Caliper Life Sciences, Villebon-sur-Yvette, France) as previously described (Logeart-Avramoglou *et al.*, 2010). For this purpose, the mice were anesthetized by delivery of 3% isoflurane (Iso-Vet®; Piramal HealthCare, Northumberland, UK) in oxygen, and 100 mg/kg D-luciferin was injected intramuscularly in the area of the hydrogel locations. The mice were then placed in a ventral position inside the detection chamber of the bioluminescence system and maintained under anesthesia. Images were taken every 5 minutes during a 1 hour period of time. A region of interest surrounding each hydrogel was manually defined on each image and the photon flux emitted by each hydrogel was quantified using the *Living Image*® 3.2 software (Caliper Life Sciences). For each mouse and time point tested, the highest BioLuminescence Intensity (BLI) signal was retained.

The percentage of viable cells post implantation was determined as the photon fluxes measured at each time point tested normalized with the respective BLI signal acquired the day after surgery (*i.e.*, day-1 of data acquisition). On days 7 and 14, the mice (6 animals for each time point) were sacrificed (using an overdose of intracardiac pentobarbital delivery (Dolethal®; Vetoquinol, Paris, France), and the hydrogels were explanted, fixed in 4% paraformaldehyde, and embedded in paraffin. All prepared paraffin sections were processed for human beta-2-microglobulin immunodetected (hβ2-MG, a membrane protein that stains human cells), using the Envision+ kit (Dako, Les Ulis, France) and a polyclonal rabbit anti-hβ2-MG (1/1000, Novocastra, Nanterre, France) as the primary antibody as previously described (Becquart *et al.*, 2010).

Assessment of the proangiogenic potential of hMSCs-containing-hydrogels

Each 125 μL hydrogel (loaded with 1.25×10^6 hMSCs) was encapsulated at the center of a silicone tube (Silicone DIA; 4mm internal diameter, 6 mm external diameter, 16 mm height ; Weber Métaux, France) and between 2 layers (3 mm height) of low-concentrated fibrin (1mg/mL, without aprotinin) gel filled from the top and from the bottom of each silicone tube edges. The fibrin on the edges fully degraded 24 hours after implantation.

To visualize the blood vessels formation driven by hMSC-contained hydrogels, the mice were injected with a radio-opaque polymer compound (Microfil®, Flowtech, Carver, MA, USA) at either 14 or 21 days post-implantation. For this purpose, each animal was deeply anesthetized and the skin from the thorax and the rib cage was incised to access the heart. The left ventricle was catheterized using a 20G canula (BD Venflon, Beckson Dickinson Infusion, Sweden) and the right atrium was cut for blood removal. Each animal was first perfused with isotonic saline (50 mL) containing heparin (100 UI/mL) using a pump (at 6 mL/min flow rate) for 6 min to rinse blood from the vasculature, and then manually perfused with 14 mL of Microfil® (prepared according to the manufacturer's instructions using 6.3 mL of Microfil®, 7 mL of the specific diluent, and 0.7 mL of the specific curing agent). During filling, the flow of the injection was approximately 2 mL/min to force the Microfil® into the capillary networks without extravasation into the surrounding tissue. The perfused euthanized animals were stored at 4 °C overnight to allow polymerization of the Microfil® and then the silicone tubes containing the hydrogels were explanted and fixed in 4% paraformaldehyde overnight. The specimen were then imaged using a Skyscan172 micro-CT-scanner (Bruker, Evere, Belgium) with voltage 80 kV, current 100 mA, exposure for 85 ms and 0.3 degree

rotation step settings, without any filter. The images obtained had 10 μm pixel size. The scanned images were then reconstructed as a stack of slices of each sample using Nrecon software, 16 bits (Bruker, Evere, Belgium). The volumes of interest (VOI; 2 per sample) were set as cylinders overlapping the internal diameter of the silicone tube (4 mm), between the 1st and the 3rd mm height from the top and from the bottom of the silicone tube edges. Indeed, as some hMSC-containing hydrogels tested partly resorbed after implantation, the aforementioned method allowed to rigorously defining similar volume of interest for each sample. New blood vessel volume was reported as the amount of binarized object-volume measured within the designated volume of interest within the threshold gray values 120-255 on CTan software (Bruker). Values regarding new blood vessel thickness and numbers were calculated using the aforementioned thresholds.

Statistical analyses

Numerical data were expressed as means \pm standard deviation (SD). Statistical analyses were performed using commercially available software (GraphPad 6.0 Software, California Corporation, USA). Each of the *in vitro* experiments was conducted in triplicate and repeated on, at least, three separate occasions. Each of the *in vivo* experiments was conducted using at least 6 samples for each group of hydrogels tested, and repeated on two separate occasions. Data were analyzed statistically using analysis of variance and the Bonferroni post-*hoc* test, and the Mann-Whitney test for parametric and non-parametric tests, respectively. For all analyses, the confidence interval was set at 95% and the significant level at $p < 0.05$.

RESULTS

The starch/AMG hydrogels produce glucose

We first determined whether the starch/AMG hydrogels (Figure 8-1a) produce glucose. To this aim, hydrogels were prepared with increasing concentration of starch (from 0.1% to 2% w/v) and 1.95 mg/mL AMG (*i.e.*, standard AMG concentration). Production of glucose inside the hydrogels was then monitored using a glucose microelectrode biosensor placed in the center of each hydrogel. In the presence of AMG, the starch hydrogels produced glucose as a function of starch concentration (Figure 8-1b). In the closed system used in this study, production of glucose plateaued within 400 to 600 seconds and was maximal ($p < 0.05$) with the highest (2%) starch concentration ($3.76 \pm$

0.31 mM; Figure 8-1b). Moreover, the initial kinetics (V_i) of the starch/AMG reaction was maximal ($p < 0.05$) with the 2% starch concentration. In contrast, in the absence of AMG, there was no detectable glucose production within the aforementioned time frame (Figure 8-1b).

The kinetics of glucose release out of both control hydrogels and the starch 2% hydrogels in the presence or absence of AMG was next investigated for 14 days. To this aim, the hydrogels were immersed into glucose-free and serum-free α -MEM medium and the amount of released glucose in the respective supernatants was monitored. Media were not changed during the period of the experiment. In this open model system, release of glucose from the starch 2%/AMG hydrogels was observed over the entire period of the study; this result suggested that the starch 2%/AMG hydrogels delivered glucose over at least a 14 day-period. Interestingly, in the absence of AMG, a spontaneous glucose release was observed in the starch 2% hydrogels, which plateaued at 7 days; the amount of glucose released from AMG-free starch hydrogels was, however, 1.8 times less than that released from the AMG-containing starch hydrogels ($p < 0.05$; Figure 8-1c).

We also explored the production of glucose within the starch 2%/AMG hydrogels as a function of the AMG concentration. In this case, production of glucose inside these hydrogels was linearly proportional with the concentration of AMG ($R^2 = 0.977$ at 180 s ; Figure 8-1d).

We also investigated *in vivo* glucose production from the hydrogels of interest to the present study. To this aim, starch 2%/AMG and starch 2% hydrogels were implanted ectopically in a mouse model. At day 7 and 14 post-implantation, the glucose concentrations monitored in the post-mortem hydrogels were compared to the ones measured in implanted control hydrogels (glucose-free and 5.5 mM glucose hydrogels). At both time points tested, the glucose concentration was higher inside the starch 2%/AMG hydrogels ($p < 0.05$) compared to results obtained with the starch 2% hydrogels (4.6 and 9.4 fold-increased), glucose-free hydrogels (3.9 and 2.9 fold-increased), and 5.5 mM glucose hydrogels (2.3 and 2.7 fold-increased) (Figure 8-1e).

The starch/AMG hydrogels improve the survival of hMSCs in near-ischemic conditions in vitro

We next determined whether the tested starch/AMG hydrogels sustained the viability of hMSCs under near-ischemic conditions. To this aim, hydrogels prepared with increasing concentrations of starch and with (Figure 8-2a1) or without (Figure 8-2a2)

standard AMG concentration, were loaded with 10^5 hMSCs and maintained in glucose-free and serum-free α -MEM media under near-ischemic conditions. At days 7 and 14, the hydrogels were degraded and the number of viable hMSCs was assessed using flow cytometer (Figure 8-2a1, 2a2). At both prescribed time points, maximal survival of hMSCs was achieved in the starch/AMG hydrogels containing the highest (2%) amount of starch ($p < 0.05$). At day 14, the hMSCs only survived inside the starch 2%/AMG hydrogels. Moreover, and compared to results obtained from the starch/AMG hydrogels, the number of viable cells in the starch hydrogels without AMG was drastically reduced at day 7 with almost no viable hMSCs remaining at day 14 for all starch concentrations tested (Figure 8-2a1 vs. 2a2). At day 7, the number of viable cells inside the starch/AMG hydrogels was linearly proportional to the initial starch concentration ($R^2 = 0.984$) (Figure 8-2b). These results provided evidence of the critical role of AMG in achieving the glucose concentrations required for ensuring long-term survival of hMSCs.

In order to determine whether the glucose released from the hydrogels was responsible for the survival of hMSCs under near-ischemic conditions, 2-Deoxy-D-Glucose (a competitive inhibitor for the production of glucose-6-phosphate from glucose at the phosphoglucosomerase level) was added to the starch 2%/AMG hydrogels, glucose-free, and 5.5 mM glucose hydrogels loaded with 10^5 hMSCs. Massive cell death was observed in all the conditions tested within 3 days of exposure to the aforementioned inhibitor (Figure 8-2c). These results provided evidence that indeed the glucose release from the starch was responsible for the ensuring the survival of hMSCs under the tested conditions. To further investigate the viability of the hMSCs in the starch 2%/AMG hydrogels after 14 days of exposure to near ischemic conditions, hMSCs recovered from the hydrogels using trypsin digestion were seeded onto tissue-culture plates and their proliferative ability was compared to that obtained from naive hMSCs (controls) over 10 days. The cell doubling time of these two populations was similar ($p > 0.05$) (Figure 8-2d). Moreover, these two cell populations were indistinguishable in terms of CD marker expression: specifically, both cell populations were positive for CD 73, CD90, CD105 and negative for CD45 (Figure 8-2e).

Taken together, these data provided evidence that the starch/AMG hydrogels produced glucose for “fueling” hMSCs thus enhancing their survival while retaining their proliferative potential in ischemic environmental conditions. Since the most effective hydrogel for enhancing hMSC survival was the starch 2%/AMG one, this concentration was used for the rest of the present study.

The starch 2%/AMG hydrogels are more effective than glucose hydrogels in extending the in vitro survival of hMSCs in near-ischemic conditions

We compared the efficacy of starch 2%/AMG hydrogels to that of glucose hydrogels prepared by loading free 5.5 mM glucose in fibrin hydrogels in order to determine which hydrogel environment enhanced survival of hMSCs in near-ischemic conditions. The hMSCs loaded in the 5.5 mM glucose hydrogels exhibited improved survival when compared to hMSCs loaded in glucose-free hydrogels under near-ischemic conditions at day 7; hardly any viable hMSCs were present in the glucose-free and in the 5.5 mM glucose hydrogels at day 14 (Figure 8-3a, 3b). In contrast, the number of viable hMSCs loaded in the starch 2%/AMG hydrogels increased by 5-fold when compared to the cells loaded in glucose hydrogels at day 7 of culture. At day 14, viable hMSCs were only observed in the starch 2%/AMG hydrogels (Figure 8-3b).

Overall, these results demonstrate that, compared to results obtained with the 5.5 mM glucose hydrogels, the survival of hMSCs in near-ischemic conditions was improved when the cells were dispersed within the starch 2%/AMG hydrogels.

The starch/AMG hydrogels are more effective than glucose hydrogels in enhancing the chemotactic and proangiogenic functions of hMSCs in near-ischemic conditions

Since the chemo-attraction of progenitors (such as MSCs) and endothelial cells are relevant aspects in the success of MSC-based regenerative medicine, we compared the chemotactic potential of supernatant CM collected from hMSCs seeded into either glucose-free or 5.5 mM glucose hydrogels to the one of supernatant CM collected from MSCs seeded into starch 2%/AMG hydrogels after exposure to near-ischemic conditions for 7 and 14 consecutive days. The respective CM-mediated chemo-attraction of either hMSCs or HUVECs was assessed using the Boyden chamber migration test.

Compared to CM from hMSCs seeded in glucose-free and in 5.5 mM glucose hydrogels, CM from hMSCs seeded in starch 2%/AMG hydrogels promoted significant ($p < 0.05$) increase in the chemotactic potential of hMSCs (9.3- and 2.0-fold increase, respectively, after 7 days; 4.8- and 2.7- fold increase, respectively, after 14 days; Figure 8-4a, 4b). Moreover, the concentrations of the chemotactic mediators MCP-1, MIF, MIP-2, ENA 78 and IP-10 were significantly ($p < 0.05$) increased in CM from hMSCs seeded in starch 2%/AMG hydrogels when compared to results obtained using CM from glucose-free and 5.5 mM glucose hydrogels for 14 days (Figure 8-4c).

Compared to HUVECs responses to CM from hMSCs seeded in either glucose-free or 5.5 mM glucose hydrogels, the CM from hMSCs seeded in starch 2%/AMG hydrogels promoted a significant ($p < 0.05$) increase in their chemotactic potential towards the HUVECs (4.2- and 2.2-fold increase, respectively after 7 days; 3.7- and 3.7-fold increase, respectively, after 14 days; Figure 8-4d, 4e). Moreover, the concentrations of the proangiogenic mediators VEGF-A, VEGF-C, VEGF-D, ANGPT-1, ANGPT-2, ANG and FGF-BASIC were significantly ($p < 0.05$) increased in the CM from hMSCs seeded in starch 2%/AMG hydrogels compared to results obtained using CM from glucose-free and 5.5 mM glucose hydrogels for 14 days (Figure 8-4f). Altogether, these data provided evidence that the starch 2%/AMG hydrogels promoted the *in vitro* chemotactic and proangiogenic functions of hMSCs in near-ischemic conditions.

The starch/AMG hydrogels are more effective than glucose hydrogels to increase the in vivo survival of hMSCs after subcutaneous implantation

To extend the results of the *in vitro* studies (Figure 8-3) to the *in vivo* setting, we assessed the potential of starch 2%/AMG hydrogels in enhancing survival of embedded hMSCs in an ectopic transplantation mouse model, *i.e.*, an avascular model.

Luc-ZSGreen-hMSCs embedded within the starch 2%/AMG hydrogels exhibited significant ($p < 0.005$) increase in BLI signal compared to the signals obtained with either the glucose-free or the glucose hydrogels (3.4 and 2.8 fold increase, respectively, after 7 days, and 2.9 and 1.9 fold increase, respectively, after 14 days) (Figure 8-5a, 5b). To validate this trend, the numbers of hβ2-MG-positive cells were counted on histological sections of hydrogels explanted at both day-7 and day-14 post subcutaneous implantation (Figure 8-5c). A significant ($p < 0.05$) enhanced number of positive hβ2-MG-cells in the starch 2%/AMG hydrogels was observed when compared to the results obtained from cells in the glucose-free hydrogels (7.3-fold increase after 7 days, and 7.2-fold increase after 14 days; Figure 8-5d).

Taken together, these data provided evidence that the starch 2%/AMG hydrogels enhanced MSC survival after ectopic implantation in mice for up to 14 days.

The starch/AMG hydrogels are more effective than glucose hydrogels in increasing the proangiogenic functions of hMSCs after implantation

The proangiogenic functions of hMSCs were investigated in an ectopic transplantation mouse model. To this aim, hMSCs were loaded into either glucose-free,

5.5 mM glucose, or starch 2%/AMG hydrogels and the volume occupied by new blood vessels adjacent to these gels was quantified after 14 and 21 days (Figure 8-6a). The new blood vessels in the vicinity of the starch 2%/AMG hydrogels filled a 2.9- and 4.0-fold increased volume ($p < 0.01$) compared to results obtained from glucose-free hydrogels at days 14 and 21 post-implantation, and a 3.4 and 2.3-fold increased volume ($p < 0.01$) compared to results obtained from 5.5 mM glucose hydrogels at the aforementioned time points post-implantation, respectively (Figure 8-6b, 6c). In contrast, the new blood vessel diameter and blood vessel number in either starch 2%/AMG hydrogels, glucose-free, or 5.5 mM glucose-hydrogels were similar (supplementary data, Table 8-1).

Overall, these data demonstrate that the starch 2%/AMG hydrogels promoted the proangiogenic function of hMSCs after implantation.

DISCUSSION

Upon implantation, maintenance of viable and functional hMSCs in the 3D environment of tissue constructs is the most critical challenge for developing successful tissue constructs of clinically-relevant volume for regenerative medicine applications. To achieve this important aim, we previously identified that the presence of glucose is sufficient to meet MSC metabolic requirements in near ischemia and ensure MSC survival upon implantation. Delivering glucose at physiologic level over at least 2 weeks post-implantation is a true challenge because glucose is a small, uncharged and highly water-soluble molecule diffusing rapidly whose high concentrations can disturb the osmotic pressure and cause cell lysis. To overcome this limitation, we designed and developed a starch/AMG hydrogel that contained AMG, an enzyme that enabled to supply sufficient levels of glucose from starch to ensure the survival and paracrine functionality of hMSC when transplanted *in vivo*.

The present report provides evidence that starch/AMG hydrogels release glucose as a function of starch and AMG concentrations (Figure 8-1b, 1d). Moreover, starch/AMG hydrogels can release glucose for a period of at least 14 days both *in vitro* (Figure 8-1c) and *in vivo* (Figure 8-1e). A very important result was the finding that the delivered glucose content within the starch/AMG hydrogels was in the physiological range (from 5.5 to 27 mM) and was significantly ($p < 0.05$) increased compared to contents obtained in the control 5.5 mM glucose hydrogels (Figure 8-1e). An unexpected finding of the present study was, however, that starch hydrogels alone (i.e., without AMG)

spontaneously released glucose (Figure 8-1c). A possible explanation for this finding is the presence in starch used in the present study of trace amounts of enzymes that degrade starch. This spontaneous release of glucose from the starch hydrogels, however, was insufficient to enhance hMSC survival (Figure 8-2a, 2b) further supporting the use of AMG to achieve production of glucose at levels that ensure survival of hMSCs post-implantation.

Starch/AMG hydrogels significantly ($p < 0.05$) extended the survival of hMSCs in near ischemia *in vitro*. Specifically, hMSCs loaded into starch/AMG hydrogels exhibited a 33- and 80-fold increase in cell viability when compared to results from glucose-free hydrogels at days 7 and 14, respectively (Figure 8-3b). Another important result was that, loading either adipose-derived hMSCs (ADSCs) or human myoblasts in starch/AMG hydrogels also resulted in at least a 13- and 125-fold increase of cell survival compared to results from glucose-free hydrogels in near ischemia at days 7 and 14 (Supplementary Figure 8-7a, 7b). Most importantly, the starch/AMG hydrogels outperformed 5.5 mM glucose hydrogels as demonstrated by the observed 5- and 650-fold increase in hMSC survival at day 7 and 14, respectively, when exposed to near ischemia (Figure 8-3b). Glycolysis was the main energy-provider pathway to hMSCs loaded into starch/AMG hydrogels under near-ischemic conditions as blocking this pathway using 2-Deoxy-D-glucose resulted in massive cell death within 3 days (Figure 8-2c). These results confirmed previous results obtained by our group and others showing that MSCs exclusively rely on the glycolytic pathways under hypoxia (Deschepper et al., 2013; Moya et al., 2017;). The positive role of glucose released from starch/AMG hydrogels on hMSC survival was further substantiated by the demonstration that hMSCs released from starch/AMG hydrogels after 14 days exposure in near ischemia exhibited similar proliferative ability and CD marker expression with naïve hMSCs cultured at 21% O₂ (Figure 8-2d, 2e). Most importantly, after implantation in an ectopic mouse model, hMSCs embedded into starch/AMG hydrogels exhibited a 3.5- and 2.9- fold increase when compared to glucose-free hydrogels at days 7 and 14 (Figure 8-5a, 5b). These data were further confirmed by counting hMSCs on histological sections (Figure 8-5c, 5d). Moreover, a 2.8- and 1.7-fold increase in hMSC viability was observed when starch/AMG hydrogels were compared to 5.5 mM glucose hydrogels at days 7 and 14 (Figure 8-5a, 5b). Increasing the glucose concentration to 27 mM in the glucose hydrogels did not improve hMSC survival (data not shown). The results of the present study also revealed a 4-fold decrease in hMSC number between day 7 and 14 when these

cells were loaded into starch/AMG hydrogels (Figure 8-5b). A possible explanation for this outcome is that the activity of AMG decreased between days 7 and 14 post-implantation. This conjecture is reinforced by the observation that the glucose concentration of cell-free starch/AMG hydrogels implanted in an ectopic mouse model decreased of 26% between day 7 and 14 post-implantation (Figure 8-1e). Alternatively, the decrease in hMSC number may reflect the occurrence of an inflammatory response that induced hMSC death as demonstrated previously using xenogenic myoblasts in a mouse model (Rinsch et al., 2001). Future studies, including either enzyme immobilization or encapsulation to preserve AMG activity (Helena et al., 2009) or the use of immunosuppressors to decrease the inflammatory response (Rinsch et al., 2001) may be required to discern between these competing hypotheses.

The beneficial effects of hMSCs when used as regenerative medicine “tools” are predominantly due to the multitude of bioactive mediators secreted by these cells upon implantation. For this reason, we investigated whether the increase in hMSC survival led to an increase in hMSC paracrine functions. Indeed, CM from hMSCs loaded into starch/AMG hydrogels and exposed to near-ischemic for 7 and 14 days led to a 2- to 4-fold increase in their chemotactic potential towards hMSCs (Figure 8-4a, 4b) and HUVECs (Figure 8-4d, 4e) compared to results obtained using the 5.5 mM glucose hydrogels. These data are consistent with the higher concentrations of chemotactic and pro-angiogenic growth factors present in the CM from starch/AMG hydrogels when compared to pertinent results from the 5.5 mM glucose hydrogels (Figure 8-4c, 4f). These observations are further substantiated by our *in vivo* findings that revealed a 3.3- and 2.2-fold increase in the volume of peripheral new blood vessel induced in the starch/AMG hydrogels loaded with hMSCs compared to pertinent results obtained from 5.5 mM glucose hydrogels at 14 and 21 days post-implantation (Figure 8-6a, 6b).

Overall, the starch/AMG hydrogels provide a powerful platform for the production of glucose to “fuel” hMSCs and improve their survival and paracrine functions post-implantation. Resorption of such systems can be tailored to the targeted regenerative medicine applications by selecting the glucose polymer (e.g., starch *versus* maltodextrins), its biophysical properties, the enzyme (e.g., a single enzyme *versus* a cocktail of starch-degrading enzymes), concentration and delivery mode (Mickova et al., 2012).

CONCLUSION

The massive hMSC death post-implantation, occurring in avascular microenvironments depleted of oxygen and nutrients, is a major roadblock to successful advances of regenerative medicine. Various approaches, including development of oxygen-delivery scaffolds, have been proposed by scientists to address this issue. hMSCs loaded in such systems, however, exhibited improved cell viability for only up to 3 days without enhancing the new blood vessel formation in an avascular model (Cook et al., 2015; Lee et al., 2015). In the present study, we develop for the first time a nutritive hydrogel system which produces glucose at physiological ranges necessary to fuel hMSCs, and thus improve cell survival for at least 14 days under near-ischemic conditions and after implantation in a subcutaneous model. Moreover, the novel starch/AMG hydrogel promotes hMSC paracrine functions, evidenced by increasing new blood vessel formation for at least 21 days after implantation; such outcomes provide nutrients (especially glucose) and oxygen into the avascular post-implantation microenvironment. Looking forward, and given the current wide interest in the use of stem cells for regenerative medicine applications, the novel inbuilt system of glucose delivery has the potential of improved tissue engineering and regenerative methodologies, which enhance cell survival and functionality after implantation.

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AUTHOR CONTRIBUTIONS

DC designed and executed all the *in vitro* and post-implantation experiments of the present study, collected and analyzed data, and wrote the manuscript. LG assisted in conducting the *in vivo* experiments regarding neovascularization and assisted in collecting data. PJ, MA and BP assisted in designing and collecting data regarding the *in vitro* experiments about MSC survival. MS and MA designed microelectrode biosensors for glucose assessment in the hydrogels tested. DA assisted in the surgery experiments. MH assisted in collecting and analyzing pertinent data from the Boyden chambers experiments. VJT provided myoblasts. BJ, GA, PE and L-GV designed the first prototype of the nutritive hydrogel. PE and PH managed the project, provided financial support, wrote parts of, and edited the manuscript. All authors critically read and approved the present manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

PJ, PE, L-GV and PH disclose that, the novel enzyme-controlled, nutritive hydrogels used in the present project, was patented globally (EP14306700; 2014). The other authors confirmed no potential conflicts of interest.

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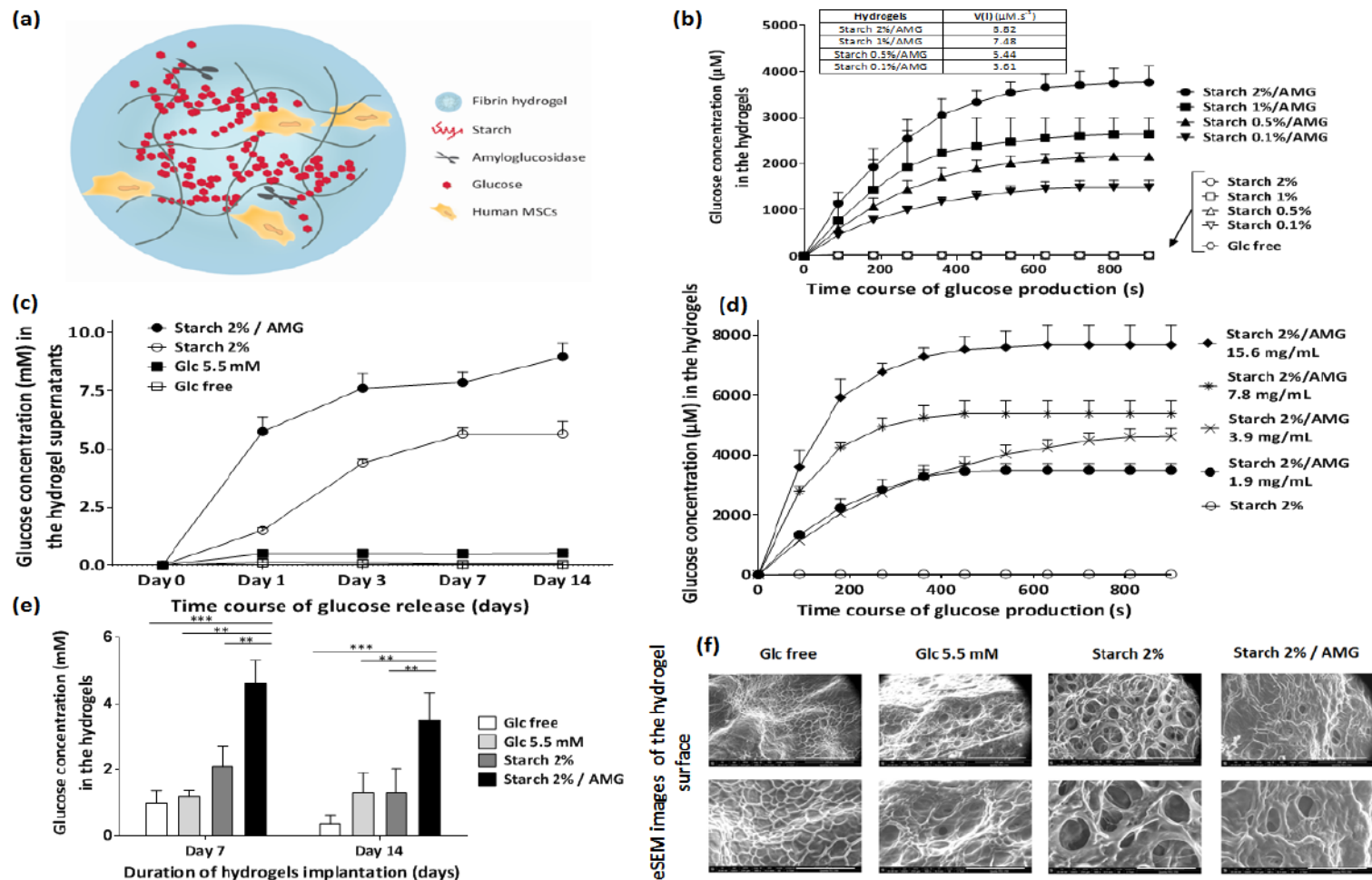


Figure 8-1 The starch/AMG hydrogels produce glucose. (a). Schematic (not to scale) of the components of the novel nutritive hydrogel. hMSCs were encapsulated in fibrin scaffolds. Starch (a polymer of glucose) was hydrolyzed by an AMG enzyme, which produced glucose to fuel hMSCs. (b). Production of glucose in starch hydrogels loaded in either the presence or absence of AMG 1.95 mg/mL, was monitored (using a glucose microelectrode biosensor) during the first 12 minutes (n=9). No glucose was monitored in all starch hydrogels without AMG and in glucose-free reservoirs. (c). Release of glucose from either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch hydrogels in the presence or absence of AMG 1.95 mg/mL. In this case, the glucose reservoirs and hydrogels were immersed into 1 mL glucose and serum-free MEM medium. Glucose released from the glucose reservoirs and from the hydrogels was monitored using biomedical ARCHITECT C8000 for 14 days (n=6). No glucose was monitored in media from the glucose-free reservoirs. (d). Production of glucose in starch hydrogels loaded with increasing concentrations of AMG was monitored (using a glucose microelectrode biosensor) during the first 12 minutes (n=9). (e). Glucose concentration was monitored inside either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch hydrogels loaded in the presence or absence of AMG 1.95 mg/mL, after subcutaneous implantation in nude mice for 7 and 14 days (n=9). (f). Representative eSEM micrographs of the external architecture of either the glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch hydrogels loaded in the presence or absence of AMG, following treatment with liquid nitrogen for 30 seconds. Scale bars: 200 μm (for eSEM images in the top row), 50 μm (for eSEM images in bottom row). Data are mean \pm standard deviation. Glucose-free reservoirs and starch hydrogels without AMG were used as negative controls. ** p < 0.01; *** p < 0.001 compared to glucose production results obtained from the starch 2%/AMG hydrogels (Mann-Whitney test). Abbreviations: AMG = amyloglucosidase; Glc = glucose; eSEM = environmental scanning electron micrographs.

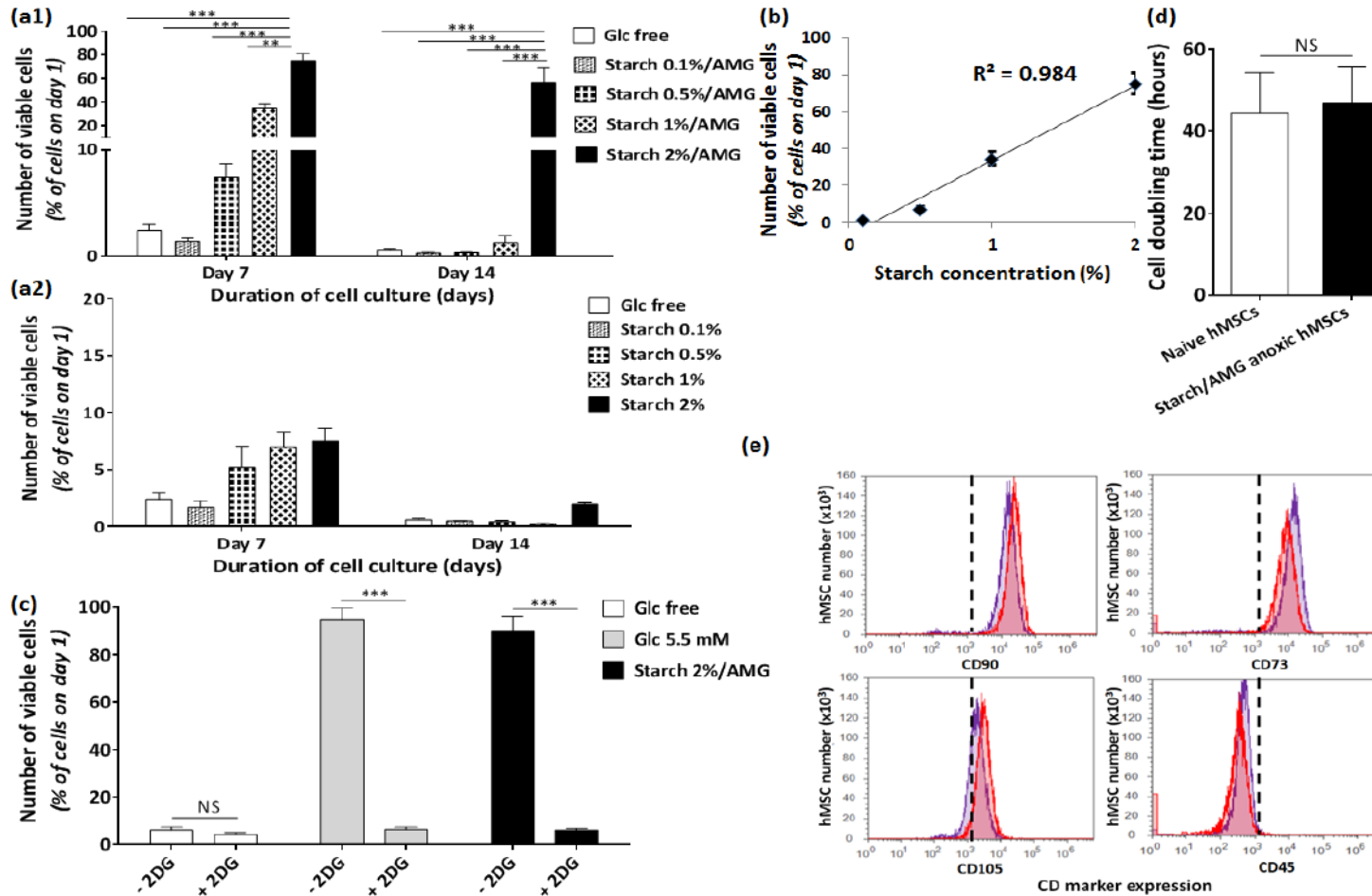


Figure 8-2 The starch/AMG hydrogel environment extends survival of MSCs in near-anoxia conditions. (a1 and a2). Viability of hMSCs seeded in either glucose-free reservoirs or starch hydrogels loaded in the presence (a1) or absence (a2) of AMG, after exposure to near-anoxia (0.1% O₂) conditions for 7 and 14 days were analyzed using flow cytometry (n=3). (b). Linear proportionality relation ($R^2 = 0.984$) between the number of viable cells maintained in the starch/AMG hydrogels under near-anoxia for 7 days as a function of the initial starch concentrations (n=3). (c). Viability of hMSCs seeded in hydrogels and exposed to near-anoxia conditions in either the presence or absence of 2DG (2-Deoxy-D-Glucose) for 3 days. At that time, the hydrogels were digested and the hMSCs were counted using the Malassez technique and excluding dead hMSCs stained with trypan blue (n = 3). (d). Doubling time of hMSCs in the starch 2%/AMG hydrogels after exposure to near-anoxia conditions for 14 days (i.e., Starch/AMG anoxic hMSCs), transferred back to standard cell culture conditions for 10 days, and compared to the viability results of hMSCs maintained under standard cell culture conditions (i.e., naive hMSCs) (n=6). (e). Flow cytometry characterization of hMSCs either maintained in the starch 2%/AMG hydrogels under near-anoxia (purple histogram) or cultured under standard conditions (red histogram) for 14 days. Data are mean \pm standard deviation. hMSCs contained in glucose-free reservoirs and starch hydrogels without AMG were used as negative controls for the results showed in frames (a1, a2 and b). hMSCs contained in glucose-free reservoirs were used as negative controls for frame (c). Naive hMSCs were used as positive controls for frame (d). NS: $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to cell viability results obtained from cells contained in the starch 2%/AMG hydrogels (Mann-Whitney test). Abbreviations: hMSCs = human mesenchymal stem cells; AMG = amyloglucosidase; Glc = glucose; HE = Hoescht 33342; PI = Propidium Iodide; -2 DG = absence of 2-Deoxy-D-glucose; +2 DG = presence of 2-Deoxy-D-glucose.

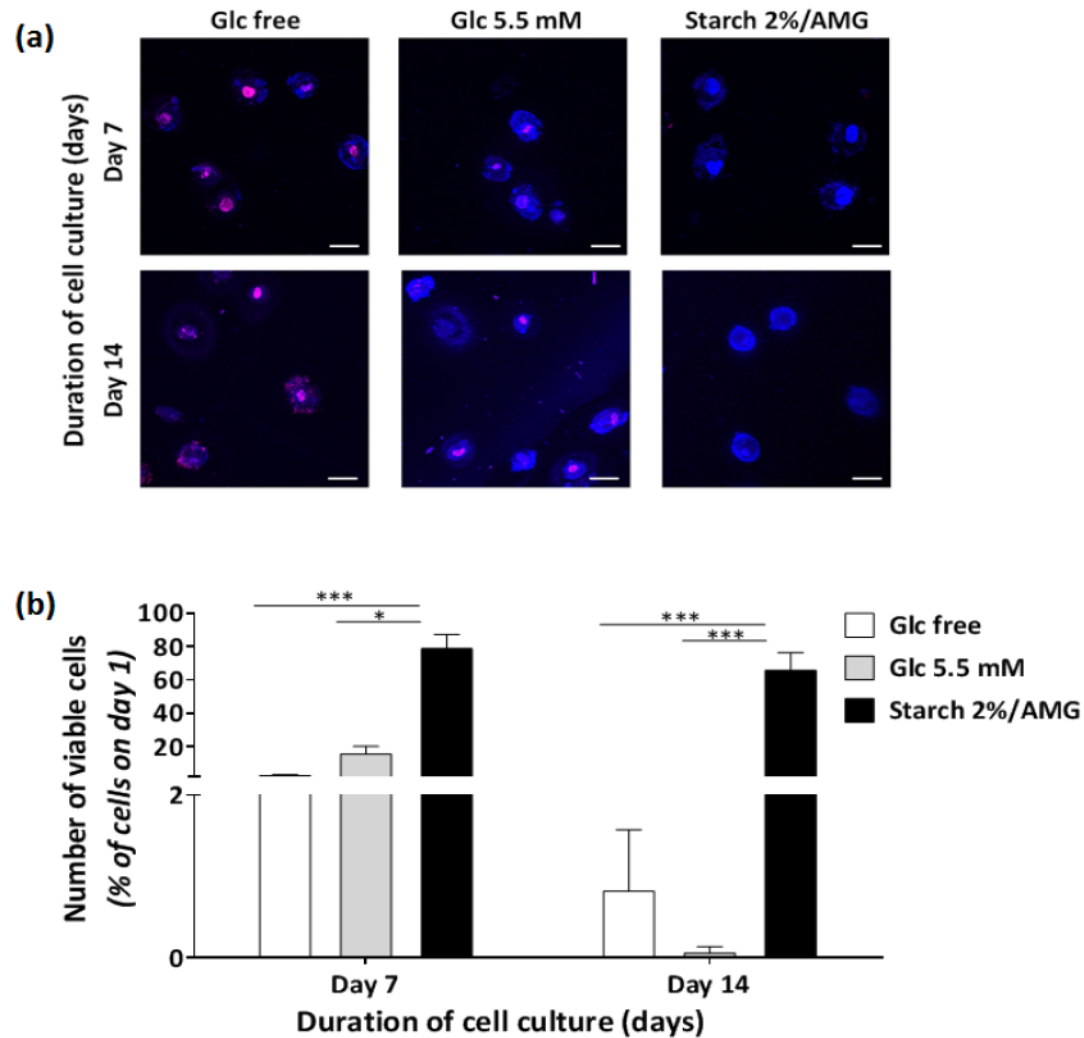


Figure 8-3 The starch 2%/AMG hydrogels are more effective than glucose reservoirs in extending the *in vitro* survival of hMSCs in near-anoxia conditions. (a). Representative confocal images of hMSCs either in glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels after exposure to near-anoxia conditions (0.1% O₂) for 7 and 14 days. These cells were stained with Hoescht 33342 (HE; cell nuclei in blue) and propidium iodide (PI; dead cells in red) stained Scale bar = 20 μm. (n=3). (b). Viability of hMSCs seeded in either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels, after exposure to near-anoxia conditions for 7 and 14 days. The cells were stained with HE and PI and analyzed using flow cytometry (n=9). Cell viability was expressed as the number of viable cells on days 7 and day 14 normalized by the respective viable cell number at day 1. Data are mean ± standard deviation. hMSCs contained in the glucose-free reservoirs were used as negative controls. *p < 0.05; *** p < 0.001 compared to cell viability results obtained from cells in the starch 2%/AMG hydrogels (Mann-Whitney test). Abbreviations: hMSCs = human mesenchymal stem cells; AMG = amyloglucosidase; Glc = glucose; HE = Hoescht 33342; PI = Propidium Iodide.

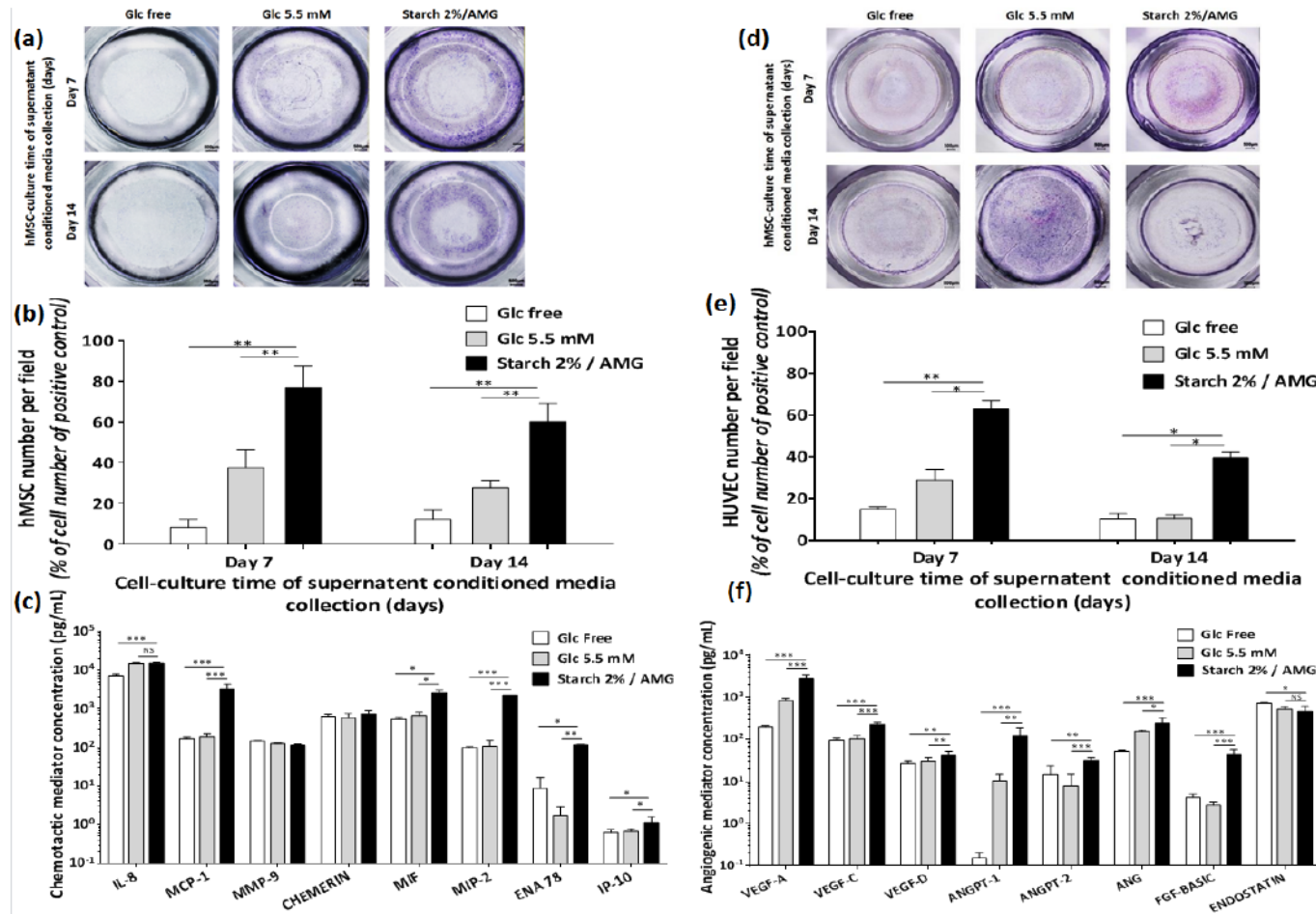


Figure 8-4 The starch/AMG hydrogels are more effective than glucose reservoirs in enhancing the chemotactic functions of hMSCs in near-anoxia conditions. (a). Representative light micrographs and (b) quantification of hMSC migration in Boyden chambers in response to conditioned media from hMSCs seeded in either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels, after exposure to near-anoxia conditions for 7 and 14 days. Scale bar = 500 μ m. (n=6). (c) Concentrations of the chemotactic mediators released by hMSCs maintained into either glucose free-reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels, after exposure to near-anoxia conditions for 14 days. (n=6). (d). Representative light micrographs and (e) quantification of HUVEC migration in Boyden chambers in response to conditioned media from hMSCs seeded in either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels, after exposure to near-anoxia conditions for 7 and 14 days. Scale bar = 500 μ m. (n=3). (f) Concentrations of the proangiogenic mediators released by hMSCs maintained into either glucose free-reservoirs, 5.5 mM glucose, or starch 2%/AMG hydrogels, after exposure to near-anoxia conditions for 14 days. (n=6). Fresh glucose-free MEM and FBS-free medium was used as negative control. Cell migration was expressed as the number of cells which had migrated across the Boyden chamber membrane normalized by the results obtained from the positive control. Data are mean \pm standard deviation. NS: $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to cell-chemotaxis results obtained from the supernatant CM from starch 2%/AMG hydrogels (Mann-Whitney test). Abbreviations: hMSCs = human mesenchymal stem cells; AMG = amyloglucosidase; Glc = glucose; MEM = minimum essential media; FBS = fetal bovine serum; IL = interleukin; MCP, monocyte chemoattractant protein; MMP = matrix metalloproteinase; MIF = macrophage inhibitory factor; MIP = macrophage inflammatory protein; ENA = epithelial neutrophil activating protein; IP = interferon gamma-induced protein; VEGF = vascular endothelial growth factor; ANGPT = angiopoietin; ANG = angiogenin; FGF-BASIC = basic fibroblast growth factor.

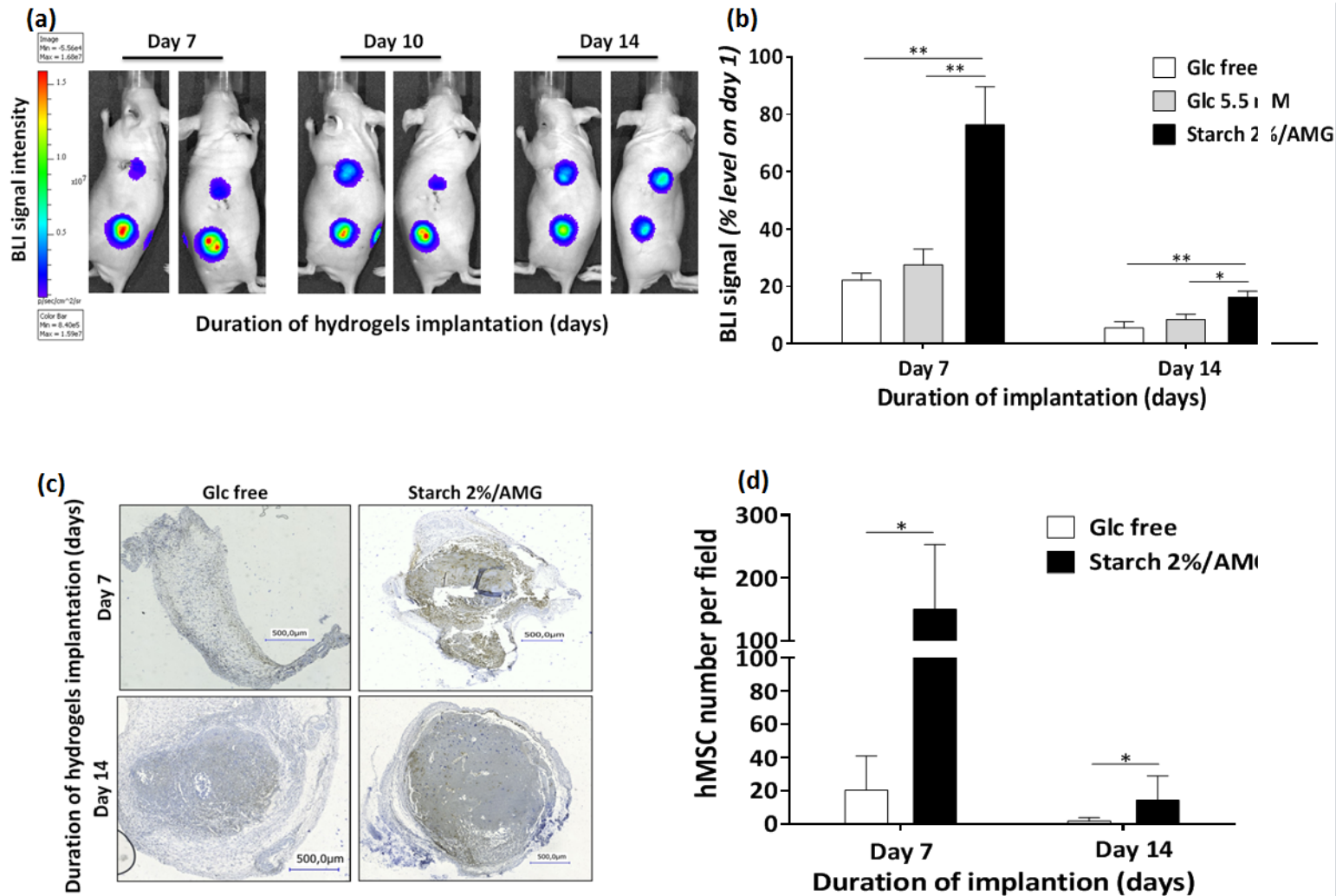


Figure 8-5 The starch/AMG hydrogels are more effective than glucose reservoirs in increasing the *in vivo* survival of hMSCs after subcutaneous implantation. (a). Noninvasive, *in vivo* bioluminescence imaging of Luc-ZSGreen-hMSCs seeded in either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels, after subcutaneous implantation in nude mice for 7, 10 and 14 days (n=9). (a). Quantification of the bioluminescent signal detected in the hydrogels described in frame (a). The percentage of surviving cells was determined by photon fluxes at each time point tested and normalized to respective results obtained for each implant at day 1 (n=12). Data are mean ± standard deviation. * p < 0.05 compared to respective results obtained from the starch 2%/AMG hydrogels (Mann-Whitney test). (c). Representative immunohistology results and (d) quantification of viable Luc-ZSGreen-hMSCs seeded in either glucose-free reservoirs or starch 2%/AMG hydrogels after 7 and 14 days of subcutaneous implantation in nude mice. Stain: β2-microglobulin. Scale bar = 500 μm. (n=3). Data are mean ± standard deviation. Luc-ZS Green-hMSCs containing glucose-free reservoirs were used as negative control. * p < 0.05; ** p < 0.01 compared to respective results obtained from the starch 2%/AMG hydrogels. The numerical data were analyzed using a One-way ANOVA followed by Bonferroni's post hoc test. Abbreviations: Luc-ZS Green-hMSCs = human mesenchymal stem cells transduced using a lentiviral vector encoding for the firefly luciferase and the ZSGreen proteins; AMG = amyloglucosidase; Glc = glucose; BLI = bioluminescence intensity

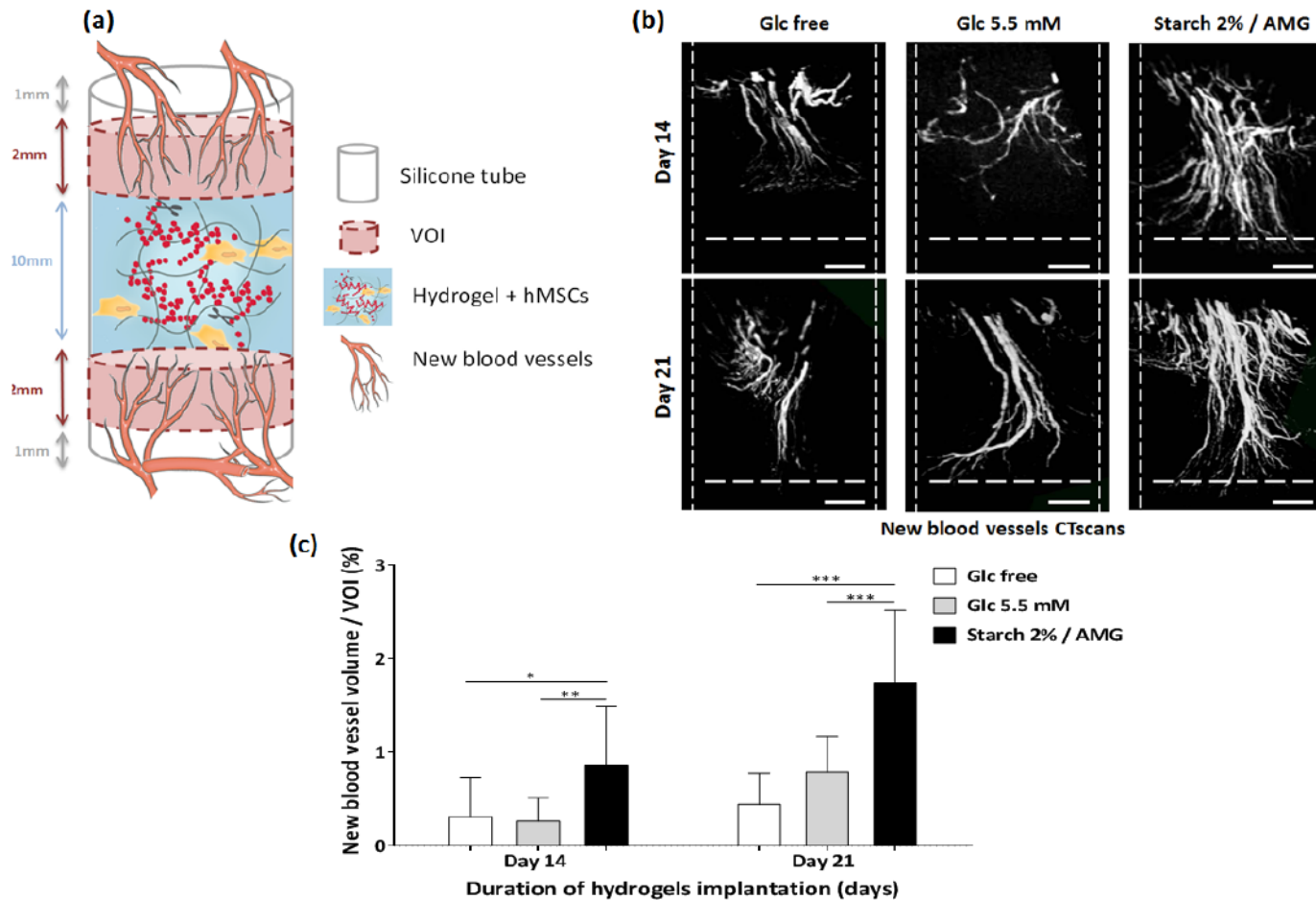


Figure 8-6 The starch/AMG hydrogels are more effective than glucose reservoirs in increasing the *in vivo* proangiogenic functions of hMSCs after subcutaneous implantation. (a): Schematic (not to scale) of the model designed to assess the proangiogenic potential of hMSC-containing hydrogels. Hydrogels were loaded with 1.25×10^6 hMSCs and encapsulated at the center of a silicone tube. After 14 and 21 days implantation in an ectopic mouse model, new blood vessels were quantified into the volumes of interest (VOI; 2 per samples), defined as cylinders overlapping the silicone tube, between the 1st and the 3rd mm height from the top and from the bottom of the silicone tube edges. **(b) and (c):** Micro-CT vasculature analysis in the vicinity of either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels after 14 and 21 days of ectopic implantation in mice. **(b).** Representative 3D reconstructions (using CT-Vox software) of the new blood vessels into the VOIs defined in the vicinity of either glucose-free reservoirs, 5.5 mM glucose reservoirs or starch 2%/AMG hydrogels after subcutaneous implantation in nude mice for 14 and 21 days. All glucose reservoirs and hydrogels had been loaded with $1.25 \cdot 10^6$ hMSCs and encapsulated into a silicone tube. After 14 and 21 days of ectopic implantation, Microfil® (a radiopaque polymer) was perfused and the samples were scanned using *in vitro* μ CT (Skyscan 1772). Vertical dotted lines represent the silicone tube sides; horizontal dotted lines represent the top of the hydrogel. Scale bar = 500 μ m. (n=16). **(c).** New blood vessel volume was reported as percentage (%) of the VOI which was analyzed using CTAn software. (n=16). *p < 0.05; **p < 0.01; ***p < 0.001 compared to respective results obtained from the starch 2%/AMG hydrogels. The numerical data were analyzed using a One-way ANOVA followed by Bonferroni's post hoc test. Abbreviations: hMSCs = human mesenchymal stem cells; AMG = amyloglucosidase; Glc = glucose; VOI = volume of interest.

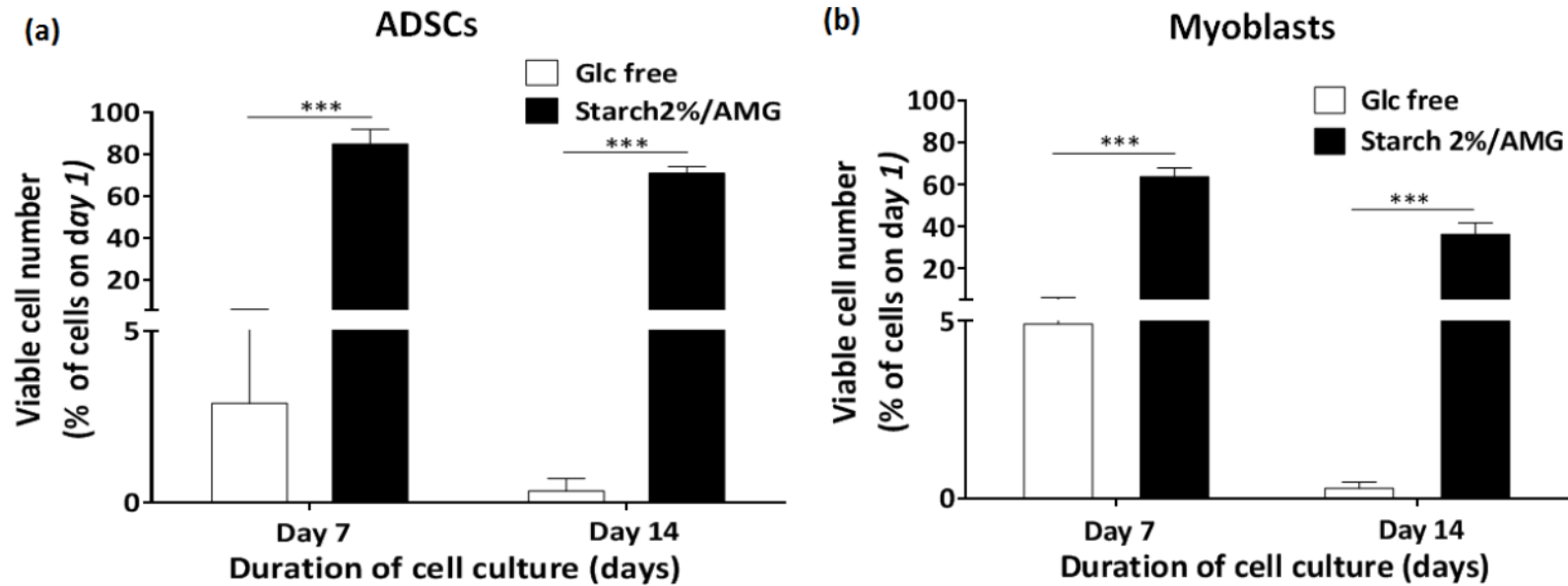


Figure 8-7 The starch 2%/AMG hydrogels are more effective than glucose-free reservoirs in extending the *in vitro* survival of ADSCs and myoblasts in near-anoxia conditions. (a). Viability of ADSCs and (b) myoblasts seeded in either glucose-free reservoirs or starch 2%/AMG hydrogels after exposure to near-anoxia conditions (0.1% O₂) for 7 and 14 days. At the prescribed time points, the cells were stained with HE (cell nuclear in blue) and PI (dead cell in red), and analyzed using flow cytometry (n=3). Cell viability was expressed as the number of viable cells normalized by the respective viable cell number at day 1. Data are mean ± standard deviation. ADSCs contained in glucose-free reservoirs were used as negative controls for (a). Myoblasts contained in glucose-free reservoirs were used as negative controls for (b). *** p < 0.001 compared to the respective results obtained from the starch 2%/AMG hydrogels. The numerical data were analyzed using a One-way ANOVA followed by Bonferroni's post hoc test. Abbreviations: ADSCs = adipose-derived stem cells; AMG = Amyloglucosidase; HE = Hoescht 33342; PI = propidium iodide.

Table 8-1 The diameters and numbers of the new blood vessels in starch 2%/AMG hydrogels, glucose-free reservoirs, and glucose-reservoirs were similar after implantation in ectopic mouse model. New blood vessel numbers and diameters were analyzed using CTAn into volumes of interest defined into either glucose-free reservoirs, 5.5 mM glucose reservoirs, or starch 2%/AMG hydrogels (n=16). NS: $p > 0.05$ compared to respective results obtained from the starch 2%/AMG hydrogels. The numerical data were analyzed using a One-way ANOVA followed by Bonferroni's post hoc test. Abbreviations: hMSCs = human mesenchymal stem cells; AMG = amyloglucosidase; Glc = glucose; NS = not significant.

Type of hydrogel	New blood vessel Numbers (mm^{-1})			New blood vessel Diameter (μm)		
	Glc Free	Glc 5.5mM	Starch 2%/AMG	Glc Free	Glc 5.5mM	Starch 2%/AMG
DAY 14	0.85 \pm 0.59	0.59 \pm 0.38	0.98 (NS) \pm 0.82	36 \pm 21	33 \pm 12	45 (NS) \pm 14
DAY 21	0.141 \pm 0.72	0.161 \pm 0.56	0.199 (NS) \pm 0.62	57 \pm 17	54 \pm 12	51 (NS) \pm 16

DISCUSSION

The DISCUSSION section provides both interpretation of the results obtained from the doctoral research project and perspectives and limitations.

9 General Discussion

The rapid and massive MSC death post-implantation, caused by the hostile ischemic microenvironment these cells encounter, is the major roadblock for MSCs clinical applications. The results of the present investigation/study provided evidence that (i) supply of exogenous glucose to hMSCs enhances their survival and functionalities (e.g., angiogenesis) both under near-anoxia conditions *in vitro* and in an ectopic mouse model *in vivo*; (ii) regulating hMSCs ER stress under an ischemic environment is a plausible mechanism regarding the impact of glucose on hMSCs, and (iii) delivery of glucose *via* an enzyme-controlled, nutritive starch/AMG hydrogel improves MSC survival and angiogenesis post-implantation in an ectopic mouse model.

9.1 Key parameters in developing a physiologically-relevant *in vitro* ischemic model

An *in vitro* ischemic model exposing MSCs to physiologically-relevant conditions is important to study the cellular damage MSCs encounter post-implantation. Research on ischemia pathology has primarily focused on understanding the impact of impaired oxygen delivery. Low oxygen levels (specifically, various levels of hypoxia to anoxia) is undoubtedly a major contributor to the ischemic insult. Other ischemia-associated insults are also significantly taken into account. For example, in the case of ischemic renal tissue, the ischemic microenvironment is characterized by low oxygen levels and limited nutrient availability, accumulation of impeded waste removal, and increased, local carbon dioxide content (hypercapnia) (Russ, Haberstroh and Rundell 2007) (Figure 9-1). Ideally, all aforementioned *in vivo* insults should be incorporated into an *in vitro*, ischemic model because each one may potentially contribute to the cellular changes observed post-implantation.

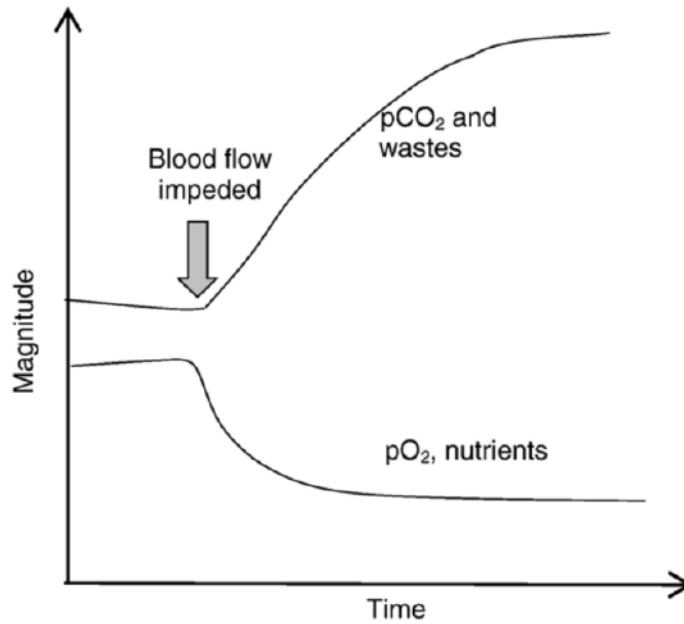


Figure 9-1 **Qualitative changes in renal tissue oxygen and carbon dioxide partial pressures during ischemia** based on data from Nelimarkka and Niinikoski, (1984). Image from (Russ, Haberstroh and Rundell 2007)

9.1.1 Low Oxygen Levels

Hypoxic/anoxic conditions exist within ischemic tissue due to the inadequate oxygen supply that induces cells to shift from aerobic to anaerobic metabolism. The oxygen levels chosen for the *in vitro* ischemic model should be the oxygen levels to which MSC are exposed post-implantation. Compared to the atmospheric oxygen level (21%; normoxia), most researchers investigating the impact of ischemia on MSC metabolism consider oxygen levels between 1% and 10% as hypoxic conditions (Das, et al. 2010) (Rosová, et al. 2008). The oxygen levels measured in normal mammalian tissue (e.g., lung, liver, kidney, brain, eye, and bone marrow) *in vivo* range from 0.5% to 14% (physioxia) (Jagannathan, Cuddapah and Costa 2016). Even though MSCs in virtually all tissues are present in perivascular niches in close proximity to vascular structures, the various tissues (where MSCs are found) exhibit oxygen levels ranging from 1% to 8% (Figure 9-2) (Mohyeldin, Garzón-Muvdi and Quiñones-Hinojosa 2010) (Ivanovic 2009) (Holzwarth, et al. 2010) (Chow, et al. 2001) (Spencer, et al. 2014). In other words, the oxygen levels used in these *in vitro* models reflect physioxic rather than the hypoxic conditions encountered by MSCs in ischemic tissues. To address this issue, previous studies at the BO3A Laboratories investigated and reported the oxygen level that best simulates the oxygen tension encountered by hMSCs loaded into hydrogel using an

ectopic mouse model. They established that hMSCs located at the core of hydrogels were exposed to oxygen levels of 0.1% at day 1 and 3 post-implantation. In these conditions, MSCs exhibited increased expression of HIF-1 α and LDH-A (Moya, Paquet, et al. 2018). In addition, the levels of HIF-1 α and LDH-A from hMSCs cultured *in vitro* without glucose and serum under 0.1% O₂ were identical to those of hMSCs dispersed within fibrin hydrogels and implanted in the ectopic model (Moya, Paquet, et al. 2018). We, therefore, employed this oxygen level of 0.1% for the *in vitro* studies carried out during this thesis project.

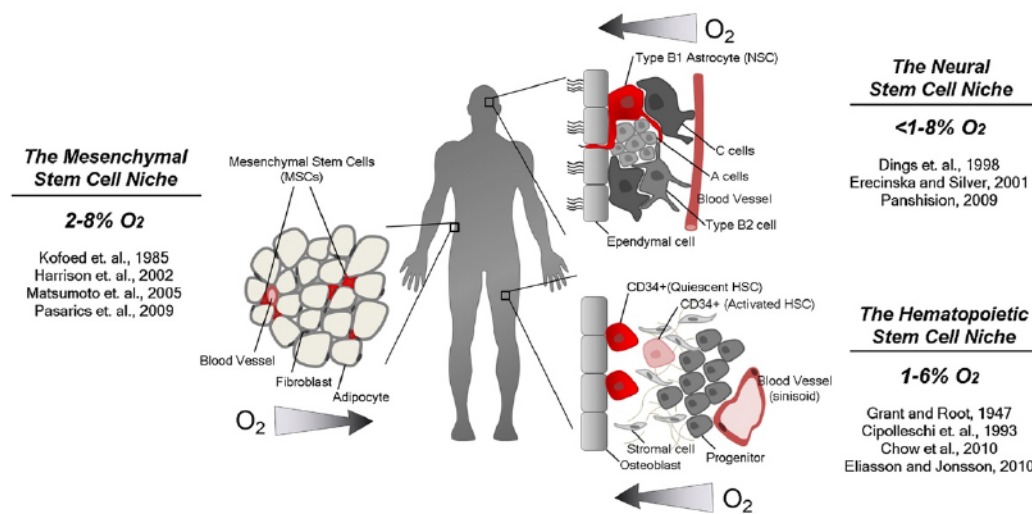


Figure 9-2 **Low Oxygen Tension Measurements in Various Stem Cell Compartments.** Image from (Mohyeldin, Garzón-Muvdi and Quiñones-Hinojosa 2010)

9.1.2 Limited Nutrient Availability and Waste Removal Impairment

Tissue ischemia severely reduces the supply of critically-needed metabolic substrates, such as glucose and ATP, as well as results in buildup of toxic, metabolic end-products. Glucose was identified as the crucial metabolic substrate hMSCs need to produce energy in the form of ATP *via* glycolysis, in order to survive under near-anoxia conditions *in vitro* (Moya, Paquet, et al. 2018). Mammalian cells can also use amino acids, in particular, either glutamine (*via* glutaminolysis) or serine (*via* serinolysis) for energy production. The aforementioned, alternative metabolic pathways, however, are not used by hMSCs under *in vitro*, near-anoxia conditions. In fact, MSCs under near-anoxia exclusively utilize anaerobic glycolysis, which results in accumulation of released lactate due to impairment of the cell metabolic waste removal (Moya, Paquet, et al. 2018).

In conclusion, it is reasonable to hypothesize that, at least, depletion of glucose should be incorporated into the *in vitro* ischemic model. In addition, the supernatant

media should not be renewed for the duration of the experiments (3 - 14 days) in order to simulate accumulation of cell metabolic waste in an ischemic microenvironment post-implantation in an ectopic model.

9.1.3 Hypercapnia

In vivo evidence reported in the scientific literature provided evidence that ischemic hypercapnia occurs in various organs, including the kidney, brain, heart, and the intestinal tract (Johnson and Weil 1991). To date, however, no study has integrated the increased local carbon dioxide content into the *in vitro* ischemic model. The present study did not use hypercapnia to simulate the *in vivo* ischemic situation. In the future, however, *in vitro* models should take this parameter into consideration in order to best simulate the *in vivo* situation.

9.2 The Underlying Mechanism of Glucose Improving hMSCs Therapeutic Potential

In Article I, we confirmed *in vitro* that the supply of exogenous glucose sustained hMSCs survival under near-anoxia for up to 14 days (Figure 7-1) and improved hMSC-mediated angiogenesis potential (Figure 7-2). Most importantly, these positive effects of glucose on MSCs survival and angiogenesis were also confirmed in an *in vivo* ectopic mouse model (Figure 7-3). Last but not least, this study determined that glucose regulated the ER stress of hMSCs under near-anoxia and improved nascent protein biosynthesis. These data suggest that glucose may be an important modulator of MSC paracrine functions as it may ultimately induce secretion of bioactive chemical compounds such as select growth factors.

9.2.1 Glucose is a Critical Metabolic Substrate for hMSCs Under Near-Anoxia

The findings of the present study provided evidence that exogenous glucose maintained hMSC survival under near-anoxia conditions (Figure 7-1). These results also corroborate reports of previous studies that, under near-anoxia conditions, MSCs obtain their energy exclusively from the use of glucose *via* glycolysis; this result was confirmed when either glycolysis inhibitors were used or by unsuccessful attempts at replacing glucose by alternative substrates such as serine, and glutamine (Moya, Paquet, et al. 2018). Taken together, these data established that glucose is the key parameter for

producing energy *via* glycolysis and for providing the support needed for MSCs survival under near-anoxia.

9.2.2 Glucose is Important for hMSC-Mediated Angiogenesis Post-implantation

Angiogenesis (*i.e.*, formation of an interconnected network of functional blood vessels) is a fundamental biological requirement for tissue repair post injury; this arrangement, provides circulating blood and, thus, supply of oxygen and nutrients as well as concomitant removal of cell metabolic waste. The present study, focused on MSC-mediated angiogenesis and provided evidence that glucose improves hMSC-mediated angiogenesis under near-anoxia conditions both *in vitro* and *in vivo* (Deschepper, et al. 2013). As shown in (Figure 7-2), CM collected from hMSCs cultured with glucose promoted significant ($p < 0.05$) increased HUVECs migration and tubular structure formation when compared to the one collected from hMSCs cultured without glucose. In addition, compared to hMSCs cultured without glucose, hMSCs cultured in the presence of glucose released significantly ($p < 0.05$) higher amounts of Angiogenin, VEGF-A, VEGF-C, Angiopoietin-1, Endostatin, and CCL2 (Figure 7-2). These *in vitro* results provided evidence that supplying glucose to hMSCs under near-anoxia improved MSC-mediated angiogenesis by increasing several pro-angiogenic growth factors release into the supernatant culture media. One unanticipated finding was that the amount of FGF-b, which is known to act synergistically with VEGF to promote angiogenesis, was significantly ($p < 0.05$) higher in the CM collected from hMSCs cultured in the absence of glucose than in the supernatant CM from hMSCs cultured in the presence of glucose. At this point, a study of the time-course is needed to determine whether this trend of increased FGF-b secretion by dying MSCs is maintained over time (Kshitiz, Ellison, et al. 2019). Alternatively, MSCs contain a major depository of bioactive FGF-b, which is passively released into the supernatant culture media either upon cell death or following sub-lethal cell injury but not by active secretion (Aizman, et al. 2015). It is possible that increased MSC death in the absence of glucose results in a robust release of the intracellular depository of active FGF-b into the supernatant culture media. The released FGF-b from MSCs upon cell death may participate, like the production of apoptotic extracellular vesicles, mitochondria, and other signals, in the emerging hypothesis of dying stem cell hypothesis as a novel mechanism for explaining the role of MSC therapy (Fu, et

al. 2021) (Galleu, et al. 2017) (Preda, et al. 2021) (Weiss and Dahlke 2019) (Weiss, et al. 2020) (Thum, et al. 2005).

The present study also evaluated the role exogenous glucose on the hMSC-mediated angiogenesis *in vivo* using an ectopic mouse model. A well-defined hierarchal blood vessel branching network was developed within the region of interest surrounding the cell-containing hydrogels loaded with glucose (Figure 7-3A). In contrast, this hierarchal branching pattern was disrupted in both the cell-containing hydrogels without glucose and in the cell-free hydrogels loaded with glucose. Furthermore, the implanted hMSC-containing hydrogels loaded with glucose exhibited increased ($p < 0.05$) volume of newly-formed blood vessels compared to the hMSC-containing hydrogels without glucose (Figure 7-3B). In contrast, the volume of newly-formed blood vessels in the cell-containing hydrogels without glucose and in the cell-free hydrogels loaded with glucose were similar and minimal. Last, histological analysis revealed abundant CD31-antibody-positive endothelial cells that had infiltrated into the implants and mostly co-localized with the blood vessels (Figure 7-3C). All these *in vivo* results provided evidence that hMSCs mediate new blood vessel formation, and that the exogenous glucose supply enhances this MSC-mediated angiogenesis.

9.2.3 Glucose Improves hMSC-mediated Angiogenesis by Regulating ER Stress

The absence of glucose significantly ($p < 0.05$) decreased nascent protein biosynthesis of hMSCs at day 1 and suppressed it at day 3 under near-anoxia (Figure 7-4A). This result provided evidence that hMSCs cultured in the absence of glucose under near-anoxia encountered a higher level of ER stress since the arrest of protein biosynthesis is a hallmark of ER stress. The results of the present study are in agreement with those reported in the literature (Jeyapalan, et al. 2007) (Kejiou, et al. 2019) (Weber, Fresard and Veach 1989) (David and Avi Dor 1975) (Ravid, Diamant and Avi-Dor 1980), that glucose availability stimulates protein synthesis in various other cells and tissues. The stimulatory effect of glucose may be primarily attributed to the energy supply because cell-free protein synthesis occurred when energized by energy substrates (Wang and Zhang 2009). Suppression of nascent protein biosynthesis was corroborated with the exhaustion of glycolytic reserves and ATP content in hMSCs cultured in the absence of glucose under near-anoxia for 1 and 3 days, respectively (Moya, Paquet, et al. 2018). Another important finding from the present study was that hMSCs cultured in the absence

of glucose under near-anoxia exhibited significantly ($p < 0.05$) higher mRNA expression of CHOP and GRP78 and increased level of phosphorylated eIF2 α and CHOP protein expression compared to hMSCs cultured in the presence of glucose (*Figure 7-4*). The higher expression of CHOP may explain the increased cell death and the relatively low angiogenic potential of hMSCs cultured in the absence of glucose observed before in the present study. The aforementioned results and observations support the hypothesis of the present study that the exogenous glucose supply exerts positive regulation of hMSC survival and their angiogenic potential through modulation of ER stress under ischemic conditions.

9.3 Nutritive Hydrogel: A Strategy of Interest in TE

Previous studies (Deschepper, et al. 2013) (Moya, Paquet, et al. 2018) as well as the present study (Article I) provide evidence that exogenous glucose supply improves the survival and promotes the angiogenic potential of hMSC exposed to ischemia. These data strongly suggest that supplying glucose to MSCs is an attractive strategy to improve their therapeutic potential. Because glucose, a small and highly diffusible molecule, cannot remain within implants for long periods of time, ensuring long-term glucose supply requires a supra-physiological dose of glucose, which inevitably leads to increased osmotic pressure and, ultimately, cell lysis. For this reason, one of the most important objectives of the present study was to develop a glucose reservoir that could continuously deliver glucose to MSCs in the concentration range needed to maintain MSC survival and functionalities.

9.3.1 A Concept of Nutritive Hydrogel

The results of the present study provide valuable information regarding the constituents for a nutritive hydrogel, scaffold for MSC implantation. This novel nutritive three-dimensional substrate is a fibrin hydrogel (acting as a temporary scaffold), starch and AMG. The principle of this nutritive hydrogel is that starch is hydrolyzed by AMG, allowing production of glucose at a physiological range (*Figure 8-1*). The glucose molecules released from starch fuels MSCs seeded within the hydrogel thus improving MSC survival, functionalities, and, ultimately, their therapeutic potential.

9.3.2 The Nutritive Hydrogel Improves hMSC Survival Under Ischemic Conditions

The novel, nutritive hydrogel improves hMSC survival *in vitro* and *in vivo*. hMSCs seeded dispersed within starch/AMG-free hydrogels died massively within three days post inoculation (Figure 8-2). In contrast, hMSCs seeded in the starch/AMG-containing hydrogels exhibited a survival rate greater than 70% after 7 and 14 days under near-anoxia conditions (Figure 8-2). In addition, starch/AMG hydrogels supported hMSC survival under near anoxia more effectively than 5.5 mM glucose hydrogels under near-anoxia (Figure 8-3). Moreover, starch/AMG hydrogels were more effective than glucose-free and 5.5 mM glucose hydrogels in supporting hMSCs survival in an ectopic mouse model (Figure 8-5).

Massive cell death post implantation is not a problem exclusive to MSCs, but pertains to various other cells used in tissue engineering, such as hepatocytes (Vacanti et al., 1988), chondrocytes (Judas, et al. 2007), cardiomyocytes (Reinecke and Murry 2002) (Laflamme, et al. 2007), or myoblasts (Fan, et al. 1996) (Hodgetts and Grounds 2003). In order to broaden the scope of application of the nutritive hydrogel for tissue engineering applications, *in vitro* survival of ADSCs and myoblasts, seeded within the novel, nutritive hydrogels and cultured under near-anoxia conditions, was investigated. After 14 days in starch/AMG hydrogels under near-anoxia conditions, ADSCs and myoblasts showed a survival rate greater than 70% and 50%, respectively (Figure 8-7). In contrast, in glucose-free hydrogels, more than 90% of ADSC and myoblasts under near-anoxia died at day 7 (Figure 8-7). These encouraging observations obtained *in vitro* still need to be confirmed *in vivo* in an animal model.

The present study also provided evidence that the nutritive hydrogel represents a strategy of potential in improving survival of MSCs, whose post implantation energy metabolism depends on glucose availability and glycolysis. The need for additional nutrients to further optimize cell survival and functionalities, however, cannot be excluded. In addition, other cell types may have different nutritional needs. For these reasons, caution must be exercised when the results of the present study are interpreted and extended to other cell types. The nutritional needs of each cell type used in tissue engineering must be characterized using high throughput assays, in order to tailor nutritive hydrogels to the specific nutrient needs of specific cells.

9.3.3 The Nutritive Hydrogel Improves hMSC-Mediated Angiogenesis

The pro-survival effects of nutritive hydrogels on cells under near-anoxia is essential but insufficient to optimize MSC functionalities. In fact, MSCs must survive and actively participate in the process of new tissue formation through their paracrine functions, particularly their angiogenic potential.

When hMSCs, seeded within nutritive starch/AMG hydrogels and cultured under near-anoxia, they secreted significantly ($p < 0.05$) higher amount of pro-chemotactic factors (IL-8, MCP-1, MIF, ENA-78, MIP-2, and IP-10) and pro-angiogenic factors (VEGF-A, VEGF-C, VEGF-D, ANGPT-1, ANGPT-2, ANG, AND FGF-b) compared to respective results obtained when the hMSCs were seeded within either glucose-free or glucose hydrogels (Figure 8-4). These results are corroborated by the higher chemotactic potential exhibited by CM collected from hMSCs seeded within nutritive starch/AMG hydrogels towards HUVECs and hMSCs (Figure 8-4) when compared to respective results obtained from control hydrogels. Most importantly, these *in vitro* observations were in agreement with the results obtained from *in vivo* studies (Figure 8-6). In fact, a significant ($p < 0.05$) increase in the volume of newly-formed blood vessels was obtained in the nutritive starch/AMG hydrogels seeded with hMSCs compared to pertinent results obtained from either glucose-free or glucose hydrogels (Figure 8-6).

For all the aforementioned reasons, the nutritive starch/AMG hydrogel represents a strategy of interest for improving MSC-mediated angiogenesis post-implantation.

9.4 Limitations of the Thesis Study

While conducting the research presented in this thesis, several limitations became obvious, need to be acknowledged, and are stated below.

- The present study did not characterize the time course of the bioactive factor release (Article I and II). For this purpose, the content of the supernatant CM obtained from hMSCs exposed to near-anoxia at various time intervals, (specifically, after 8 hours as well as after 1, 2, 3, 7, and 14 days) should be analyzed.
- The critical bioactive factor(s) which are responsible for the observed cell migration and tubular structure formation are yet to be determined. A study using neutralizing antibodies (e.g., anti-VEGF-A, anti-MCP-1) to block the bioactivity of these chemical compounds is needed. (Article I and II)

- Elucidation of the details regarding the effect of glucose in regulating ER stress of hMSCs, thus hMSC-mediated angiogenesis, is also needed (Article I). A highly sensitive assay monitoring the processing of proteins through the secretory pathway and the ER stress in real time could use naturally secreted luciferase (Gluc) (Badr, et al. 2007), as well as ER stress inducers and inhibitors. The results of such studies will contribute valuable information to the current understanding of the correlation between glucose supply, ER stress of hMSCs, and the mediated angiogenesis.
- The role of glucose in the UPR branches (specifically, IRE1 α and ATF6) has not been studied (Article I). Further research focusing on the expression and functions of IRE1 and ATF6 is needed in order to gain a comprehensive understanding of the effects of glucose on ER stress in hMSCs.
- The performance of the novel, nutritive hydrogel for long-term glucose production must be investigated (Article II). Measurement of the released glucose concentrations from the novel, nutritive hydrogels at longer time intervals, (such as 1 or 2 months), will provide the definite information needed to determine whether this nutritive hydrogel can be used for long-term glucose delivery *in vivo*.
- The starch employed in the novel, nutritive hydrogels was not completely resorbed at the end of the pertinent experiments; such an outcome may have a negative impact on tissue repair and regeneration (Article II). For this reason, further research regarding the degradation rates of various glucose polymers is required. The therapeutic efficacy of the novel, nutritive hydrogel will be enhanced by identifying and using a glucose polymer with a degradation rate that is synchronous with the ingrowth rate of regenerating tissues.
- Because the novel, nutritive hydrogel used in the present study contained fibrin, substantial amount of amino acids was released during degradation of this construct. It is unknown whether these released amino acids are taken up by hMSCs and whether they affect hMSC survival and functionalities. Formulation and use of isotope-labeled fibrin hydrogels could provide the information needed to resolve this issue.

CONCLUDING REMARKS

*"Being a graduate student is like becoming all of the Seven Dwarves. In the beginning you're **Dopey** and **Bashful**. In the middle, you are usually sick (**Sneezy**), tired (**Sleepy**), and irritable (**Grumpy**). But at the end, they call you **Doc**, and then you're **Happy**."*

- Ronald T. Azuma

10 Restatement of aims

The present research investigation focused on the role of glucose on MSC-mediated angiogenesis and developed a novel, glucose-delivery, nutritive hydrogel for MSC-mediated tissue engineering applications.

10.1 Summary of Major Findings

- The exogenous glucose supply plays a crucial role in hMSC survival and promotes angiogenic potential under ischemic conditions post-implantation.
- The modulation of ER stress of hMSCs by exogenous glucose supplying is a possible mechanism for hMSC survival and hMSC-mediated angiogenesis.
- The novel, nutritive starch/AMG hydrogel produces glucose in a physiological range.
- The novel, nutritive starch/AMG hydrogel milieu improves hMSCs survival and paracrine function pertinent to angiogenesis post implantation.

10.2 Future Perspectives

The intriguing results of the present study justify further investigation in order to elucidate the underlying mechanism(s) regarding the role of glucose in MSCs survival and functionalities post-implantation, as well as to optimize the novel, nutritive hydrogel as a three-dimensional substrate for various MSC applications. In addition to nutrient deprivation, the inflammatory response and oxidative stress play significant roles in *in*

in vivo ischemic cascades. Since immunomodulation plays a crucial role in tissue repair, the function of glucose in MSC-mediated immunomodulation and redox homeostasis is needed in order to fully determine the beneficial effects of glucose supply in MSCs-based therapies. As far as the novel, nutritive hydrogel is concerned, the main aspects which must be taken into consideration when optimizing their use in tissue engineering applications include (but are not limited) to the biophysical properties of hydrogel, choice of glucose polymer (e.g., starch *versus* maltodextrins), choice of enzyme (e.g., a single enzyme *versus* a combination of several starch-degrading enzymes), injectability, biocompatibility, and delivery mode. These aspects are at the frontier of MSC-mediated tissue engineering and need further research investigations.

10.3 Significance of This Thesis

MSCs are promising candidates for tissue engineering applications, including bone regeneration, cartilage repair, wound healing, *etc.* The rapid and massive cell death observed in the ischemic microenvironment impedes MSC use for several applications including tissue engineering ones. Accepting that absence of glucose is the culprit for MSC death post-implantation, the present study provided new insight into the role of glucose in MSC-mediated angiogenesis post-implantation. Improved understanding of the role of nutrients in MSC fate and functionalities can be used to develop nutritive hydrogels for MSC-mediated tissue engineering applications.

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APPENDIX

A-1 Publication, Presentation, and Funding

Journal Publications

1. **Luo G**, Larochette N, Denoeud C, Wosinski P, Potier E, Petite H. **Glucose Promotes Transplanted Human Mesenchymal Stem Cell Survival and Paracrine Effects by Regulating Endoplasmic Reticulum Stress.** (Manuscript in preparation)
2. Denoeud C, **Luo G**, Paquet J, Boisselier J, , Moya A, Diallo A, Marinesco S, Meiller A, Becquart P, Moussi H, Vilquin J-T, Logeart-Avramoglou D, Gand A, Larreta-Garde V, Pauthe E, Potier E, Petite H. **Enzyme-Controlled, Nutritive Hydrogel for Mesenchymal Stem Cell Survival and Paracrine Functions.** (Manuscript in preparation)
3. Salazar-Noratto GE, **Luo G**, Denoeud C, Padrona M, Moya A, Bensidhoum M, Bizios R, Potier E, Logeart-Avramoglou D, Petite H. **Understanding and leveraging cell metabolism to enhance mesenchymal stem cell transplantation survival in tissue engineering and regenerative medicine applications.** Stem Cells. 2020 Jan;38(1):22-33.
4. **Luo G**, Wosinski P, Bizios R, Potier E, Petite H. **The critical role of glucose metabolism in mesenchymal stem cell mediated tissue engineering.** (Manuscript in preparation)

Conferences Proceedings

1. **Luo G**, Salazar-Noratto GE, Potier E, Petite H. **Engineering Bone with Mesenchymal Stem Cells: Challenges and Obstacles.** Biomedical and Health Research, Volume 80: Stem Cells and Regenerative Medicine. 2021; 60-66

Presentations

1. **Luo G.**, Denoeud C., Paquet J., Larochette N., Potier E., Petite H., Mesenchymal stem cell survival in near-anoxia and functions pertinent to angiogenesis rely on the presence of glucose, 9th International Symposium Europe-China on Stem Cells and Regenerative Medicine, Strasbourg, 2019 (*Oral presentation*)
2. Denoeud C., Paquet J., Boisselier J., Moya A., Diallo A., **Luo G.**, Marinesco S., Meiller A., Larreta-Garde V., Pauthe E., Potier E., Petite H., Enzyme-controlled, starch-based hydrogels for mesenchymal stromal cell survival and paracrine functions, TERMIS, Greece, 2019 (*Poster presentation*)
3. Denoeud C., Paquet J., Boisselier J., Moya A., Diallo A., **Luo G.**, Marinesco S., Meiller A., Larreta-Garde V., Pauthe E., Potier E., Petite H. Enzyme controlled, starch- based hydrogels for mesenchymal stem cell survival and paracrine functions, ORS, Austin, 2019 (*Poster presentation*)
4. **Luo G.**, Denoeud C., Paquet J., Boisselier J., Gand A., Moya A., Diallo A., Marinesco S., Meiller A., Becquart P., Moussi H., Vilquin J., Larreta-Garde V., Pauthe E., Potier E., Petite H., Enzyme-controlled, nutritive hydrogel for mesenchymal stromal cell survival and paracrine functions, ORS, Phoenix, 2020 (*Oral presentation*)
5. **Luo G.**, Denoeud C., Larochette N., Potier E., Petite H., Mesenchymal stem cell survival in near-anoxia and functions pertinent to angiogenesis rely on the presence of glucose, ORS, Phoenix, 2020 (*Poster presentation*)
6. **Luo G.**, Denoeud C., Larochette N., Potier E., Petite H., Glucose promotes mesenchymal stem cell paracrine functions pertinent to angiogenesis by regulating unfolded protein response, ISSCR VIRTUAL, 2020 (*Poster presentation*)

Funding

1. China Scholarship Council
2. La Fondation pour la Recherche Médicale

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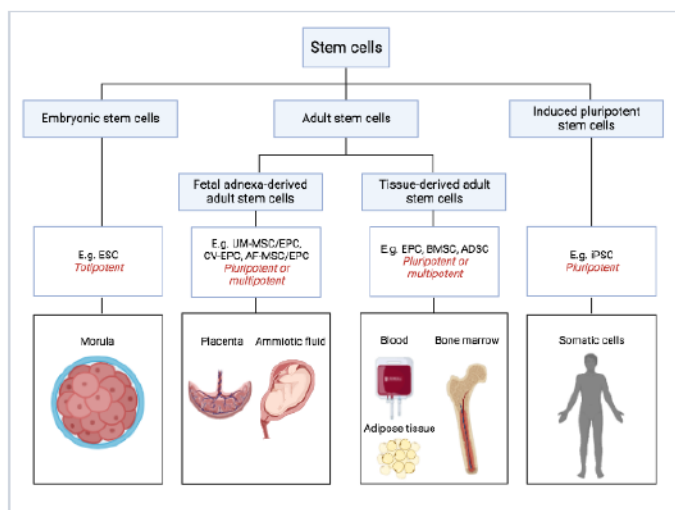
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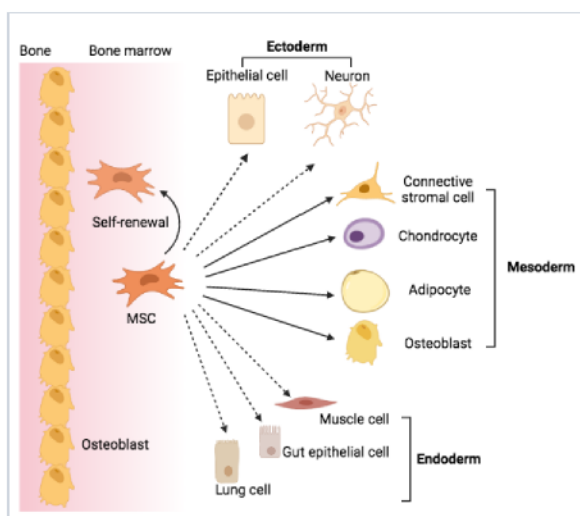
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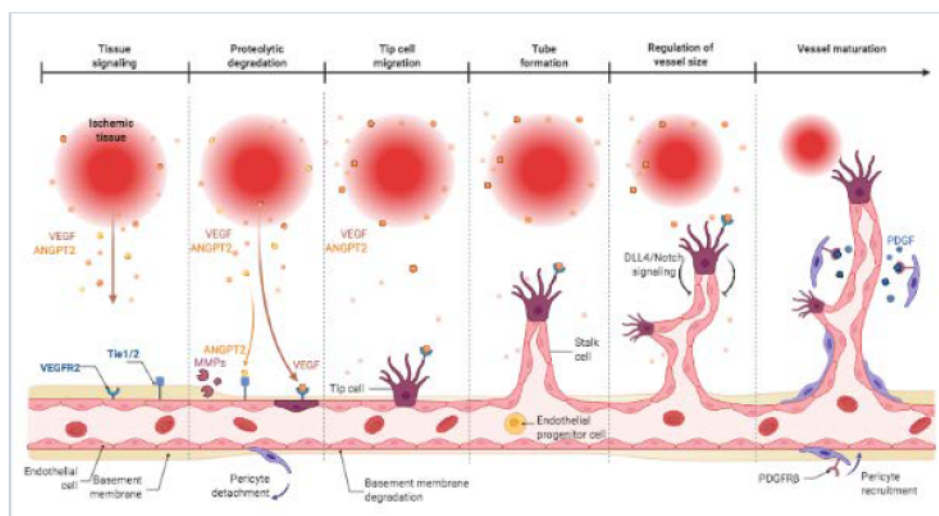
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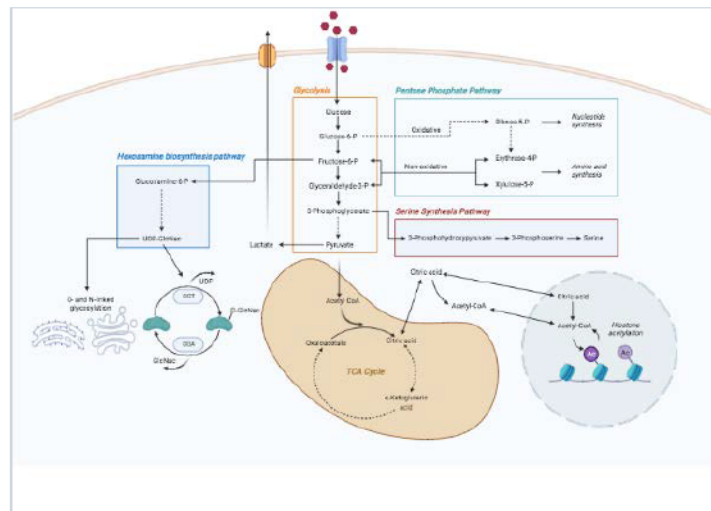
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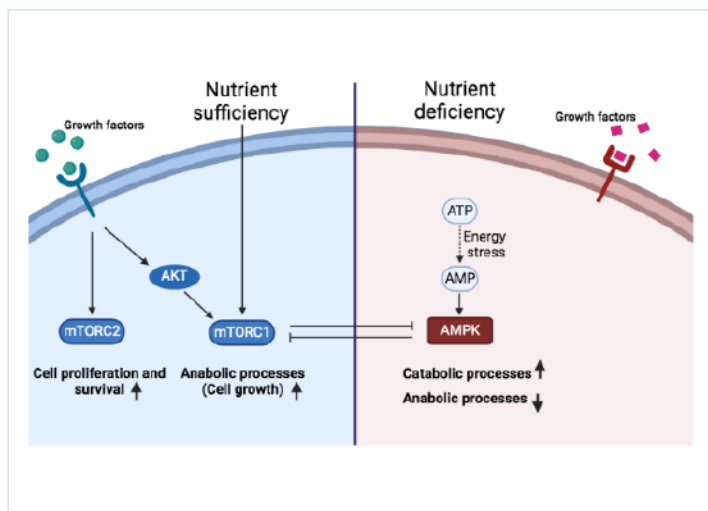
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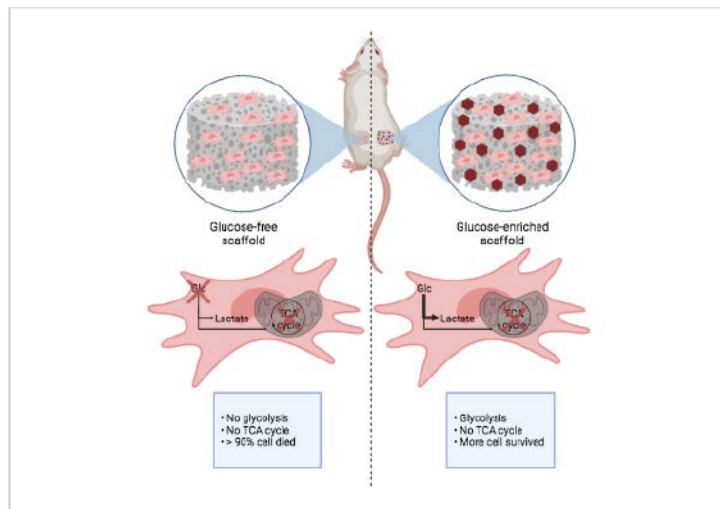
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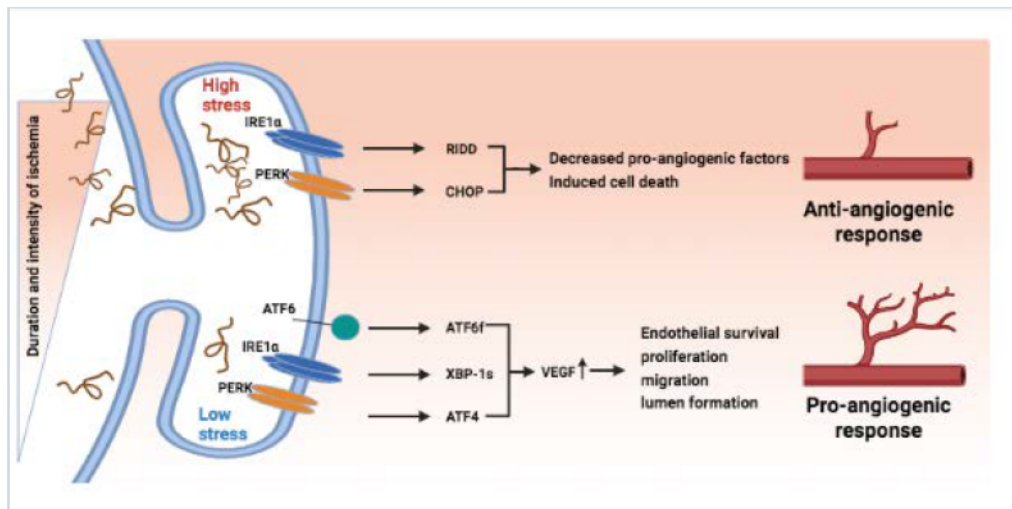
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