HUMAN BONE MARROW-MESENCHYMAL STEM CELLS DIFFERENTIATION INTO BRAIN-LIKE ENDOTHELIAL CELLS

by

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ABSTRACT

HUMAN BONE MARROW-MESENCHYMAL STEM CELLS DIFFERENTIATION INTO BRAIN-LIKE ENDOTHELIAL CELLS

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Human pluripotent stem cells (hPSCs) have been frequently utilized to produce robust monolayers of brain microvascular endothelial cells (BMECs). During the differentiation process, hPSCs are to first differentiate into mesenchymal lineage cells. Accordingly, Mesenchymal stem cells obtained from human bone marrow (BM-MSCs) may offer a method for producing fully functioning human BMECs that may be utilized to construct the blood-brain barrier (BBB) for study aims. Looking through the literature, it was shown that BM-MSCs may differentiate into a range of cell types, endothelial cells (ECs) included. However, there is no strategy for the conversion of BM-MSCs into endothelial cells with brain characteristics, which precludes their wide applications. Therefore, we developed a new protocol for brain-like endothelial cells (BLECs) differentiation from BM-MSCs, inspired by embryologically developmental procedures and previously published iPSCs-BMECs protocols. To develop the differentiation protocol, we optimized the seeding densities of BM-MSCs and the components of the differentiation medium by using three different differentiation media: Endopan, EGM-2, and IMDM. Then, in order to enhance the development of BLECs, we looked into adding retinoic acid (RA) to the differentiation media at various concentrations. Aside from that, we examined the control of the hypoxic environment during endothelial cell differentiation by chemical HIF-1α regulators, cobalt chloride ($CoCl_2$), and sodium sulfite (Na_2SO_3) to mimic the embryological developmental environment and observed

significantly increased expression of brain endothelial cell markers. Different combinations of the basal media used to formulate the expansion and differentiation media were shown to impact how differentiated cells behaved, with IMDM found to favor BLEC differentiation over LG-DMEM. The effect of using animal-derived serum (fetal bovine serum-FBS) and synthetic serum, B27, during differentiation, was also tested, and FBS proved to be more effective than B27, in particular when the differentiation media was supplemented with $CoCl_2$ and Na_2SO_3. The use of the IMDM medium in conjunction with the addition of 3 μM RA shortened the differentiation time of BM-MSCs into BLECs from 14 to 9 days. The addition of 200 μM $CoCl_2$ for two days of differentiation followed by standard differentiation medium or the addition of 4 mM Na_2SO_3 throughout the differentiation period enhanced occludin, CD-31, ZO-1, and claudin-5 expressions in BLECs. In addition, we could prove the functionality of the BLECs by tube structure formation when cultured on Matrigel. In conclusion, we have provided a protocol for the differentiation of BM-MSCs into BLECs that can be used to construct fully functional, physiologically relevant, human cell-based BBB models for the study of brain-related diseases and the testing of various novel drugs for the treatment of neurological disorders.

ÖZET

İNSAN KEMİK İLİĞİ-MEZENKİMAL KÖK HÜCRELERİNİN BEYİN BENZERİ ENDOTEL HÜCRELERİNE FARKLILAŞTIRILMASI

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Anahtar Kelimeler: İnsan Kemik İliği-Mezenkimal Kök Hücreleri, Beyin Benzeri Endotel Hücreleri, Farklılaşma, Retinoik Asit, Hipoksi, Tüp Oluşumu Testi

İnsan pluripotent kök hücreleri (iPKH'ler), beyin mikrovasküler endotel hücrelerinin (BMEH'ler) sağlam tek tabakalarını üretmek için sıklıkla kullanılmıştır. Farklılaşma süreci sırasında, hPSC'ler ilk olarak mezenkimal kök hücrelerine farklılaşacaktır. Buna göre, insan kemik iliğinden (Kİ-MKH'ler) elde edilen mezenkimal kök hücreler, çalışma için kan-beyin bariyerini (KBB) inşa etmek üzere kullanılabilecek tam işlevli insan beyni mikrovasküler endotelyal hücrelerini (BMEH'ler) üretmek için bir yöntem sunabilir. Literatüre bakıldığında, Kİ-MKH'lerin endotel hücreleri (EH'ler) dahil olmak üzere bir dizi hücre tipine farklılaşabileceği gösterilmiştir. Bununla birlikte, geniş uygulamalarını engelleyen Kİ-MKH'lerin beyin özelliklerine sahip endotel hücrelerine dönüştürülmesine yönelik bir strateji yoktur. Bu nedenle, embriyolojik olarak gelişimsel prosedürlerden ve daha önce yayınlanan iPKH-BMEHs protokollerinden esinlenerek Kİ-MKH'lerden beyin benzeri endotel hücrelerine (BBEH) farklılaşması için yeni bir protokol geliştirdik. Farklılaşma protokolünü geliştirmek için, Kİ-MKH'lerin hücre ekim yoğunluklarını ve farklılaşma ortamının bileşenlerini üç farklı farklılaşma ortamı kullanarak optimize ettik: Endopan, EGM-2 ve IMDM. Daha sonra, beyin benzeri endotel hücrelerinin gelişimini artırmak için farklılaşma ortamına çeşitli konsantrasyonlarda retinoik asit (RA) uyguladık. Bunun dışında, embriyolojik gelişim ortamını taklit etmek için kimyasal HIF-1α düzenleyiciler, kobalt klorür ($CoCl_2$) ve sodyum sülfit (Na_2SO_3) ile endotel hücre farklılaşması sırasında hipoksik ortamın kontrolünü inceledik ve beyin endotel hücre

belirteçlerinin ekspresyonunda önemli ölçüde artış gözlemledik. Büyüme ve farklılaşma ortamını formüle etmek için kullanılan bazal ortamın farklı kombinasyonlarının, farklılaşmış hücrelerin nasıl davrandığı üzerinde bir etkiye sahip olduğu gösterildi; IMDM'nin LG-DMEM'e göre BBEH farklılaşmasını desteklediği bulundu. Farklılaşma sırasında hayvandan türetilmiş serum (fetal sığır serumu-FSS) ve sentetik serum B27 kullanmanın etkisi de test edildi ve FBS'nin, özellikle farklılaşma ortamı $CoCl_2$ ve Na_2SO_3 ile desteklendiğinde B27'den daha etkili olduğu kanıtlandı. IMDM ortamının 3 μM RA eklenmesiyle birlikte kullanılması, Kİ-MKH'lerin BBEH'lere farklılaşma süresini 14 günden 9 güne kısaltmıştır. İki günlük farklılaşma için 200 μM $CoCl_2$ ilavesi ve ardından standart farklılaşma ortamı veya farklılaşma süresi boyunca 4 mM Na_2SO_3 ilavesi, BBEH'lerde occludin, CD-31, ZO-1, claudin-5 ekspresyonlarını arttırdı. Ek olarak, Matrigel'de kültürlendiğinde tüp yapısı oluşumuyla BBEH'lerin işlevselliğini kanıtlayabiliriz. Sonuç olarak, beyinle ilgili hastalıkların incelenmesi, nörolojik bozuklukların tedavisinde çeşitli yeni ilaçların test edilmesi için tamamen işlevsel, fizyolojik, insan hücre tabanlı KBB modelleri oluşturmak için kullanılabilecek Kİ-MKH'lerin BBEH'lere farklılaştırılması için bir protokol sağladık.

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I am dedicating this piece to my cherished daughter, Tülin. I sincerely hope you will forgive me for the extended periods you have been apart from me, but I can tell you that everything I am doing is for your benefit and the benefit of your future self-esteem. Finally, but certainly not least, I want to convey my profound thanks to my husband, Islam, for all the efforts he supported me in earning this degree. I can never express my gratitude to you enough for all the efforts you have put in to allow me to achieve this. Your generosity and endurance throughout the past several years, during which we have lived our lives in thirty-hour intervals, are almost incomprehensible. I'm grateful you are my staunchest ally, dearest friend, and most prominent supporter. You are essential to this master's existence. You being my person makes me really grateful.

For my daughter, Tülin, who deserves the best in the world

Yomna Soliman Mohammed Soliman Tolba

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1. Human Central Nervous System (CNS) Health and Disease: The Function of the Blood-Brain Barrier

Annually, neurological conditions, including Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD), are diagnosed in more than 3 million adult Americans (Borlongan et al., 2013). Just 9 of these diseases are projected to cost Americans $789 billion (in 2014 USD) in health care costs. Costs would dramatically rise as a result of the population almost doubling by 2050, from 43.1 million to 83.7 million (Gooch et al., 2017). Although chronic diseases impose an ever-increasing burden on individuals and society, their origins are often still a mystery. Numerous neurological diseases have been linked to blood-brain barrier (BBB) dysfunction, like amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson's disease (PD) (Engelhardt & Ransohoff, 2012; Garbuzova-Davis et al., 2011; Zlokovic, 2014). Therefore, comprehension of the BBB's biology and pathology will aid in discovering new therapeutic targets that may be employed to treat neurological disorders.

1.1.1. The Neurovascular Unit (NVU)

We must examine the cellular and molecular architecture of the BBB in detail if we are to comprehend its structure and the method of transport through it. The neurovascular unit (NVU) is a structural network that includes neurons and their surrounding cells, including pericytes, glial cells such as oligodendrocytes, astrocytes, and microglia, and vascular cells like vascular smooth muscle cells (SMCs) and brain endothelial cells (Zlokovic, 2014). Brain endothelial cells (BECs), which are highly occluded at the

capillary level, surround the capillary tube initially and share the basement membrane layer with pericytes, a structure with astrocytic endfeet and neurons. At the arteriolar level, BECs are separated from pericytes and astrocytes by arteriolar SMCs, which in turn are connected to neuronal innervation. The BBB, which comprises a monolayer of BECs surrounded by astrocytes and pericytes, is the most important and core part of the NVU. It extends along the capillary and arteriolar axis, dividing them and what they convey from the brain to blood or the other way around (Sweeney et al., 2019). Essential functions of these cells include cell-matrix interactions, neurogenesis, angiogenesis, and neurotransmitter control. Most importantly, however, maintaining the integrity and function of the BBB and constantly regulating the volume of cerebral blood flow (CBF) entering the brain is a critical role of the NVU in ensuring normal brain function and preventing any brain diseases (Yu et al., 2020). Indeed, researchers have identified a strong link between BBB dysfunction and breakdown and the prevalence of several neurodegenerative diseases such as AD, PD, and ALS (Sweeney et al., 2019).

1.1.2. Blood-Brain Barrier (BBB)

It is important to briefly explain the normal physiology of the BBB, how it works, and the cells that make it up. Paul Ehrlich and Edwin Goldmann were the first to demonstrate a specific barrier between the CNS and the circulatory system in 1885 and 1913, respectively. It then took Stern and Gaultier years to name it the “blood-brain barrier” (BBB) in 1922 (Stern & Gautier, 1922; Yarong He, Yao Yao, Stella E Tsirka, 2014). Due to its dynamic morphology, the BBB, also known as the blood-brain interface (BBI), is the complex structure by which the brain maintains its functionality and normal behavior. It has several characteristic features, including the absence of fenestrae, a negative membrane charge, a conserved thickness in the BEC monolayer, and the lowest activity of the pinocytosis mechanism (Bagchi et al., 2019). Nevertheless, it's crucial to remember that these properties are not intrinsic but acquired and well-established by the endothelium for orchestrated interaction with the vasculature during CNS development, as evidenced by several developmental studies (Andreone et al., 2015; Carmeliet & Tessier-Lavigne, 2005; Eichmann & Thomas, 2013).

Regarding the physiology of the human brain, the circulatory system is essential for nutrition and oxygen to reach neurons. In fact, the human brain receives 20% of the heart's overall output while making up only 2% of the body's total mass. In addition, the brain uses about 20% of the body's total oxygen and 25% of its glucose on a daily basis (Zlokovic, 2008). These fuels and nutrients are delivered directly to the CNS on demand, as it has no reservoir of circulating energy substrates and metabolites for spontaneous

consumption (Sweeney et al., 2019). Therefore, it is critical to consider a specialized barrier that maintains brain homeostasis by filtering out red blood cells (RBCs), leukocytes, pathogens, and neurotoxic and vasculotoxic plasma components responsible for neuronal dysfunction and impairment. This unique property of the BBB is termed selective permeability and is achieved with the help of several BEC biomarkers, including tight and adherens junctions, which we will discuss below. Thus, it is likely that increased BBB permeability combined with decreased CBF leads to the accumulation of a neurotoxic substance called β-amyloid (Aβ) in the brain, which most studies show very often leads to in AD, as reviewed in Vinters et al. paper (Vinters, 2015). Moreover, Montagne and colleagues pursued a novel approach to detect and quantify leakage of plasma proteins into the brain of AD patients using a contrast-enhanced magnetic resonance imaging (MRI) protocol that recorded a greater permeability of the BBB in AD patients with aging hippocampus compared with healthy controls (Montagne et al., 2015).

1.1.3. Brain Endothelial Cells (BECs)

In addition to pericytes and astrocytes, a monolayer of BECs is primarily the main component of the BBB. They exhibit specific junctional properties and interact with neurons, glial cells, and the basement membrane (BM) underlying the BECs with the help of other regulatory proteins and biomarkers to build the network of such a barrier (Reed et al., 2019). The BECs wrapped around the blood vessels form the actual physical backbone of the BBB, and the primary intact cell membrane has substantially tighter junctions than the continuous endothelium of peripheral vasculature (Ayloo & Gu, 2019). These junctions, along with adherens junctions, are critical for proper CNS function. Critical characteristics of BECs include a minimum level of transcytosis, absence of fenestrae, precise regulation of ion balance by solute carriers, transport of macromolecules via specific carrier proteins or transporters targeted for ubiquitination, and expression of a few breast cancer resistance proteins (BCRs) and multidrug resistance proteins (MRPs) (Reed et al., 2019; Zlokovic, 2014).

Moreover, BECs have higher energy requirements than other cells in the BBB because they have the most significant number of mitochondria to generate sufficient biological energy needed for controlled solute transport across the barrier (Abdullahi et al., 2018), which increases the tendency for an inflammatory response mediated by high levels of oxidative damage and cytotoxicity. Tight junctions (TJs) are large multiprotein complexes anchored to the membrane of adjacent BECs to seal them together and form a continuous and impermeable barrier. The selective permeability of the endothelium to impede the free flow of water and dissolved substances is demonstrated by the high trans-

endothelial resistance (TEER) value of 1500 to 2000 cm^2 (Butt et al., 1990; Hollmann et al., 2017). TJs are formed primarily by endothelial-specific claudin family proteins such as claudin-5, occludins, tricellulins, and junctional adhesion molecules (JAMs). Zona occludens (ZO) family proteins bridge these proteins and the cytoskeleton microfilaments, as shown in Figure 1.1 (Abdullahi et al., 2018; Liebner et al., 2018).

Claudin-5 is the predominant 20 to 24 kDa protein that binds the cells together to a high degree and limits paracellular diffusion. Technically, ablation of claudin-5 appears to cause early postnatal edema and lethality in the mouse brain (Engelhardt & Liebner, 2014). Other isoforms, such as claudin-1 (Pfeiffer et al., 2011) and claudin-3 (Liebner et al., 2008), which may contribute to the barrier property, can also be expressed. Occludin also promotes paracellular permeability through higher-order dimerization and oligomerization. Regarding preserving the BBB, tricellulin is a poorly understood substance, except that, as the name implies, it is positioned at the junction of three cells to limit the passage of macromolecules (Haseloff et al., 2015; Reinhold & Rittner, 2017). JAMs help regulate the transmigration of immune cells like leukocytes, neutrophils, and macrophages (Sladojevic et al., 2014). Moreover, migration or complete loss of JAMs from the endothelium has devastating effects on BBB properties (X.-S. Wang et al., 2014).

In general, TJ synthesis and production in developing brain capillaries are induced and orchestrated *in vivo* by the Wnt/β-catenin signaling. However, dysfunction of the Wnt/β-catenin protein complex results in BBB impairment, which is commonly in relation to various brain disorders such as AD, PD, brain tumors, multiple sclerosis, and stroke (Laksitorini et al., 2019). Laksitorini et al.'s work showed that external activation of Wnt signaling promoted the BBB phenotype in *in vitro* BEC cultures and further decreased paracellular permeability in cerebral microvasculature. All these TJs are attached to intracellular ZOs that bind to cytoplasmic filaments to keep the structure robust and tight. On the other hand, BECs express AJs, which are specialized cellular homophilic interactions made with cadherin molecules intracellularly linked to actin filaments. AJs are critical components of cell-cell interactions and mediate cell maturation (Tietz & Engelhardt, 2015). By encouraging complex formation with the vascular endothelial growth factor receptor-2 (VEGFR-2) via the phosphoinositide 3-kinase (PI3K) signaling pathway, cadherins are crucial for endothelial integrity (Abdullahi et al., 2018).

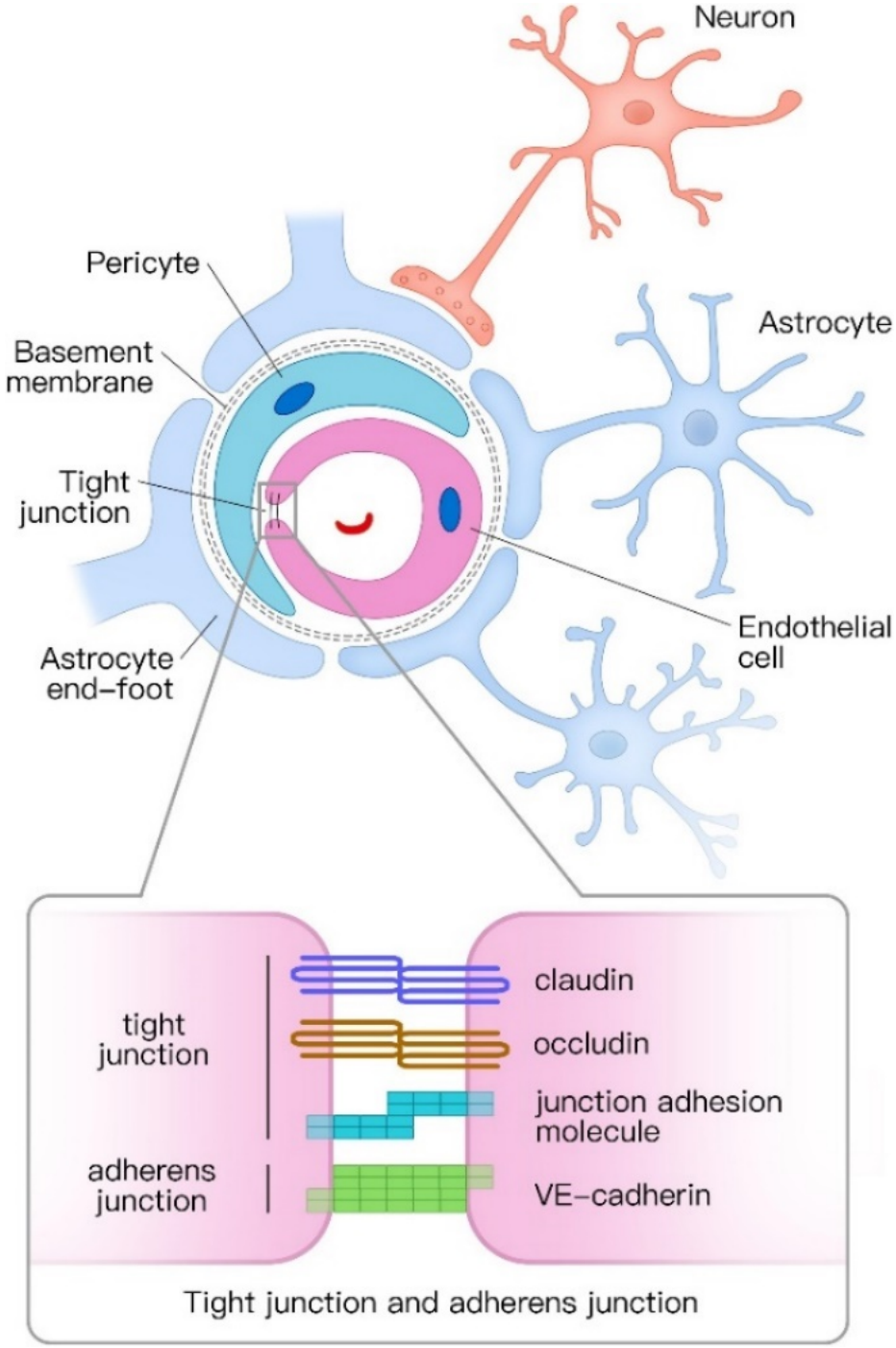

Figure 1.1. An illustration of the tight and adherens junctions in the neurovascular unit (NVU). Vascular, glial, and neuronal cells comprise the NVU, pericytes, oligodendroglia, and vSMCs. Pericytes and astrocyte end-feet encircle endothelial tubes. Endothelial cells are connected in close proximity by adherens and tight junctions. Most of the tight junctions are made up of occludin, claudin, and junctional adhesion molecules, whereas the adherens junction is mostly made up of vascular endothelial (VE) cadherin. NVU is the neurovascular unit. Reproduced with permission from (Yu et al., 2020), Copyright the Creative Commons Attribution (CC BY 4.0) license.

Additionally, the BBB expresses four catenin isoforms, such that β-catenin connects vascular endothelial cadherins (VE-cadherins), the most prominent cadherin member in the BECs, to α-catenin via its cytoplasmic tail to bind to actin filaments. N-cadherins are the second most predominant member of the cadherin family and facilitate the interaction between the endothelial layer and the pericytes covering it. Other biomarkers expressed by BECs are biological transporters. These are the solute-carrier transporters (SLCs) and the ATP-binding cassette (ABC) family, which significantly influence the permeation of endogenous and exogenous substances into the CNS (Sanchez-Covarrubias et al., 2014). ABC transporters primarily consist of P-glycoprotein (P-GP), BCRPs, and MRPs. Each of them has a specific role in the membrane: P-GPs organize the passage of chemically diverse compounds, MRPs have a role in the efflux of anionic medicines and the conjugated metabolites of them, while BCRP functions in overlap with P-GP and somehow acts synergistically to control the brain transport of drugs and can also interact with various organic compounds (Abdullahi et al., 2018).

1.1.4. Development of BECs

Early in development, it becomes clear that the BBB is more specialized than the peripheral endothelium. The perineural vascular plexus, which is formed by migrating mesodermal angioblasts covering the neural tube, starts the formation of the cerebral vasculature (Engelhardt, 2003). Subsequently, the vascular sprouts invade the nearby neuroectoderm via vascular endothelial growth factor (VEGF) concentration gradients (Raab, 2004). Although not exclusive to the CNS, this VEGF-mediated signaling is crucial for healthy vascular growth throughout the body (Shalaby et al., 1995). However, CNS angiogenesis, but not non-CNS angiogenesis, has been shown to require Wnt/β-catenin signaling (Daneman et al., 2009; Stenman et al., 2008). Neural progenitor cells express Wnt7a and Wnt7b in the neural tube's ventral region and the growing forebrain. These ligands block β-catenin deterioration and activate β-catenin targeted genes, such as solute carrier family two member 1 (SLC2A1) (Daneman et al., 2009). This occurs when they bind to Frizzled (Fzd) receptors in the growing vasculature. The downstream activation of death receptor 6 (DR6) and tumor necrosis factor receptor superfamily, member 19, also known as TNFRSF19 and TROY, which further promotes vascular sprouting, provides more proof that Wnt signaling specifically affects angiogenesis in the CNS (Tam et al., 2012).

Last but not least, in contrast to how the Wnt/β-catenin signaling cascade governs angiogenesis in the CNS, the orphan G protein-coupled receptor (GPR124) provides cell-autonomous control over vascular branching in the CNS (Kuhnert et al., 2010). excision

of GPR124 in mice resulted in significant brain hemorrhage, which in turn caused embryonic death. Further study translating Wnt7a/Wnt7b signaling for CNS angiogenesis revealed GPR124 and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) to be essential components in this signaling cascade (Cho et al., 2017). In light of this, the unique development of the CNS vasculature is influenced by both cell-autonomous and non-cell-autonomous mechanisms.

For the development and upkeep of BBB properties in BECs, interactions between the various cells that make up the neurovascular unit (NVU), such as astrocytes, pericytes, microglia, and neurons, are essential (Obermeier et al., 2013). Astrocytes release Sonic Hedgehog (Shh), which aids in forming and maintaining powerful tight junctions. The paracellular leak is enhanced when this route is impaired (Alvarez et al., 2011). However, pericytes reduce vascular permeability and immune cell infiltration by preventing the expression of genes, including angiopoietin-2 (ANGPT2), plasmalemma vesicle-associated protein (PLVAP), intercellular adhesion molecule 1 (ICAM1), and activated leukocyte cell adhesion molecule (ALCAM), and others, in BECs (Armulik et al., 2010). The function that microglia play in neuroinflammation is drawing more attention to the interactions between the BBB and microglia (Thurgur & Pinteaux, 2019), and a better understanding of these interactions is expected to be essential for the development and/or discovery of therapeutics against neuroinflammation.

Improvements in transcriptional sequencing techniques, notably RNA sequencing at both the mass and single-cell levels, have clearly highlighted the molecular heterogeneity of the BBB, both locally and throughout the vascular tree (Noumbissi et al., 2018). Even though the expression of a number of cell adhesion molecules and transporters seems to be heavily influenced by spatial identity, other aspects of the BBB, including the large tight junctions created by occludin and claudin-5, seem to be preserved in these regions (Vanlandewijck et al., 2018). For instance, Yousef et al. observed that in a latest study, aged mice's hippocampus had a clustering of vascular cell adhesion molecule 1 (VCAM1) expression in the endothelial cells of arteries and veins but not significantly in capillaries (Yousef et al., 2019). However, the chemical profiles of these two clusters differed significantly.

Genes associated with migration and proliferation were more highly expressed in vascular VCAM1+ populations. Cytokine receptor expression has been associated with inflammation and served to identify venous VCAM1+ populations (Yousef et al., 2019). It is yet unknown how much NVU member interactions affect how transcriptional activity varies based on location. Future development of BBB-targeting drugs will depend on a thorough understanding of these specific molecular patterns, the alterations in BMEC activity they cause, and the role of NVU contacts in regulating these characteristics.

1.1.5. Dysfunction of BBB

Numerous neurodegenerative diseases, such as multiple sclerosis (Friese et al., 2014), traumatic brain injury (Barzó et al., 1996; Korn et al., 2005; Sandoval & Witt, 2008; Strbian et al., 2008), HD (Drouin-Ouellet et al., 2015), ischemic stroke (Rosell et al., 2008), and normal neurovascular aging (Montagne et al., 2015), are associated with BBB dysfunction. When the BBB is not dysfunctional, the therapeutic distribution of drugs is impeded because many drugs are hydrophilic, have large molecular sizes, or act as efflux transporter substrates (Abbott, 2013; Muldoon et al., 2007). Enhanced transcellular and paracellular permeability, as shown in ischemic stroke (Knowland et al., 2014), and reduced expression and efflux transporter activity, as seen in AD and PD, are frequent signs of BBB breakdown (Bauer et al., 2008; R. Park et al., 2014; Vogelgesang et al., 2002). This deregulation alters the quantities of ions and macromolecules in the brain, such as albumin and neurotransmitters, which impairs synaptic and axonal communication and exacerbates inflammation (Abbott et al., 2006).

The critical functions of BECs in the pathophysiology of neurodegenerative disorders have been highlighted since BBB disruption arises as a prevalent issue in the early stages of neurodegenerative diseases. Understanding how BEC works is a potential first step in understanding the processes by which early vascular dysfunction leads to the progression of neurodegeneration as the BBB has evolved from a passive diffusion barrier to a modulator of central-peripheral interactions, and so has our knowledge of it. BECs perform two seemingly contradictory purposes: they protect the vulnerable brain from toxins and serve as an interface to continuously receive and release signals, maintaining and controlling the homeostasis of the brain. The majority of prior research on neurodegenerative illnesses has concentrated on the loss of barrier functions, and the active control of BECs has received much too little attention. Here, we outline a protocol for creating BECs from a particular kind of stem cells known as MSCs. By using this approach, it will be possible to investigate the evidence for BECs malfunction in neurodegenerative disorders and investigate how BEC signals contribute to the pathogenesis of these conditions.

1.2. Stem Cells and Mesenchymal Stem Cells (MSCs)

Human MSCs provide an unmatched opportunity to investigate BBB modulation and malfunction due to their potential for substantial self-renewal and differentiation into diverse cell types seen in the human body. Prior to now, human MSCs have not been transformed into endothelial cells that demonstrate traits of the BBB *in vivo*, such as the production of nutrient transporters, tight junction proteins, and active efflux transporters. Therefore, we sought to differentiate the first MSCs into endothelial cells that exhibit features of the brain capillaries. According to induced pluripotent stem cell (iPSC) differentiation methods, iPSCs are transformed into mesodermal lineage cells, which can later be transformed into BMECs. Therefore, we thought of using MSCs directly and differentiating them into BMECs. To do this, we should understand these stem cells, their properties, their tissues of origin, as well as their capacity to develop into endothelial cells.

1.2.1. MSCs Roots

Mesenchymal stromal cells (MSCs) or mesenchymal stem cells (MSCs) first became known in 1968, when Friedenstein and his colleagues found that bone marrow (BM) featured a type of cell that could form bone structures when ectopically transplanted into a mouse kidney (Friedenstein et al., 1966). Consequently, they named these osteogenic stem cells (Friedenstein et al., 1968). In 1970, the same laboratory succeeded in isolating from BM a type of fibroblast known as the colony-forming unit (CFU-F), which has the ability to differentiate into osteocytes, chondrocytes, and adipocytes (Friedenstein et al., 1970). After discovering CFU-Fs, Friedenstein attempted to perform the same experiment of ectopic transplantation with CFU-Fs in 1974. The results were exactly as expected because when ectopically injected into a mouse's kidney, these cells were capable of forming bone elements (Friedenstein et al., 1974).

Until 1991, this cell type was not referred to as one of the stem cells. Caplan then named them and observed their bone and cartilage differentiation(Caplan, 1991). After their stem cell property was established, there was considerable doubt about their stem cell status (Bianco et al., 2008). Because of this, several laboratories assigned them various names, such as bone marrow stromal cells, multipotent stromal cells, mesenchymal stromal cells, mesodermal stem cells, and many others. Recently, Caplan recommended renaming them

"Medicinal Signaling Cells" to emphasize their role in tissue engineering, as they secrete many factors and elements that facilitate healing and new tissue formation (Caplan, 2017).

Pittenger demonstrated their multipotency in 1999 (Pittenger et al., 1999). Until that point, MSCs were only known to develop into the three lineages of cartilage, bone, and adipose tissues, together known as the traditional differentiation lineages of MSCs. Three years later, Jiang discovered new non-mesodermal lineages that can differentiate from MSCs, such as ectodermal and endodermal lineages, demonstrating the multipotency of MSCs (Jiang et al., 2002). Examples of these lineages include cardiomyocytes, endothelia, epithelia, pancreatic B-cells, hepatocytes, and neurons(Afflerbach et al., 2020).

1.2.2. MSCs Characteristics and Different Sources

The following characteristics are required for MSCs to confront the International Society for Cellular Therapy criteria: 1) they must express CD-105, CD-73, and CD-90 surface markers; 2) they must not express CD-45, 34, 14, 11b, 79a, 19, and human leukocyte antigen-DR isotype (HLA-DR) surface markers; 3) they must be plastically adherent in cultures; and 4) they must be able to differentiate into the three classical mesodermal lineages (osteoblasts, chondroblasts, and adipocytes) (Dominici et al., 2006). STRO-1, CD106, and CD146 (bone marrow stromal-1 antigen, vascular cell adhesion molecule 1/CD106, and melanoma cell adhesion molecule/CD146, respectively) are three markers of BM-derived MSCs that have recently been discovered (Gronthos et al., 2003; Sacchetti et al., 2007; Simmons & Torok-Storb, 1991).

1.2.3. Origin of MSCs

During the developmental phase of the embryo, the mesoderm, the third germ layer, forms the adult connective tissue, including BM, bones, ligaments, cartilage, and tendons(Caplan, 1991). One hypothesis is that BM-stromal cells are derived from embryonic mesenchymal cells (of mesodermal origin) because they both express mutual markers, including osteopontin, vimentin, fibronectin, and laminin-B1. However, their absolute origin is unclear.(Dennis & Charbord, 2002) Another hypothesis relates to the ectodermal origin of MSCs, from which SRY-box transcription factor 1 (SOX-1)-

expressing cells arise during embryonic neuroectoderm and neural crest formation. SOX1-expressing cells are thought to originate because these cells are found on BM and exhibit features of MSCs. However, during development, the cells residing on BM appear to be replaced by other unknown populations (Takashima et al., 2007). A study on BM of mouse embryos clarifies that BM has two cell populations: one with a mesodermal origin that proliferates intensively, forms bony structures, and does not express nestin. The second has an ectodermal origin, does not divide, does not form bony structures, but does express nestin. Both populations are MSCs, but their origin affects their role, function, and marker expression in adult tissues (Isern et al., 2014).

1.2.4. Niches of MSCs

The niche of stem cells is where they reside undifferentiated (Schofield, 1978). Although the location of MSCs is unknown, it has been hypothesized that it is in the blood vessels, given the presence of all mesenchymal sources. More importantly, cells residing in the perivascular spaces of blood vessels, called pericytes, which have been isolated from specific tissues like skeletal muscles, placenta, pancreas, adipose tissue, CD146+ cells, platelet-derived growth factor receptor beta (PDGF-Rβ)+ cells, neuron-glial antigen 2 (NG2)+ cells, and alkaline phosphatase (ALP)+ cells, are thought to be precursors of MSCs because they give rise to cell populations that meet the criteria of MSCs (Crisan et al., 2008). In parallel with Freidenstein's findings, CD146+ cells have been shown to be responsible for forming bone structures when MSCs are ectopically transplanted to another site(Sacchetti et al., 2007).

In an experiment on a damaged rodent tooth, MSCs were shown to originate from pericytes in an intermediate phase before becoming odontoblasts. On the contrary, in the same experiment, another odontoblast originated from a different MSC origin but not from pericytes that had migrated from blood vessels into the damaged site (Feng et al., 2011). There has been evidence that MSCs from various sources can migrate from blood vessels, express CD34, lack CD-31 and CD145 expression, and satisfy other MSC requirements, such as the capacity to *in vitro* differentiate into PCs (Covas et al., 2008; da Silva Meirelles et al., 2015).

MSCs are expressing some features making them a suitable candidate for constructing *in vitro* models, such as having an autologous source, self-renewal ability, multipotency, various sources, including breast milk, BM, placenta, adipose tissue, Wharton's jelly, tooth pulp, endometrium, and dermis (Dzobo, 2021), and posing no ethical issues when compared to embryonic stem cells (ESCs). Most importantly, MSCs, when introduced

into the body for treatment, do not cause allergic rejections(Afflerbach et al., 2020). Moreover, the age of the subject at the time MSCs were obtained is a unique characteristic of MSCs; for example, if MSCs were obtained at the fetal stage, they would proliferate more rapidly, but if MSCs were obtained from adult tissue, they would form more colonies(Andrzejewska et al., 2019).

Going over the literature, we couldn't find any direct differentiation protocol of MSCs into BLECs. However, we were able to find that one of the multilineage differentiation pathways of MSCs is their differentiation into ECs. Also, Qian et al. paper claimed a protocol for hPSCs differentiation into BLECs, with hPSCs being first to differentiate into mesodermal lineage cell that is then differentiated into BLECs. Here, we used MSCs as an intermediate mesoderm-derived cells and directly differentiated them into BLECs.

1.3. Endothelial Cell Differentiation from MSCs

MSCs can be differentiated into ECs by either directly or indirectly co-culturing them with other cell types or certain coatings. MSCs have been co-cultured with endothelial progenitor cells (EPCs) (Ge et al., 2018), high density of MSCs (Whyte et al., 2011), lysate of HUVECs (Lozito et al., 2009)and on chemically fixed EC layers (Joddar et al., 2018), or endothelial cell matrix (ECM) (Gong et al., 2017). The co-culture methods are efficient in differentiating MSCs because they play a role in activating the Notch signaling pathway, which then activates Notch-induced VEGF-A signaling. Consequently, the produced ECs express many features of ECs, like the increase of CD-31 expression levels, the cobble-stone morphology, and the formation of Tubes on Matrigel.

Differentiation of MSCs in a differentiation medium supplemented with growth factors has been extensively investigated in the literature. Growth factors supplementations simulate the ECs development in embryos by manipulating certain ECs differentiation signaling pathways, including Hedgehog signaling and Notch signaling to regulate VEGF signaling, bone morphogenetic protein (BMP) signaling, which, via activating Smad family proteins (tumor suppressor), regulates the early vascular formation, VEGF signaling that is restricted to ECs differentiation, Hypoxia-inducible factor 1α (HIF-1α) to modulates VEGF signaling and Wnt/β-catenin signaling. In order to differentiate MSCs into ECs, Oswald et al. were the first to apply growth factors (Oswald et al., 2004).

For most studies, VEGF was the widely used growth factor in a concentration of 50ng/ml. Different types of MSCs were used, BM-MSCs (Bai et al., 2010; M. Y. Chen et al., 2009; Khaki et al., 2018; Oswald et al., 2004; Pankajakshan et al., 2013; C. Wang et al., 2018) and umbilical cord-derived MSCs (UC-MSCs)(Alviano et al., 2007; M. Y. Chen et al., 2009; Doan et al., 2014; S. Zhang et al., 2020). Some groups supplemented the differentiation medium with more growth factors like basal fibroblast growth factor (b-FGF), Insulin-like growth factor (IGF), epidermal growth factor (EGF)(M. Y. Chen et al., 2009; Doan et al., 2014; Pankajakshan et al., 2013; C. Wang et al., 2018).

Defining the constituents of the differentiation medium has been shown to be critical in iPSCs differentiation protocols (Hollmann et al., 2017); therefore, here, we started with Wang differentiation medium (IMDM) (C. Wang et al., 2018) and EGM-2 from Pankajakshan paper (Pankajakshan et al., 2013) along with other endothelial differentiation medium kit, Endopan, because all of those media are having defined growth factors. Also, IMDM and EGM-2 proved to produce highly efficient ECs with many EC markers expressions, including CD-31, Cd-34, von Willebrand factor (vWF), VEGFR, and VE-Cadherin (Pankajakshan et al., 2013; C. Wang et al., 2018).

1.4. Methods for Inducing Brain Characteristics of ECs

1.4.1. Retinoic Acid (RA)

In the literature, RA is associated with an increase in brain-like properties of ECs (Mizee et al., 2013). Adult mouse brain endothelium has been reported to exhibit the retinol signaling receptor and transporter (STRA6) but not peripheral endothelium (Bouillet et al., 1997). Additionally, research by the Lippmann group found that STRA6 expression improved throughout the differentiation of BMECs produced from iPSCs (Lippmann et al., 2012). Experiments *in vivo* revealed that BMECs exhibit retinol-binding protein and its membrane receptor STRA6 (Kawaguchi et al., 2007). RA has also been demonstrated to boost the number of BBB traits in humans (Mizee et al., 2013) and immortalized rodent BMEC lines (Hafny et al., 1997; Lechardeur Delphine, 1995). Radial glial cells, which provide the brain with RA during the embryonic cascade, are strongly linked to the developing vasculature and are essential for creating BBB characteristics in BECs during embryonic development.

RA was also shown to be crucial for the regulation of endothelial cell growth and neovascularization during vascular development in research knocking down the enzyme retinaldehyde dehydrogenase 2 (Raldh2-/-), which is necessary for the generation of active Rain the embryo (Lai et al., 2003). Studies on human fetal brain tissue after death have revealed that radial glial cells contain this enzyme. The growing cerebral vasculature also shows considerable expression of the Retinoic acid receptor β (RARβ), the primary CNS target receptor for RA signaling. Studies conducted *in vitro* have shown that various brain EC-barrier components are triggered in an RA-β and RAR-β dependent manner.

Furthermore, serum proteins and a fluorescent tracer were able to enter the developing brain when RAR activation was pharmacologically suppressed during mouse BBB development. Additionally, the expression of important BBB determinants was decreased (Mizee et al., 2013). Moreover, the Lippmann group demonstrated maturation of iPSC-derived BMEC features upon adding RA during differentiation, including increased expression of adherens junction proteins, MRP efflux activity, and barrier function (Lippmann, Al-Ahmad, et al., 2014).

It is known that blood-brain barrier development and endothelial Wnt signaling are regulated by each other (Liebner et al., 2008). In mouse embryos with aberrant endothelial RA signaling, ectopic Wnt signaling has been observed in the brain vascular tissue. The RA receptor, RAR-α, regulates β-catenin activity in BECs through protein kinase C (PKC)-dependent phosphorylation processes that target β-catenin for enzymatic digestion and transcriptional repression. RA controls vascular Wnt signaling by altering pericyte number in growing brain vasculature. Vascular Wnt signaling must be modulated by RA in order to prevent excessive pericyte recruiting, which would impede endothelial-pericyte contacts crucial for vascular integrity (Bonney et al., 2018).

RARα, RARγ, and RXRα receptors have been found to be capable of causing barrier phenotypes through specific RAR agonists. A considerable improvement in barrier integrity and an induction of VE-cadherin expression were seen after RA was administered to iPSC-derived BMECs at days 6–8 of differentiation. Additionally, VE-cadherin expression was increased, and barrier integrity was improved by RAR/RXRα costimulation to levels that replicated RA outcomes (Stebbins et al., 2018).

RA has been tested for osteogenesis (Lim & Park, 2016), neuronal differentiation (Jacob et al., 2015), angiogenesis *in vitro,* and wound repair *in vivo*. It has been demonstrated that when the differentiation medium was preconditioned with high doses of RA, RA enhanced the expression of VEGF and HIF-1α (Pourjafar et al., 2017). For the first time, here, we looked at how adding RA affected MSCs' ability to differentiate into BLECs with enhanced BBB characteristics.

1.4.2. Hypoxia and MSCs

Stem cells are one of the many cell-related processes regulated by hypoxia in the body. From embryonic development through maturity, the hypoxic environment is crucial for cell renewal and repair (Abdollahi et al., 2011). While mild hypoxia, as employed in intermittent hypoxia (IH) therapy, has neuroprotective benefits in a variety of CNS illness models, excessive hypoxia typically results in acute and chronic brain injury (Li et al., 2021). When evaluating the effects of low-oxygen environments (1–5% O_2) and normoxic (21% O_2) conditions, a number of studies were found using a variety of sources of pluripotent stem cells, such as hiPSCs cultured with or without the addition of growth factors, human embryoid bodies (hEBs), mESCs hESCs, or mEBs, showed a rise in the number of cells expressing the common endothelial markers (e.g., CD144, PECAM1, VEGFR1, Tie2), depicted endothelial-like structures, and were able to form tubule-like structures on Matrigel and uptake acetylated LDL (Podkalicka et al., 2020).

It is also shown that human iPSC-derived BLECs differentiated under hypoxic conditions exhibit *in vivo*-like functionality when cocultured with primary human pericytes and astrocytes, as indicated by their high barrier integrity, P-GP functionality, and antibody transcytosis capability in contrast to primary BECs and normoxic differentiation conditions (Morad et al., 2019; T. E. Park et al., 2019; Sahtoe et al., 2021). Under a hypoxic microenvironment, the Akt and NF-B pathways control early endothelial differentiation and its capacity to migrate (Liu et al., 2017). Co-culturing in normoxia has also been demonstrated to preserve endothelium and osteogenic differentiation markers. While limiting cell proliferation and osteogenesis, the hypoxic environment also promotes angiogenesis even after just one day of therapy (Nguyen et al., 2020).

1.4.2.1. The mechanism of oxygen sensing reveals the effect of hypoxia on MSCs

When oxygen is available in large quantities, the molecular mechanism necessary for cellular oxygen detection is triggered. HIF-1α and HIF-2α subunits, HIF-α subunits, are hydroxylated by a series of HIF-specific prolyl hydroxylase domain (PHD) enzymes on evolutionarily conserved proline residues to make them identifiable by von Hippel-Lindau protein (pVHL)-containing ubiquitin ligase complexes. The proteasome then degrades the hydroxylated HIF-α (Simon, 2016). Reduced oxygen levels block HIF-prolyl hydroxylation, which activates target genes and enables reversible HIF-α stabilization and dimerization with HIF-1β (ARNT). The family of HIF-regulated genes includes 500–1000 validated downstream effectors that control several hypoxic responses necessary for proper physiology, embryogenesis, and a number of disorders and events,

including cancer and tissue ischemia. These adaptations depend on the cellular context, as shown in Figure 1.2.

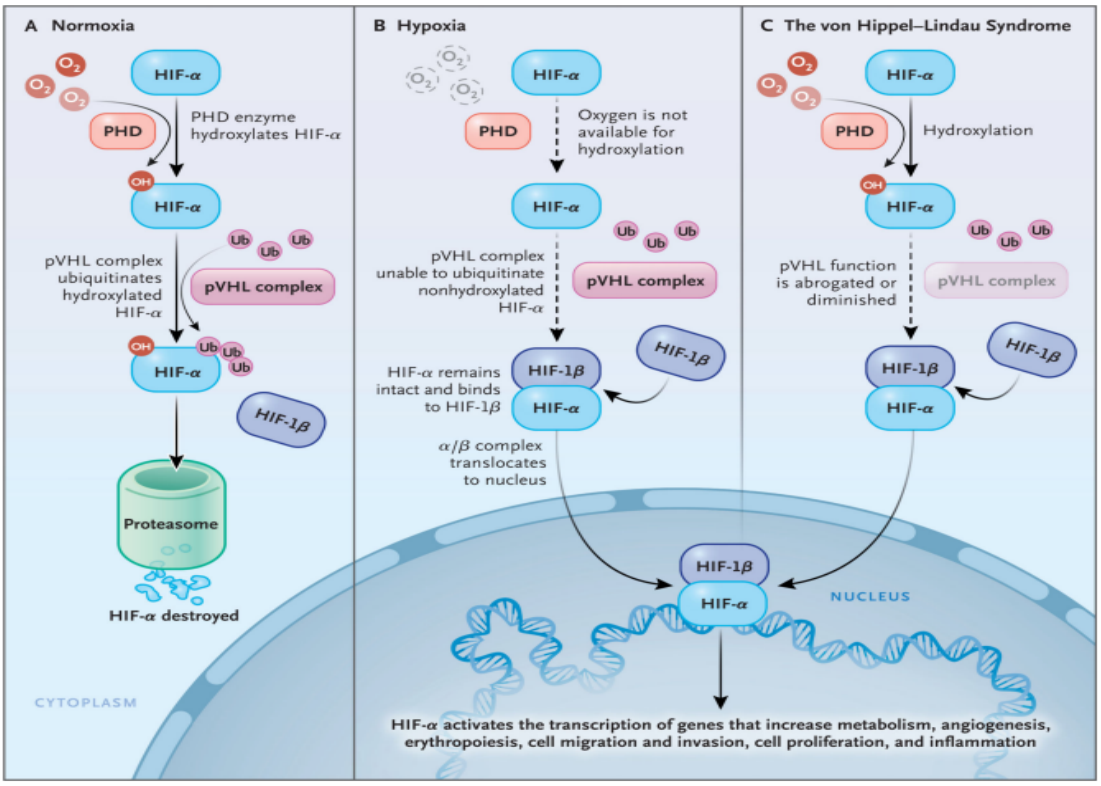

Figure 1.2. Oxygen Sensing: A Comprehensive Analysis**.** Reproduced with permission from (Simon, 2016), Copyright Massachusetts Medical Society.

1.4.2.2. Duration of hypoxia has an impact on MSCs differentiation

Many studies conducted on different types of hPSCs to test the effects of hypoxia; for example, The Pedro-Lopez group observed alterations in the expression of genes related to vasculogenesis and angiogenesis, as well as the control of vascular permeability, vasodilatation, and vasoconstriction, in hESCs cultivated under 1 percent and 5 percent O_2 for one, five, and fifteen days (Prado-Lopez et al., 2010). Another research found that early differentiation in low O_2 Concentrations (5 percent O_2) produced a significant number of ECs (White et al., 2013). Furthermore, in the Shin et al. investigation, culture under hypoxic circumstances had no impact on chromosomal integrity. However, when the culture was prolonged under harsh circumstances (1 percent O_2 over 7 or 15 days), this led to noticeably more necrosis(Shin et al., 2011).

1.4.2.3. Hypoxic pre-conditioning (HPC) of MSCs as a preliminary step prior to differentiation

The outcomes of HPC on stem cells have been inconsistent, allegedly due to variations in the oxygen content, exposure time, and passage number (Chacko et al., 2010). For stem cells to differentiate into ECs, priming the cells in hypoxia prior to differentiation is essential, according to several studies (Chacko et al., 2010; Hu et al., 2008; Muzakkir et al., 2020). For example, during the seven days of mESCs differentiation, Tsang et al. examined six variants of normoxia and hypoxia (5 percent O_2). It has been demonstrated unequivocally that the first hypoxia levels must last at least two days (Tsang et al., 2017). Furthermore, when hiPSCs were differentiated for a sum of 12 days under six days of sustained hypoxia (5 percent O_2), initiated either early in the differentiation (primed 5 percent O_2) or on days 6–12 (secondary 5 percent O_2), the expression of platelet endothelial cell adhesion molecule 1 (PECAM1) and CD144 and was at a comparable scale as being under normal O_2 Levels as contrasted to primed 5 percent O_2, resulting in tremendous increased expression of ECs markers (Kusuma et al., 2014).

1.4.2.4. Differentiation under hypoxia toward ECs: HIF-1α as a key effector

Based on the duration of hypoxia, HIF-1α seems to be variably modulated, as HIF-1α expression was visible as early as 12 hours (different from under normoxia), peaked between days 1 and 2, and then was gradually reduced by days 5 and 7, showing transient HIF-1α synthesis in spite of persistently hypoxic condition (Cameron et al., 2008; Kusuma et al., 2014). It is known that HIF-1α target genes, glucose transporter type 1 (GLUT-1) and VEGF, are increased in hypoxic environments (1% and 5% O_2) (Cameron et al., 2008). HIF-1α and ETS variant transcription factor 2 (ETV2) are necessary for hypoxia-mediated stimulation of EPCs differentiation and maturation since they both inhibited overexpression of ECs markers under hypoxia in HIF-1α-KO and ETV2-KO cells, potentially via regulating Notch1 levels (Tsang et al., 2017). By linking to reverse hypoxia-response element (HRE) sequences (rHRE), HIF-1α may potentially function as a gene expression suppressor. Curiously, the Oct4 promoter included four of these HIF-1α-specific rHRE elements, and it was shown that HIF-1α decreased Oct4 by interacting with three of them (Lee et al., 2012). Moreover, HIF-1β is essential for maintaining HIF transcriptional activity. This implies that HIF-1α and HIF-1β play a significant part in cells' development of vascular properties in hypoxia (Podkalicka et al., 2020). Additionally, it has been made clear how crucial VEGF receptor regulation is for

regulating the development of vascular networks stimulated by low oxygen tension (Han et al., 2010).

Traditionally, a hypoxia chamber is used to create hypoxia. Nevertheless, other approaches don't need specialized tools, such as the chemical modulation of HIF-1α by $CoCl_2$, the reduction of pericellular oxygen levels by raising the medium level, and the oxygen usage by an enzyme system named GOX/CAT, as shown in Figure 1.3. (Rinderknecht et al., 2021).

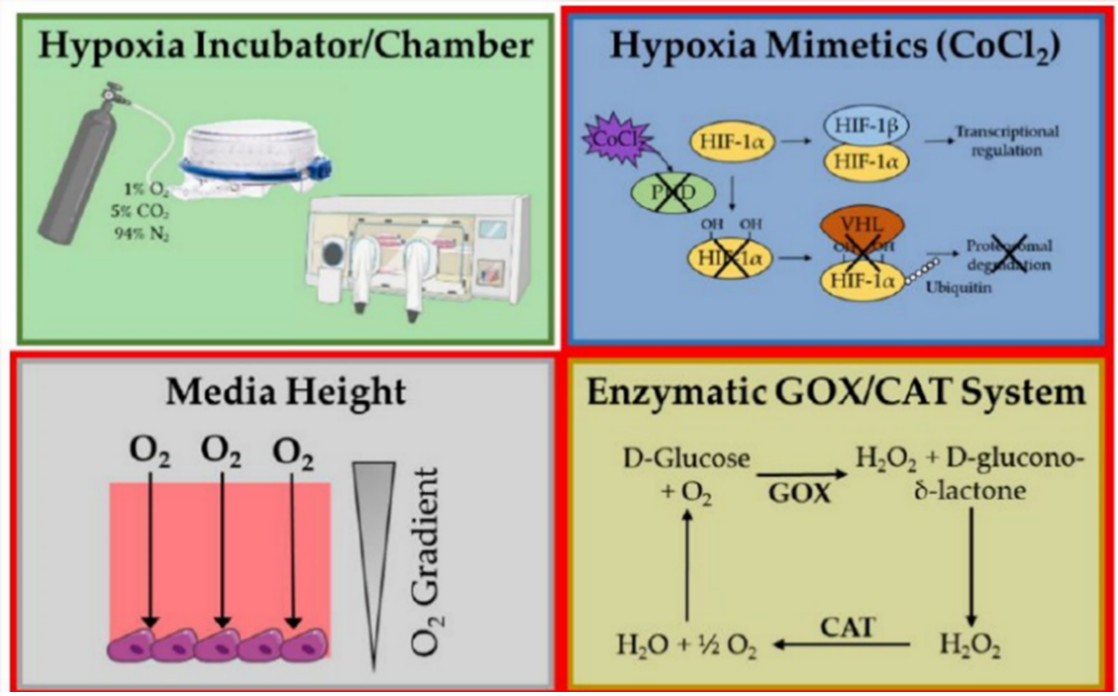

Figure 1.3. Different methods of inducing hypoxia. Reproduced with permission from (Rinderknecht et al., 2021), Copyright the Creative Commons Attribution (CC BY 4.0) license.

1.4.2.5. Differences between oxygen differential and culture conditions' effects on MSCs' fate

The effects of hypoxia have been reported to increase proliferative potential, clonogenic potential, the secretome of MSCs, and osteogenic, chondrogenic, and adipogenic differentiation (Samal et al., 2021). MSCs may come into contact with low O_2 levels based on the *in vivo* habitat (Barry & Murphy, 2004; Dionigi et al., 2014), sometimes as low as less than 1% (Boyette et al., 2014; Mas-Bargues et al., 2019). To illustrate, MSCs have O_2 concentrations of 1–7% in bone marrow (Fehrer et al., 2007a), 10–15% in

adipose tissue (Bizzarri et al., 2006; Kabon et al., 2004), 3–10% in muscle (Saltzman et al., 2003), 1–2% in cartilage (Reuther et al., 2012), 1.5–8% in umbilical cord blood and amniotic fluid (Fischer & Bavister, 1993), and 10–12% in peripheral blood (Mas-Bargues et al., 2019). Compared to the 21% O_2 detected in normoxic environments often employed in labs for MSC cultivation, this physiological O_2 concentration (physioxia) is significantly lower (Boyette et al., 2014). The activity of MSCs may be affected by the high number of free radicals resulting from these different O_2 concentrations in normoxia (Lennon et al., 2001).

Research on the application of hypoxia to MSCs has yielded wildly varying results, possibly related to the fact that different studies define hypoxia differently and the O_2 concentration range of hypoxia from 1 to 7% (Ren et al., 2006). Variations in culture systems, choice of markers, micronutrients, and growth factors make the uneven findings even more problematic (Ma et al., 2009). In these studies, differentiation is usually accompanied by either expansion in normoxia and differentiation in hypoxia or expansion in hypoxia and differentiation in normoxia. Here, we expanded cells under normoxia and differentiated cells under hypoxia with hypoxic preconditioning in the expansion medium for 24 hours.

Compared with cells cultured in normoxia, increased proliferation and colony-forming potential of hMSCs and mMSCs have been documented at O_2 concentrations of 1 to 5% (Boyette et al., 2014; dos Santos et al., 2010; Fehrer et al., 2007a; Jin et al., 2010a). Some theories postulate that the rise in proliferation may result from p16's being downregulated, which allows cells to avoid senescence (Jin et al., 2010a). When hMSCs are exposed to hypoxia, they initially show increased cell mortality within the first 1-2 hours and impairment of various physiological processes (Buravkova et al., 2014). Autophagy, which may be the initial response to hypoxia, may occur in this condition as a means of survival for hMSCs (Wu et al., 2014). However, longer periods of hypoxia exposure (at concentrations of 2–5% O_2) have been associated with higher rates of proliferative activity (Boyette et al., 2014; Fehrer et al., 2007a; Lavrentieva et al., 2010). Hypoxia not only results in the decreased regulation of p16 but also stabilizes HIF-1α, which may activate a variety of signaling pathways inside the cell, more prominently, overexpression of the anti-apoptotic protein survivin, which promotes enhanced proliferation (Chacko et al., 2010; Kiani et al., 2013).

hMSCs and mMSCs cultivated in hypoxic environments (2–5% O_2 concentrations) exhibit increased proliferation as well as increased multipotency, as demonstrated by elevated expression of early mesodermal and stemness genes such as octamer-binding transcription factor 4 (Oct-4), Sox2, and Nanog (dos Santos et al., 2010; Fehrer et al., 2007a; Valorani et al., 2010). based upon the maintenance of the undifferentiated state

and the activation of genes associated with mesodermal and non-mesodermal lineages, it has been hypothesized that MSCs might retain the properties of “true stem cells” under hypoxia (Buravkova et al., 2014; Nekanti et al., 2010). hMSCs expanded at 2% O_2 have been reported to produce more cells; however, differentiation under normoxia did not differ between hypoxic and normoxic cells in terms of adipogenic or osteogenic differentiation, but cells expanded at 3% O_2 showed greater differentiation (dos Santos et al., 2010; Fehrer et al., 2007a).

Furthermore, it’s been proven that both maintenance of the undifferentiated state of cells and promotion of greater proliferation in basal media can be achieved by a prolonged culture of hMSCs for up to 4 passages at 5% O_2. However, compared to cells expanded under normoxia, these cells showed a greater capacity for osteogenic differentiation after being grown in a hypoxic environment, indicating increased multipotency (Basciano et al., 2011).

In both *in vivo* and *in vitro* environments, oxygen concentration considerably modulates genetic and environmental factors that affect MSCs' survival and plasticity (Simon & Keith, 2008). Numerous studies have shown that MSCs can proliferate and regulate their multipotency when exposed to prolonged hypoxia (longer than 24 hours), demonstrating that low O_2 concentrations may be essential for the physiological ecosystem of MSCs. Many studies using mMSCs and hMSCs were inconclusive and reported results that varied greatly. These discrepancies may be related to species differences, exposure times to hypoxia and O_2 levels, physical restrictions, and interactions between cells and their substrates brought on by 2D or 3D culture technique differences, and growth factor differences (Fehrer et al., 2007b; Jin et al., 2010b; Samal et al., 2021). As a result, strict control of cultural conditions is necessary to get reliable findings. These factors include frequency of media changes, scaffold characteristics, stiffness, growth supplements, pH, etc. Understanding the variables and growth conditions that mediate stem cell growth and stemness is thus essential to the development of efficient strategies for *in vitro* cell proliferation without compromising their functionality and expression.

1.4.2.6. Cobalt chloride as a hypoxia-imitating Compound

Numerous studies have used $CoCl_2$ as a compound that mimics hypoxia *in vitro* by stabilizing HIF-1α, and different results have been obtained (Y. Chen et al., 2019; K. S. Kim et al., 2006; Teti et al., 2018). In one study, the capacity for chondrogenesis of MSCs obtained from various sources was examined in relation to how hypoxia affected their activity (Teti et al., 2018). The findings demonstrated that cell viability was unaffected

by the addition of $CoCl_2$ at concentrations ranging from 50 to 400 μM. The cellular origin also affected the overexpression of chondrogenic markers such as VCAN, SOX9, COL2A1, and ACAN (Teti et al., 2018). Another research examined how human exfoliated deciduous teeth (SHEDs) stem cells responded to hypoxia when cultivated with 50 μM and 100 μM $CoCl_2$. HIF-1α protein levels steadily rose with concentration without significantly increasing the toxicity. Furthermore, $CoCl_2$ reduced osteogenic differentiation while upregulating stem cell markers (Y. Chen et al., 2019).

Furthermore, chemical induction of hypoxic stress increased the permeability of the BBB when tested with hydralazine or $CoCl_2$ (50 and 100 μM; the lowest cytotoxic doses defined by a cytotoxicity test) for 24 hours. Hypoxic stress caused by hydralazine reduced the expression of ZO-1 but didn't affect occludin expression, according to tight junction protein analysis. Boosting the expression and the function of the cells' efflux transporters has also been increased as a defensive strategy (Chatard et al., 2017).

1.4.2.7. Sodium sulfite is a hypoxia-mimetic compound

Another chemical that can induce hypoxia is sodium sulfite (Na_2SO_3). Sodium Sulfite' Na_2SO_3' can scavenge O_2 molecules, and metal ions, like Co^{2+}, Zn^{2+}, and Ni^{2+}, can induce the up-regulation of HIF-1α (Collaco et al., 2006; Kaczmarek et al., 2009). Erythropoietin, B-cell lymphoma 2, and vascular endothelial growth factor A (VEGF-A) genes are all induced when a hypoxic environment activates HIF-1α. The generated hypoxic state impacts the MSCs' stemness, differentiation, and proliferation(Sart et al., 2014). Moreover, Na_2SO_3 has been used as hypoxia-mimetic in C. Elegans in concentrations ranging from 1g/L (5.5 mM) to 4 (22 mM) g/L and established hypoxia microenvironment at 2g/L (11mM) (Bin et al., 2010).

Other studies tested a mixture of $CoCl_2$ and Na_2SO_3 with defined concentrations (Ghaly & Kok, 1988; H. Kim & Kwon, 2021). A study tested mixing 10 μM or 100 μM of $CoCl_2$ with 4 mM of Na_2SO_3 and showed safety, better osteogenic differentiation, and proliferation of MSCs with more expression of VEGF and HIF-1α genes (H. Kim & Kwon, 2021).

Here, we optimized the safest concentration of both $CoCl_2$ and Na_2SO_3 and added them to the differentiation media to mimic the BBB embryonic development conditions, thus increasing the BBB characteristics of the resultant BLECs.

1.5. Synthetic and Animal-derived Serum Supplementation

For iPSCs-derived BLECs, researchers used a serum-containing medium. Then they switched to a Serum-free supplement, B27, a substitute serum used often in neuronal cultures (Brewer et al., 1993), with the advancements made by many groups in providing a protocol with higher BBB characteristics of the produced BLECs (Hollmann et al., 2017; Neal et al., 2019; Qian et al., 2017; Wilson et al., 2015). Here, we describe an unexpected benefit of adding FBS rather than switching to a serum-free component, B27, for BLECs differentiation from BM-MSCs. Oppositely to iPSCs results, B27 showed incompatibility with BM-MSCs differentiation media. These improvements in differentiation methods are anticipated to benefit the modeling of age- and disease-related decreases in BBB function utilizing BLECs produced from MSCs.

1.6. Basal Medium Effect on the Differentiation

Low glucose- Dulbecco Modified Eagle Medium (LG-DMEM) basal medium is frequently used as the basal medium for expanding bm-MSCs (Bai et al., 2010; M. Y. Chen et al., 2009; Oswald et al., 2004). some protocols used LG-DMEM as the basal medium of ECs differentiation medium (Bai et al., 2010; M. Y. Chen et al., 2009; Oswald et al., 2004), while others used IMDM (C. Wang et al., 2018), high glucose- Dulbecco Modified Eagle Medium (HG-DMEM) (Tancharoen et al., 2017), or Dulbecco Modified Eagle Medium/ F12 (DMEM/F12) (S. Zhang et al., 2020) as their basal medium for ECs differentiation. Here, we tested the effect of changing the basal medium of the differentiation medium along with the expansion medium and combining different expansion and differentiation basal media.

In this thesis, we combined MSCs and hPSCs differentiation protocols into ECs and BLECs, respectively. In other words, we used MSCs-derived ECs protocols and added some clue factors from hPSCs-derived BLECs, like RA, hypoxia-regulators, seeding density, and serum supplementation to streamline the BBB characteristics and provide a fully defined protocol for differentiating MSCs into BLECs.

2. MATERIALS AND METHODS

2.1. MSCs Expansion Medium Preparation

2.1.1. LG-DMEM

Low glucose-Dulbecco Modified Eagle Medium (LG-DMEM, Gibco) basal medium containing 10g/L glucose, NaHCO3, and supplemented with 10% heat-inactivated- fetal bovine serum (hi-FBS, Pan-biotech), 1% Penicillin-Streptomycin (Pen/strep, Pan-biotech), and 1% 200 mM L-glutamine (L-glu, Merck).

2.1.2. IMDM

Iscove's Modified Dulbecco Medium (IMDM, PAN BIOTECH) basal medium containing L-Glutamine and 3.024g/L $NaHCO_3$ and supplemented with 10% heat-inactivated- fetal bovine serum (hi-FBS, Pan-biotech), and 1% Penicillin-Streptomycin (Pen/strep, Pan-biotech). The medium was sterile-filtered and stored at 4°C.

2.2. Endothelial Differentiation Medium

2.2.1. IMDM

Iscove's Modified Dulbecco Medium (IMDM, Pan-Biotech) basal medium with L-Glutamine and 3.024g/L $NaHCO_3$ supplemented with 2% Fetal Bovine Serum (FBS, Pan-biotech), Vascular Endothelial Growth Factor (VEGF, Lonza), basic Fibroblast Growth Factor (bFGF, Lonza), Insulin-like Growth Factor (IGF, Lonza), Epidermal Growth Factor (EGF, Lonza), Ascorbic acid (AA, Lonza), Heparin (Lonza).

2.2.2. LG-DMEM

Low glucose-Dulbecco Modified Eagle Medium (LG-DMEM, Gibco) basal medium containing 10g/L glucose, NaHCO3, and supplemented with 2% Fetal Bovine Serum (FBS, Pan-biotech), Vascular Endothelial Growth Factor (VEGF, Lonza), basic Fibroblast Growth Factor (bFGF, Lonza), Insulin-like Growth Factor (IGF, Lonza), Epidermal Growth Factor (EGF, Lonza), Ascorbic acid (AA, Lonza), Heparin (Lonza).

2.2.3. Endopan

Basal Medium Endopan 300 SL with Serum substitute Pannexin SL-S supplemented with EGF (human recombinant Epidermal Growth Factor), FGF-2 (human recombinant basic Fibroblast Growth Factor), VEGF (human recombinant Vascular Endothelial Growth Factor), Vitamin C (Ascorbic Acid phosphate), R3-IGF-1 (human recombinant Insulin-like Growth Factor), GA (Gentamycin/Amphotericin), Hydrocortisone, and Heparin.

2.2.4. Endothelial Growth Medium 2 (EGM-2)

Endothelial Basal Medium-s (EBM-2, Lonza) supplemented with EGF (human recombinant Epidermal Growth Factor), FGF-2 (human recombinant basic Fibroblast Growth Factor), VEGF (human recombinant Vascular Endothelial Growth Factor), Vitamin C (Ascorbic Acid phosphate), R3-IGF-1 (human recombinant Insulin-like Growth Factor), GA (Gentamycin/Amphotericin), Hydrocortisone, and Heparin (all from Lonza bullet kit). The medium was sterile-filtered and stored at 4°C.

The first and second media were prepared as previously described (C. Wang et al., 2018)with some modifications in which we used the specified growth factors from Lonza Bullet-kit, not as described in the paper. The fourth medium was prepared as previously described.

2.3. MSCs Culture

MSCs were expanded in Low glucose-Dulbecco Modified Eagle Medium (LG-DMEM) basal medium containing 10g/L glucose, NaHCO3, and supplemented with 10% heat-inactivated- fetal bovine serum (hi-FBS), 1% Penicillin-Streptomycin (Pen/strep), and 1% 200 mM L-glutamate. When reaching 80-90% confluency, cells were washed with DPBS-/-, trypsinized by Trypsin/EDTA, and plated again in new plates. The used MSCs were in passage 21.

2.4. Differentiation of MSCs into BLECs

MSCs were seeded at different seeding densities determined according to each experiment's conditions ($1x10^4$, $7.5x10^3$, $5x10^3$, $4x10^3$, $2x10^3$ cells/well of 48-well plates) for 24 hours, hypoxic pre-conditioned in 50 μM $CoCl_2$ or 4 mM Na_2SO_3 in the expansion medium for 24 hours (if a hypoxia regulator is being tested). Then the medium was switched to differentiation medium only, according to the experiment’s conditions (Endopan, EGM-2, IMDM, LG-DMEM), or differentiation medium supplemented with

50 μM $CoCl_2$ or 4 mM Na_2SO_3 (if a hypoxia regulator is being tested) for the defined duration of each experiment with medium change frequency determined by each experiment's conditions. For the experiments when RA was tested, the medium was switched to the same differentiation medium but with 1, 3, and 10 μM RA for 48h or 72h on either Day 6 or Day 12 of differentiation.

2.5. Immunofluorescence Staining

After cells had completed their differentiation protocol, cells were checked under the light microscope (Carl Zeiss Primovert, 3841016470), washed three times with DPBS+/+ (PAN, P04-35500), fixed with 4% paraformaldehyde (PFA) (Merck, 158127)and incubated for 20 minutes in the dark at room temperature, incubated in the blocking buffer (0.015% of 30% bovine serum albumin (BSA) (Pan-Biotech, P06-1403100), 0.0001% Tween 20 (Sigma, P1379-1L) in DPBS+/+) for 1 hour in the dark at room temperature, washed three times with DPBS+/+, incubated with the primary antibody specific for ZO-1 (ProteinTech, 21773-1-AP, 1:500), CD-31 (ProteinTech, 11265-1-AP, 1:500), Occludin (ProteinTech, 27260-1-AP, 1:500) overnight in the dark at 4°C, washed three times with DPBS+/+, incubated with the Cy™3 AffiniPure Goat Anti-Rabbit IgG (H+L) secondary antibody (Jackson Immuno Research, 111-165-003, 1:1000) for 1 hour in the dark at room temperature, washed three times with DPBS+/+ and incubated each time in the dark on a shaker for 5 minutes, incubated with a ready-made solution of DAPI stain (Sigma-Aldrich, MBD0015, 1:1000) for 2 minutes in the dark, washed three times with DPBS+/+, mounted with DPBS+/+, and checked under Zeiss Vert.A1 Fluorescence microscope.

2.6. Quantification of Images by ImageJ Software

Cells were quantified for their fluorescence by ImageJ Software, where a certain area of each image was selected. The mean fluorescence intensity value was chosen from the measurement settings. Comparisons have been made by applying those steps for test images and only secondary Ab images, then subtracting the value of the only secondary Ab mean fluorescence intensity from the test mean fluorescence intensity to get the mean

fluorescence intensity value of the test images ($fF_{test} = iF_{test} - F_{only\ secondary\ Ab}$). This process was repeated for many areas, and the mean fluorescence intensity averages were calculated and compared using GraphPad Prism software.

2.7. MTT Cell Proliferation Assay

The cells were cultivated for 24 hours at 37°C with 5 percent CO_2 after being plated in a 96-well plate at a $5x10^3$ cells/ well density in LG-DMEM supplemented with 10% FBS. The cells were then exposed to different doses of $CoCl_2$ (0.005-400 μM) and Na_2SO_3 (0.025-2000 μM) for 5 and 9 days, respectively. For each sample, three duplicate wells were prepared. Control cells that had not been treated were utilized. After the treatment, 50 μl MTT was added to the cells, and they were then incubated for an additional 3–4 h. After removing the supernatant, 200 μl of Solubilization Solution was added to each well, and the plate was then stirred for 10 minutes to dissolve the crystals. An enzyme-labeling device (TECAN infinite M200 Pro) assessed the absorbance at 570 nm. The zero-absorbance point was the negative control well, which had no hypoxic agent. The assays were carried out in triplicate. Logarithmic concentration was used as the x-axis, and the cell viability percentage value (mean standard deviation) as the y-axis to create graphs of cell growth.

2.8. Tube Formation Assay

After cells were incubated in a 48-well plate containing the endothelial differentiation medium for nine days, cells were harvested with trypsin, seeded at $1x10^4$ cells/well, and incubated at 37ºC and 5% CO_2 for 24 hours in a 96-well plate precoated with 50 μl Matrigel Corning. Cells were examined under a Zeiss Vert.A1 microscope. Then, a plugin in ImageJ software was used to measure the total tube length and total segment length. Briefly, tiff formatted images were opened, and the Analyze HUVECs Phase Contrast option was chosen from the Network Analysis Menu. The data were given in an Excel sheet format and then compared by GraphPad Prism software.

2.9. Western Blot

B-Mercaptoethanol and 2x Laemmli sample buffer (Bio-rad) were combined with the lysates before being heated at 95 °C for 5 minutes. Following that, a gel produced in accordance with the instructions provided in the TGX Stain-Free FastCast Acrylamide kit was loaded with an equivalent quantity of proteins and an unstained protein marker (12 percent) (Bio-rad). Using the Wet Transfer System (Consort), proteins were transferred to a PVDF membrane and subsequently blocked for 1 hour at room temperature with 5 percent skim milk (Serva) in PBST (1 percent Tween (Sigma)). The following antibodies were used in primary antibody incubations, which were carried out overnight at + 4 °C: anti-Occludin (ProteinTech, 27260-1-AP, 1:5000), anti-Claudin-5 (Sigma-Aldrich, SAB4502981, 1:500), and anti-CD-34 (ProteinTech, 14486-1-AP, 1:500) after three PBST washes. Secondary antibodies, goat anti-rabbit IgG-HRP, were treated with the membrane for 1 hour at room temperature (ProteinTech, 1:5000). The unstained marker could be seen after a 5-minute incubation period at room temperature with streptactin HRP conjugate (Bio-Rad, 1:10000). Following washing, the image was discovered using the ChemiDoc MP Imaging System and Clarity Western ECL Substrate (Bio-Rad). Total protein normalization was utilized to quantify the bands using Image Laboratory Software Version 6.1 (Bio-Rad).

2.10. Statistical Analysis

Statistical comparisons were performed using Two-way ANOVA and One-way ANOVA. Data were expressed as mean ± standard error of the mean. P Values <0.05 were considered statistically significant. The P values indications for *, **, ***, **** were < 0.1, < 0.01, < 0.001, and < 0.0001, respectively.

3. RESULTS AND DISCUSSION

The combination of the outstanding achievements in hPSCs differentiation into BLECs and MSCs differentiation into ECs enlightened the way for this research in different ways. Going through the literature, many protocols for differentiating hPSCs into BLECs are available; some use iPSCs, hematopoietic stem cells (HSCs), or ESCs, but none use MSCs. Qian et al. paper differentiated iPSCs first into mesodermal lineage cells that are then differentiated into BLECs. Therefore, we sought to differentiate MSCs into BLECs utilizing the help of iPSCs protocols and protocols for differentiating MSCs into ECs. We aimed to mix such protocols and come up with clue factors to be used for our MSCs-derived BLECs differentiation protocol.

Earlier methods for iPSC differentiation into BLECs included planting the cells at certain densities and allowing them to grow for three days in mTeSR medium (Ludwig et al., 2006) and expanding the cells for six days in unconditioned medium (UM) to immature BMECs (Katt et al., 2016; Lippmann, Al-Ahmad, et al., 2014; Lippmann et al., 2012; Wilson et al., 2015). Basic fibroblast growth factor (bFGF) and RA were added to an EC medium in which BMECs were grown before being purified onto 24 collagen/fibronectin matrices. After then, switching to an EC medium devoid of bFGF and RA caused the barrier phenotype to appear. Twenty-four hours following this medium shift, peak barrier phenotypes were seen, and this is when the majority of experiments to determine barrier fidelity were carried out. From the moment of iPSC seeding, these techniques needed a minimum of 13 days to produce functioning, pure BLECs.

Pro-angiogenic drugs and endothelial induction supplements have recently received much attention as potential new treatments for ischemic illnesses. The principal regulator in distinguishing ECs and vascular development was thought to be VEGF (Ferrara, 2004). VEGF stimulates a number of signaling pathways involved in EC growth and differentiation (Bhakuni et al., 2016). Similar to VEGF, bFGF aids in the differentiation of MSCs into ECs (Z. Xu et al., 2013). Previous studies claimed that bFGF and VEGF work together to induce vasculogenesis in a synergistic manner (Asahara et al., 1995).

EGF has been shown to be a powerful inducer of EC motility, maturation, and vasculogenesis (Volz et al., 2017). Recent research revealed a feedback loop between EGF and VEGF production, increasing the potential that EGF stimulates angiogenesis via a VEGF-independent mechanism, perhaps by activating the PI3K and mitogen-activated protein kinase (MAPK) pathways (Mehta et al., 2007). IGF is a possible ingredient in the EC differentiation medium while being less effective than VEGF (Nicosia et al., 1994).

3.1. Culture of BM-MSCs

BM-MSCs showed fibroblast-like morphology while passaging and maintaining in the LG-DMEM expansion medium, as shown in Figure 3.1a. As the colony continued to multiply, the cells expanded parallel to one another in accordance with the "whirlpool" growth principle, as shown in Figure 1b. Cells showed higher rates of death and cell body formations with higher seeding densities, as shown in Figure 1c. When CD-105 expression levels were tested in BM-MSCs and induced cells in the IMDM differentiation medium, MSCs showed higher expression than differentiated cells, as shown in Figure 3.1d and e.

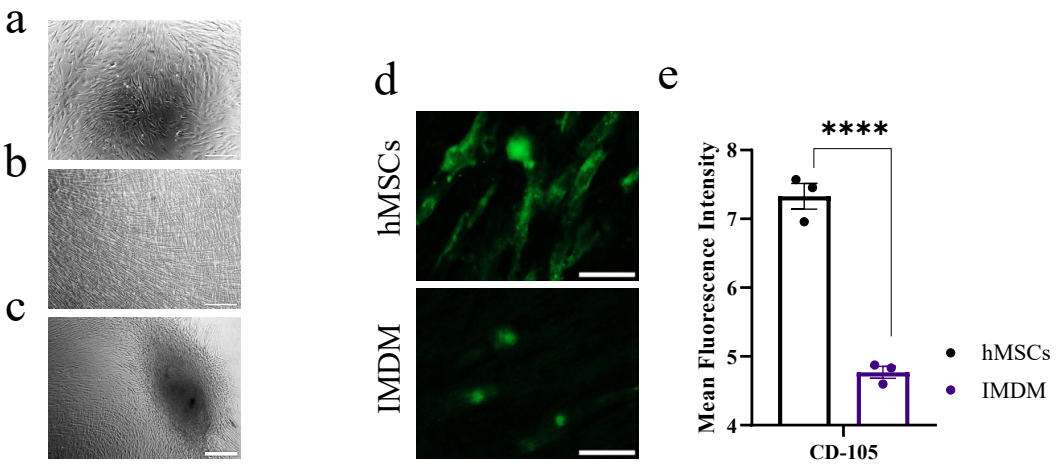

Figure 3.1 Culture, morphology, and marker expression of BM-MSCs. (a-c) Bright-field images of BM-MSCs (a) cultured in LG-DMEM expansion medium, where the whirlpool growth pattern and cell bodies crystals formation upon culture continuation are evident in (b) and (c), respectively. (d) Immunofluorescence images of BM-MSCs and the differentiated cells in the IMDM differentiation medium as $4x10^3$ cells/well of 48 well plates for nine days showed higher expression of CD-105 in BM-MSCs over the differentiated cells. (e) the fluorescence intensity quantified by Fiji software. One-way ANOVA showed significance at $P< 0.05$. All the scale bars are 100 µm.

3.1. Differentiation of BM-MSCs into BLECs

3.1.1. Different Medium Constituents Affect the Differentiation of BM-MSCs

It has been shown in the past that modifications to culture techniques and differentiation media may affect the timeframes at which some lineages, including neuroectoderm, differentiate (Chambers et al., 2009; Lippmann, Estevez-Silva, et al., 2014; Pankratz et al., 2007) and midbrain dopaminergic neurons (Chambers et al., 2009; Kawasaki et al., 2000), with cells eventually displaying the same distinguishing markers using all techniques. It is not unexpected that changes to the differentiation medium caused variable differentiation timescales. Shuffling this information together, we sought to start our experiments by comparing different media with different basal media and/or different growth supplements. Therefore, Endopan, IMDM, and EGM-2 media were used and compared for their ability to differentiate MSCs into BLECs. Different seeding densities and differentiation media have been tested as an initial step. Comparing induced cells to undifferentiated MSCs revealed clear differences. The cells started to form short spindle-shaped structures, as shown in Figure 3.2a. At the induced stage, the cells went confluent, as shown in Figure 3.2b.

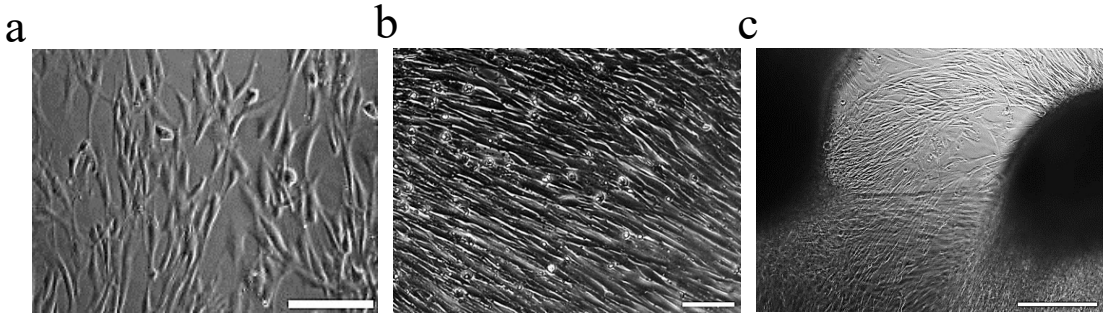

Figure 3.2. Culture and morphology of differentiated BM-MSCs. (a) Bright-field image of BM-MSCs in IMDM medium on D4 showing the short spindle. (b) higher confluency of cells along with culture continuation. (c) cell bodies crystals formation upon culture continuation on D9. The scale bar of (a) is 50 μm. The scale bars of (b and c) are 100 μm.

Firstly, complete Endopan was used as the differentiation medium. MSCs were expanded in LG-DMEM expansion medium until reaching 80-90% confluency; then, the complete Endopan differentiation medium was applied. 6-well plates were seeded with $1x10^5$, $2x10^5$, $3x10^5$, and $4x10^5$ cells/well in the expansion medium for 24 hours, and then the medium was switched to a complete Endopan differentiation medium. Cells were kept in that medium for 14 days, and the medium was changed every two days. Cells started to form crystals of cell bodies at D9 of differentiation, as shown in Figure 3.2c. Therefore, the seeding density was decreased for the cells to survive till D14.

Secondly, another different endothelial differentiation medium has been tested, the complete IMDM differentiation medium, from Wang et al. Paper (C. Wang et al., 2018). Cells were expanded in LG-DMEM expansion medium for 24 hours in a 48-well plate with $5x10^3$, $1x10^4$, $2x10^4$, and $4x10^4$ seeding densities. Two replicates for each condition and one control MSCs culture without differentiation. The medium was changed every day for 14 days. For cells in the IMDM differentiation medium, there was no noticeable growth pattern along the differentiation process. Then, immunostaining was conducted on the resultant cells from both differentiation media at D14; the results showed that both media induced cells to express ZO-1 and CD-31 surface markers.

After that, another medium for differentiation, Endothelial Growth Medium-2 (EGM-2), from Pankajakshan et al. paper (Pankajakshan et al., 2013). MSCs were seeded on a 48-well plate at $5x10^3$, $1x10^4$, $2x10^4$, and $4x10^4$ densities as two experimental replicates and one control replicate for ten days, with medium change every three days. Cells showed a pattern of growth in the differentiation medium. So, the lower seeding densities were more fitting than the higher ones, and the cells expressed ZO-1 and CD-31 as the two previously tested media.

After different seeding densities, $1x10^4$ seeding density was used to compare the previously mentioned media at D7 and D14 of differentiation with medium change daily for the expression of ZO-1 and CD-31 surface markers. IMDM showed the highest expression of ZO-1 and CD-31 on D7 of differentiation to be better than Endopan and EGM-2, as shown in Figures 3.3a and 3.3b.

Additionally, D14 results were lower than D7. Still, we owe these results to the fact that the higher seeding density and the longer duration of culture are causing the cells to quickly form cell body clusters that are easily detached while washing or changing the medium. Therefore, the following experiment was conducted with lower seeding densities ($7.5x10^3$ and $5x10^3$) to exclude the factor of flowing out of cells.

Immunofluorescence staining for CD-31 and ZO-1 was tested over the 2, 5, 8, 11, and 14 days of induction, and the three chosen differentiation media were compared for those markers' expression at two different seeding densities, $5x10^3$ cells/well and $7.5x10^3$ cells/well. ZO-1 showed the highest expression on Days 5 and 8 for the 5k seeding density with EGM-2 and on Days 8 and 14 for the $7.5x10^3$ seeding density with IMDM, as shown in Figures 3.4a and 3.5a. At the same time, CD-31 showed the highest expressions on days 8 and 14 for the 5k seeding density with IMDM and on days 5 and 11 for the $7.5x10^3$ seeding density with EGM-2 and IMDM, respectively, as shown in Figure 3.4b and 3.5b. From these results, day eight was the most promising for both markers. Also, higher expressions were noticed with higher seeding densities, but the higher seeding densities were causing the washing out of cells and the formation of cell bodies; thus, we continued with lower seeding densities. In conclusion, IMDM and EGM-2 showed more promising data than Endopan; therefore, our next experiment was conducted to compare those two media.

Moreover, it was then concluded that cells formed cell bodies faster as the seeding density increased. Also, cell detachment was evident because of the cell body formation on the wells cultured with Endopan and IMDM. While EGM2 proved more promising on Day 8, IMDM provides higher ZO-1 and CD-31 expressions among the different medium conditions used for EC differentiation. Consequently, 5k seeding density was chosen because, at higher densities, the cells form cell bodies at least on D10 of differentiation.

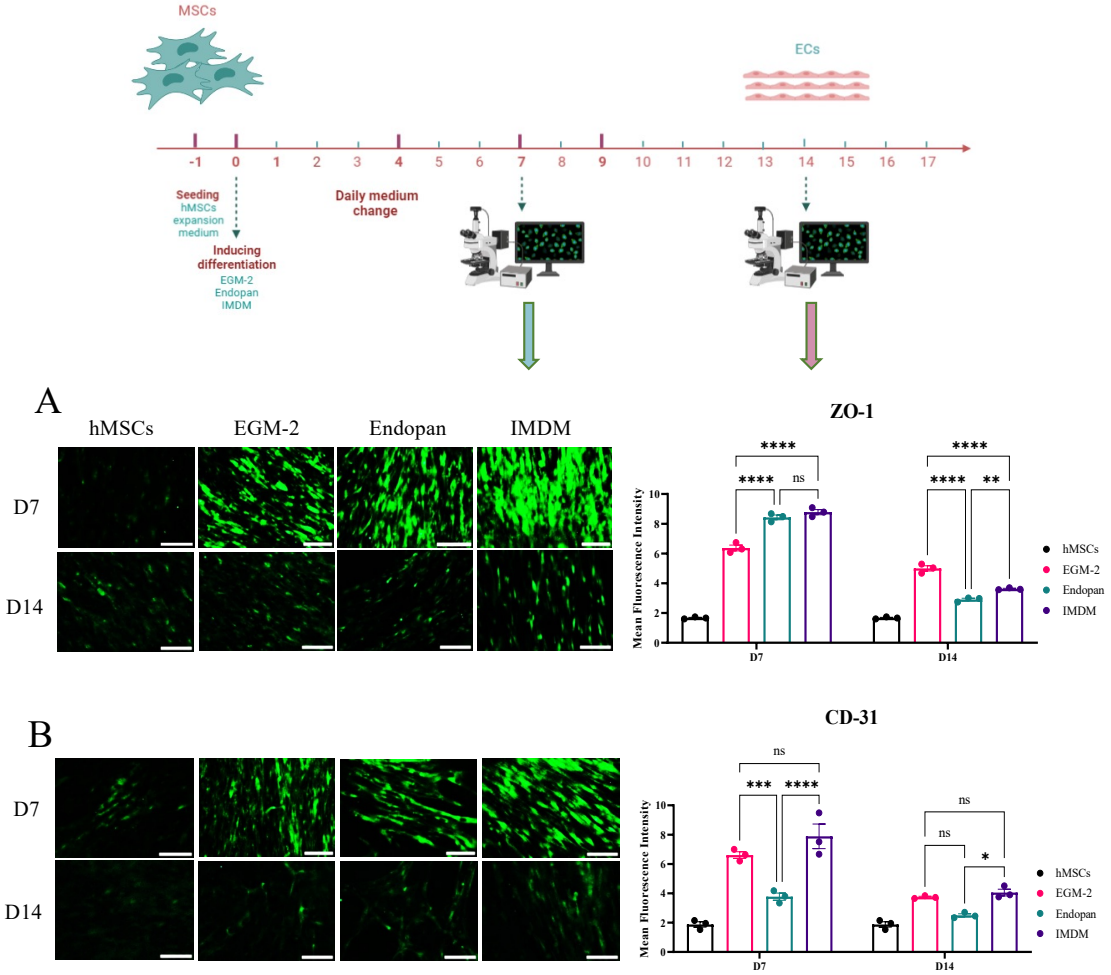

Figure 3.3. Different medium constituents affect the differentiation. BM-MSCs were cultured in different differentiation media as **1x10^4** cells/well of 48-well plates for 7 and 14 days, respectively. ZO-1 (a) and CD-31 (b) expressions were tested by immunofluorescence staining, and the fluorescence intensity was quantified by Fiji software. Two-way ANOVA showed significance at P< 0.05. All scale bars are 50 µm.

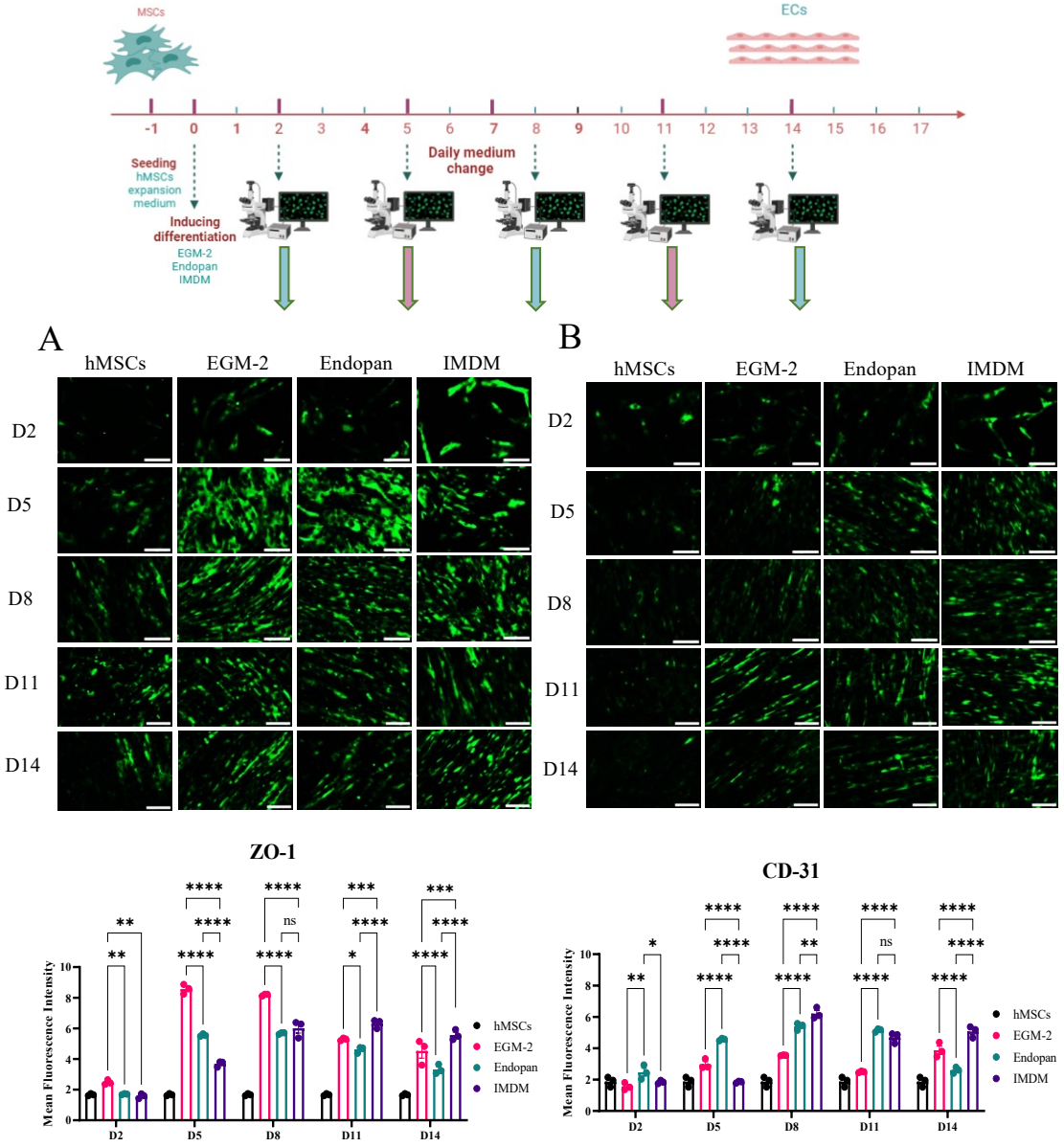

Figure 3.4. Different medium compositions and seeding densities affect the differentiation. BM-MSCs were cultured in different differentiation media as **$5x10^3$** cells/well of 48 well plates for 2, 5, 8, 11, and 14 days, respectively. ZO-1 (a) and CD-31 (b) expressions were tested by immunofluorescence staining, and the fluorescence intensity was quantified by Fiji software. Two-way ANOVA showed significance at $P< 0.05$. All the scale bars are 50 µm.

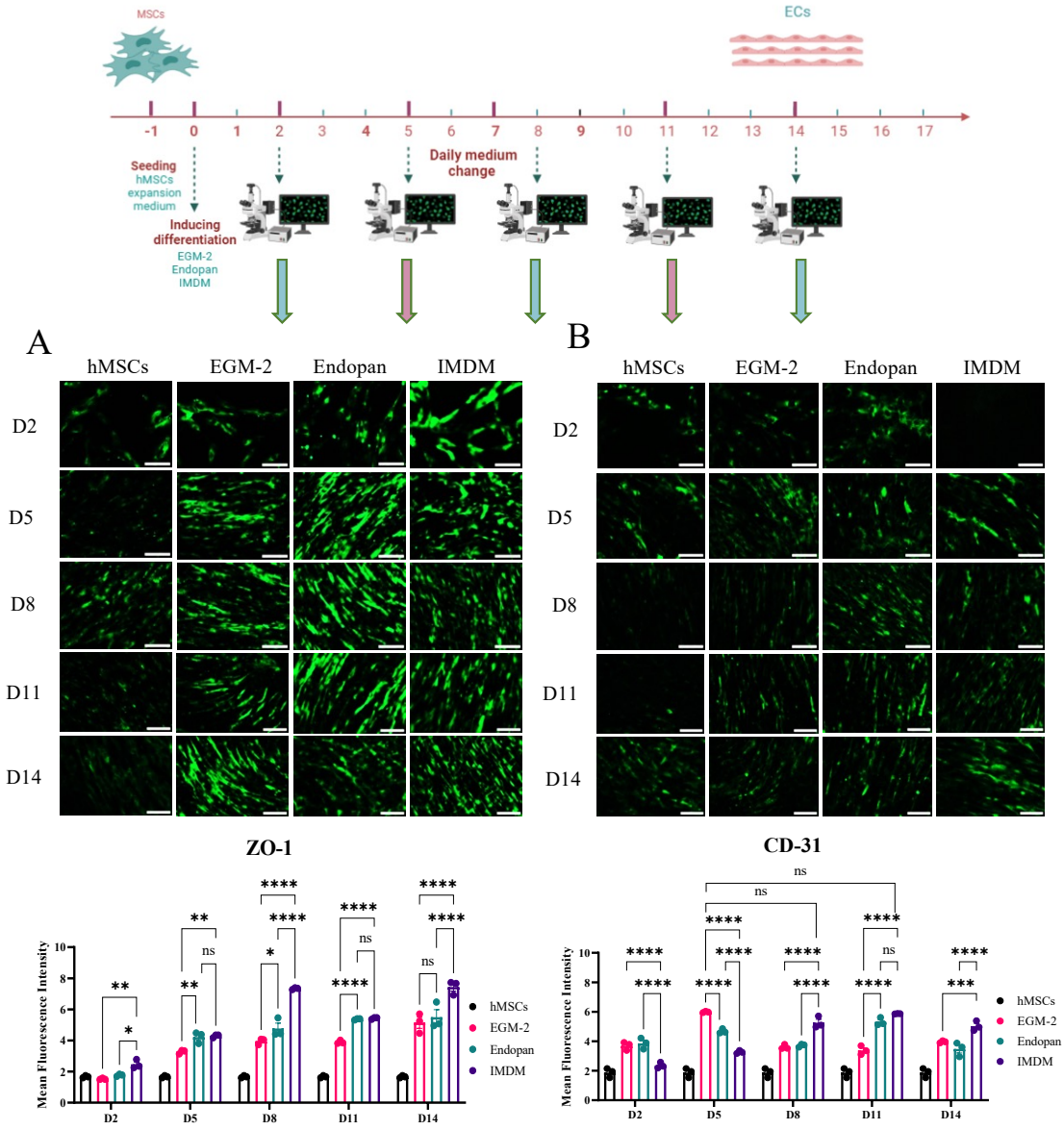

Figure 3.5. Different medium compositions and seeding densities affect the differentiation. BM-MSCs are cultured in different differentiation media as **$7.5x10^3$** cells/well of 48 well plates for 2, 5, 8, 11, and 14 days, respectively. ZO-1 (a) and CD-31 (b) expressions were tested by immunofluorescence staining, and the fluorescence intensity was quantified by Fiji software. Two-way ANOVA showed significance at P< 0.05. All the scale bars are 50 µm.

3.1.2. Medium Change Frequency Plays a Role in the Differentiation of BM-MSCs into BLECs

Knowing that MSCs are secreting secretomes with higher amounts of growth factors (Bartaula-Brevik, 2017), we sought to test whether medium change frequency every day or every three days is better for inducing the cells. For this, IMDM and EGM-2 were compared for their ability to induce differentiation with higher levels of markers expressions. A decreased seeding density of $4x10^3$ was used, and ZO-1 and CD-31 expressions showed better data with IMDM with no difference between every day and every three days medium change as shown in Figure 3.6; therefore, IMDM with medium change every three days was chosen as the best media to induce MSCs differentiation into ECs. Indeed, IMDM has been previously proven to differentiate MSCs into ECs with higher percentages of CD-31, CD-34, vWF, VE-cadherin, and VEGFR-2 expressions (C. Wang et al., 2018) as they proved that many cytokines added to the differentiation system made it more effective than just one pro-angiogenic factor (G. Zhang et al., 2008) or combination of two cytokines (M. Y. Chen et al., 2009).

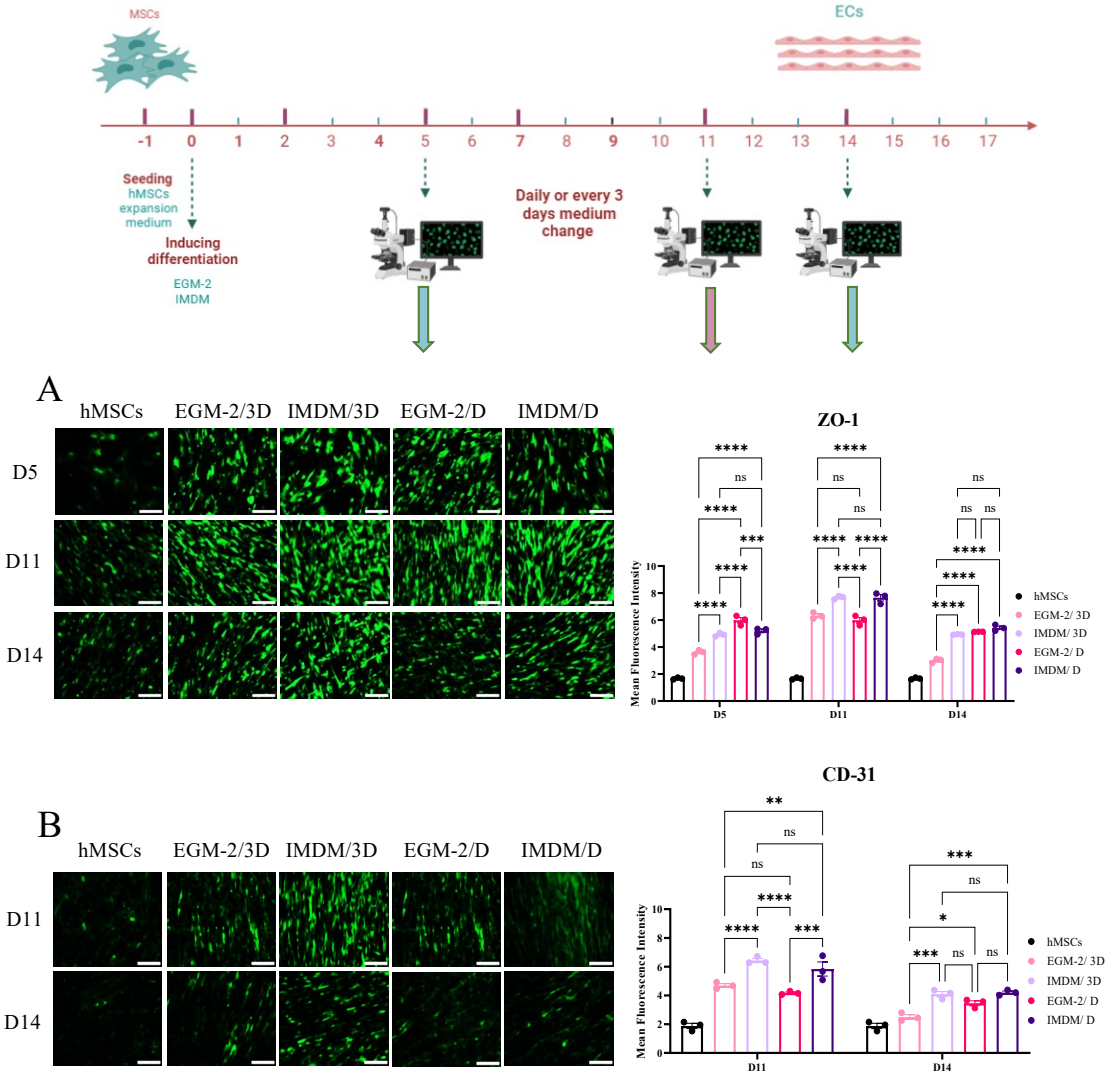

Figure 3.6. Medium change frequency plays a role in the differentiation. Medium change frequency daily and every three days were tested when BM-MSCs cultured in different differentiation media as $4x10^3$ cells/well of 48 well plates for 11 and 14 days. ZO-1 (a) and CD-31 (b) expressions were tested by immunofluorescence staining, and the fluorescence intensity was quantified by Fiji software. Two-way ANOVA showed significance at $P< 0.05$. All the scale bars are 50 µm.

3.2. Specification of ECs

3.2.1. RA Impact on MSCs Differentiation into BLECs

Next, we sought to improve the protocol by adding some clue factors used in iPSC-BLECs protocols, including retinoic acid (RA). Incorporating RA, which is released by radial glial cells in the developing CNS and is hypothesized to provide immature BMECs *in vivo* BBB features (Mizee et al., 2013), throughout the differentiation process greatly enhanced the passive barrier traits of the BMECs (Lippmann, Al-Ahmad, et al., 2014). We first tested different concentrations of RA, 1 μM, 3 μM, and 10 μM, for their ability to improve the expression levels of ZO-1 over 48h and 72h when added on day six or day 12. Three μM for both 48h and 72h showed the best results when added on day 6 of differentiation, as shown in Figure 3.7. Although the increase in the expression was not as much as we expected, the addition of RA decreased the duration period of differentiation from 14 days to 8 days. Thus, we needed to look up new factors to efficiently increase the expression of ZO-1.

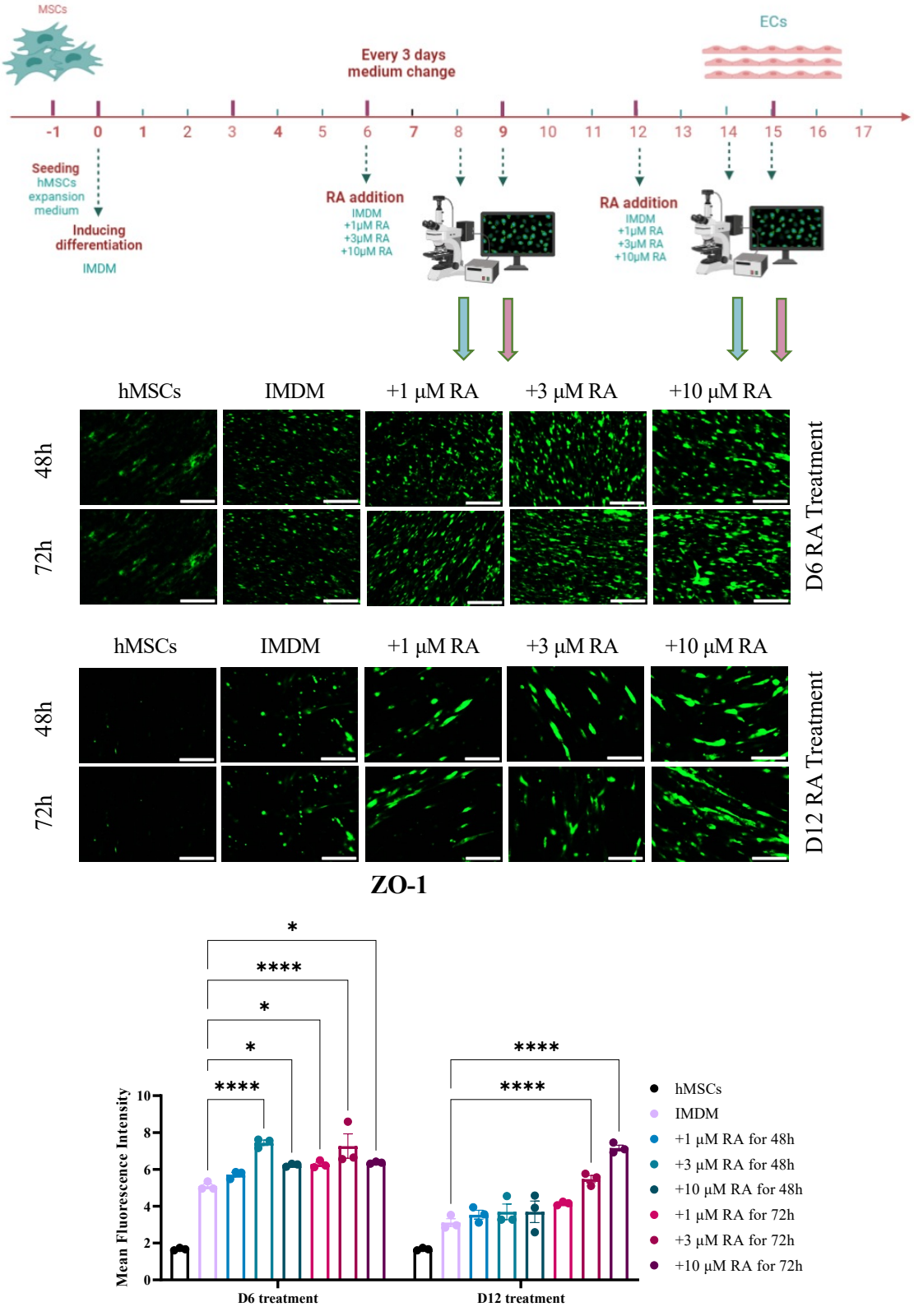

Figure 3.7. RA impact on MSCs differentiation into BLECs. BM-MSCs were cultured in IMDM differentiation medium as $4x10^3$ cells/well of 48 well plates for 9 and 14 days with the addition of 1, 3, and 10 µM RA on day six and day 12 of differentiation. The figure shows ZO-1 expressions tested by immunofluorescence staining and the fluorescence intensity quantified by Fiji software. Two-way ANOVA showed significance at $P< 0.05$. All the scale bars are 200 µm.

3.2.2. Chemical Hypoxia Induces the Differentiation of BM-MSCs into BLECs

Given that the BBB initially develops in the developing brain in an environment with low O_2 levels (1–8%) before the circulatory system is established (Abdollahi et al., 2011), and endothelial development from various stem cell origins has been found to be strongly influenced by oxygen supply (Kusuma et al., 2014; Lee et al., 2012), We proposed that comparable hypoxic settings may be used to cultivate MSC cells to produce more highly differentiated BLECs and perhaps stabilize their phenotypic. Additionally, the formation of brain microvessels depends on canonical Wnt/β-catenin signaling, and the Wnt ligands Wnt7a and Wnt7b have been linked to BBB development *in vivo* (Daneman et al., 2009; Delsing et al., 2018).

Additionally, previous *in vitro* and *in vivo* investigations have shown that the Wnt/β-catenin signaling pathways interact with the HIF-1α signaling pathways (W. Xu et al., 2017; Q. Zhang et al., 2013). By stabilizing HIF-1α, $CoCl_2$ has previously been shown to chemically imitate the consequences of hypoxia (K. S. Kim et al., 2006). It's interesting to note that, compared to normoxic circumstances, iPSCs exposed to 100 μM cobalt chloride ($CoCl_2$) for nine days of differentiation had their levels of HIF-1α protein elevated (T. E. Park et al., 2019). Overall, $CoCl_2$ has been tested over the whole differentiation period of the MSCs-BLECs differentiation protocol.

Given that the safe concentration window of $CoCl_2$ is 200 μM (Teti et al., 2018). This concentration, along with 150 μM, 100 μM, and 50 μM, were added to IMDM for the eight days of differentiation. Unfortunately, the cells couldn't survive till the end of differentiation (not shown). Moreover, many MSCs protocols that apply hypoxic conditions for the differentiation are conducting a step named “Hypoxic preconditioning (HPC)” (Chacko et al., 2010; Muzakkir et al., 2020) by adding $CoCl_2$ concentration to the expansion medium for 24h before inducing the cells in the differentiation medium. HPC is thought to induce the expression of prosurvival and pro-angiogenic markers (Chacko et al., 2010). Consequently, HPC was applied for 24h in the expansion medium with lower concentrations (25 μM, 5 μM, 1 μM, and 0.2 μM), but cells couldn't survive till the end of differentiation. More low concentrations (0.15 μM, 0.1 μM, 0.05 μM, and 0.025 μM) were tested, but their ZO-1 expression was not significant. Therefore, we considered adding the higher concentrations to IMDM for only 24h, 48h, and 72h after HPC MSCs in those concentrations for 24h. Surprisingly, ZO-1 expression levels were much higher with higher concentrations of $CoCl_2$, as shown in Figure 3.8. Collectively, MSCs were incubated with $CoCl_2$ for 72h, 24h of them in the expansion medium, and 48h in the IMDM differentiation medium.

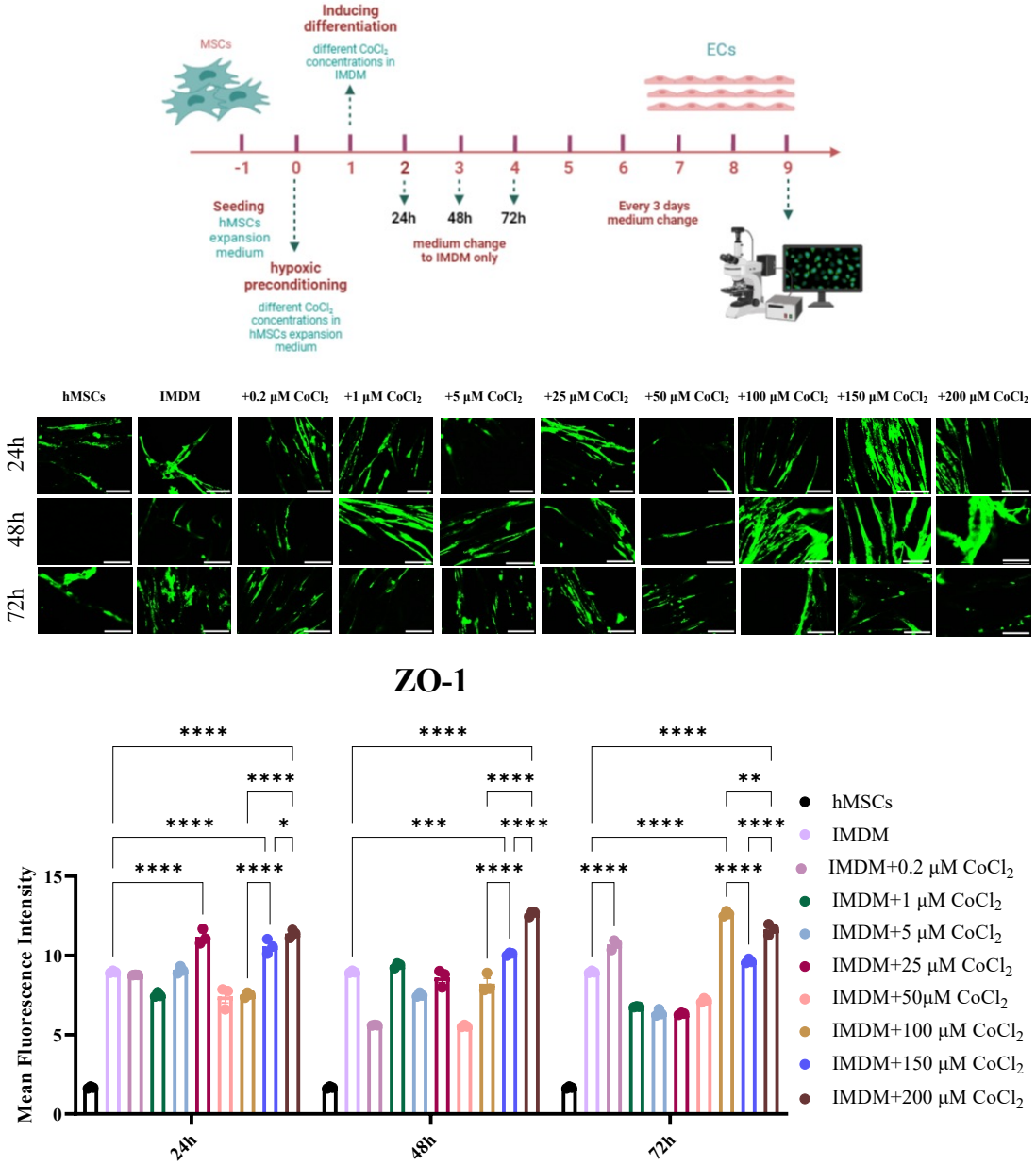

Figure 3.8. Chemical Hypoxia affects BM-MSCs differentiation. BM-MSCs were cultured in the IMDM differentiation medium as $2x10^3$ cells/well of 48 well plates for nine days. The figure shows ZO-1 expressions tested by immunofluorescence staining and the fluorescence intensity quantified by Fiji software. Two-way ANOVA showed significance at $P< 0.05$. All the scale bars are 200 µm.

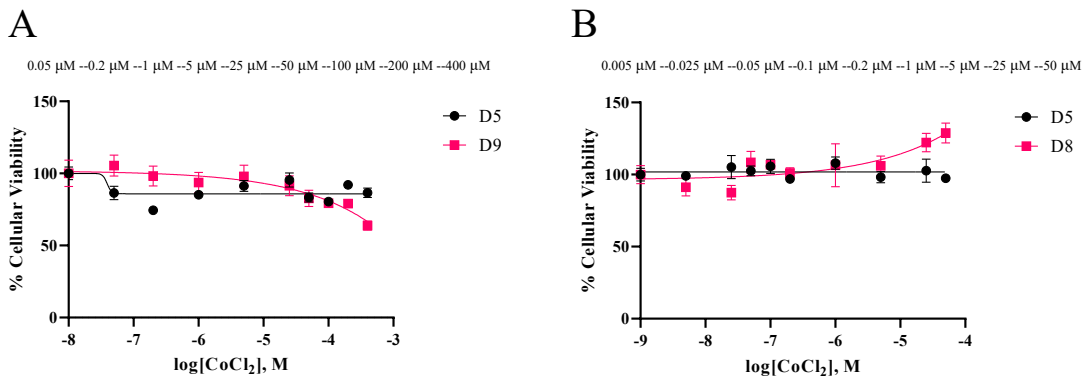

Figure 3.9. MTT Assay for $CoCl_2$ cytotoxicity. BM-MSCs were cultured in LG-DMEM expansion medium as $2x10^3$ cells/well of 96-well plates for (a) 5 and 9 days and (b) 5 and 8 days with the represented concentrations. The figure shows the non-cytotoxic safe concentrations of $CoCl_2$. The graphs are non-linear regression curves with logarithmic concentrations.

Afterward, different concentrations of $CoCl_2$ ranging from 0.5 µM to 400 µM were tested for their cellular toxicity over five and nine days of culture by MTT Assay. 50 µM showed the highest and safest concentration over the nine days of culture, as shown in Figure 3.9a. To confirm the non-cytotoxicity of 50 µM $CoCl_2$, another MTT Assay was conducted for $CoCl_2$ concentrations ranging from 0.025 µM to 50 µM. The results confirmed the safety and non-cytotoxicity of 50 µM over the nine days in the culture, as shown in Figure 3.9b.

Sodium sulfite, Na_2SO_3, is a chemical that regulates hypoxia. It is known to scavenge O_2 molecules as well as metal ions, including Co^{2+}, Zn^{2+}, and $Ni^{2+,}$ and to cause the up-regulation of HIF-1α (Collaco et al., 2006; Kaczmarek et al., 2009). The generated hypoxic state impacts the MSCs' stemness, proliferation, and differentiation(Sart et al., 2014). In addition, Na_2SO_3 has been utilized at C. elegans to mimic hypoxia in doses ranging from 5.5 mM to 22 mM, and at 11 mM, it created a hypoxic microenvironment (Bin et al., 2010). Therefore, different concentrations ranging from 2 mM to 16 mM were tested for 24h with MSCs in IMDM. High concentrations, 16 mM and 8 mM, were highly toxic to MSCs and mostly killed all the cells, while 4 mM and 2 mM were safe. Nonetheless, an MTT assay was conducted to confirm the non-cytotoxicity of Na_2SO_3 through testing different concentrations ranging from 0.025 µM to 4 mM over five days and nine days of culture, and all those concentrations proved safe and non-toxic, as shown in Figure 3.10. Therefore, the highest and safest concentration (4 mM) has been chosen to induce hypoxia.

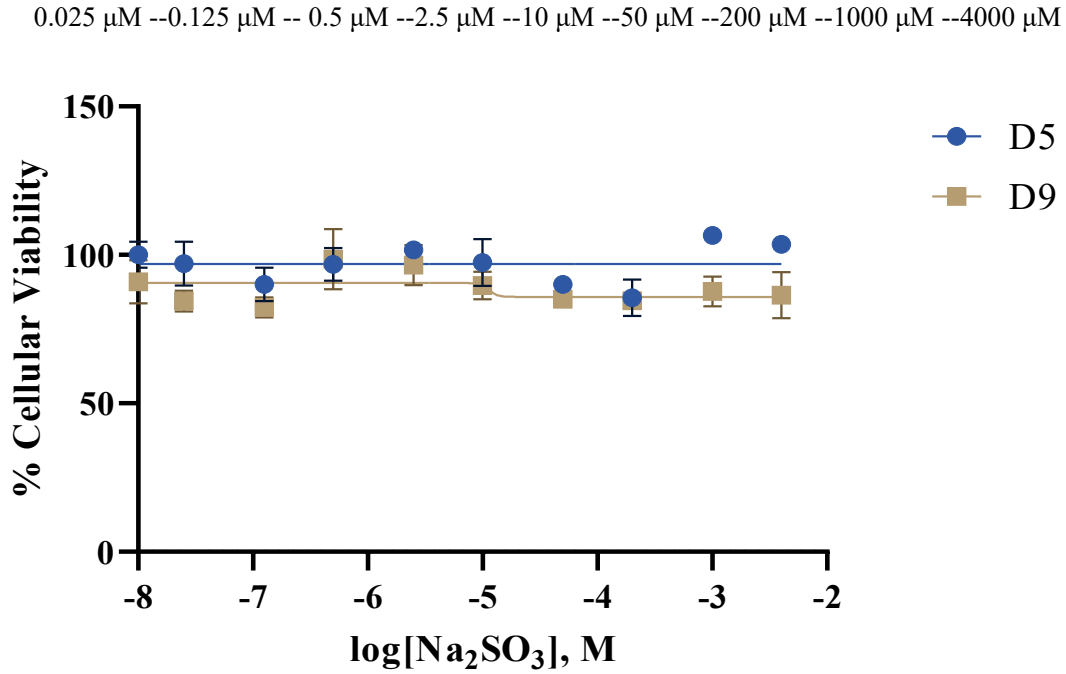

Figure 3.10. MTT Assay for Na_2SO_3 cytotoxicity. BM-MSCs were cultured in LG-DMEM expansion medium as $2x10^3$ cells/well of 96-well plates for 5 and 9 days with the represented concentrations. The figure shows the safe, non-cytotoxic concentrations of Na_2SO_3. The graphs are non-linear regression curves with logarithmic concentrations.

In a study, $CoCl_2$ and Na_2SO_3 showed a rapid formation of hypoxic conditions. MSCs proliferation was also affected by hypoxia. Moreover, the autocrine activity of VEGF, which is abundantly expressed by HIF-1α, boosts MSC growth and encourages osteogenic differentiation (Ghaly & Kok, 1988; H. Kim & Kwon, 2021). Accordingly, $CoCl_2$+Na_2SO_3 different mixtures were applied to the differentiation medium as 10 µM $CoCl_2$+4 mM $Na_2SO_3$and 100 µM $CoCl_2$+4 mM Na_2SO_3, but the cells couldn't survive in the latter mix. Therefore, separate application of those hypoxia-mimetics is better for inducing hypoxia.

3.3. Effect of Basal Media and Serum Supplementation on BM-MSCs Differentiation into BLECs

3.3.1. The Role of Different Combinations Between Expansion and Differentiation Media in Inducing the Differentiation of BM-MSCs into BLECs

In all previously conducted experiments, our expansion medium was LG-DMEM, and our differentiation medium was IMDM. We thought to establish different mixing combinations between the LG-DMEM and IMDM basal media. In other words, we used LG-DMEM once as an expansion medium and once as a differentiation medium. The same also applies to IMDM. Collectively, four combinations were formulated, LG-DMEM+IMDM, LG-DMEM+LG-DMEM, IMDM+LG-DMEM, and IMDM+IMDM, where the former and the latter abbreviations were for the used basal medium for preparing expansion and differentiation media, respectively. ZO-1 expression levels were increased significantly in the media where IMDM was the differentiation medium over the LG-DMEM differentiation medium combinations, as shown in Figure 3.11.

Here, we could prove the different mixing of expansion and differentiation media is affecting the differentiation of MSCs. Moreover, the differentiated cells in the LG-DMEM differentiation medium showed more round morphology, as shown in Figure 3.14. In contrast, cells in IMDM showed significantly higher levels of Occludin and Claudin-5, as shown in Figure 3.12. These results led us to conclude that the IMDM differentiation medium is priming the differentiation of MSCs toward BLECs. Because the IMDM+IMDM combination was more promising in the expression of ZO-1, 50 μM $CoCl_2$ and 4 mM Na_2SO_3 were added to it. $CoCl_2$ showed better ZO-1 expressions that were more concentrated at the cell's circumference, whereas Na_2SO_3 showed less expression than $CoCl_2$ but higher than the previously used combination, LG-DMEM+IMDM, as shown in Figure 3.11.

3.3.2. FBS Proved to Be Superior to Serum-Free Supplementation

Given that serum-free media has been used in some iPSC protocols and produced good results because it has been used in neuronal cultures (Brewer et al., 1993; Hollmann et al., 2017; Neal et al., 2019; Qian et al., 2017), we thought to use the B27 serum-free supplement instead of the animal-derived FBS supplement that we were using in the previous differentiation media. B27 was shown to be less effective than FBS with lower ZO-1 expressions, as shown in Figure 3.11. Also, the western blot results of Occludin were much lower than the FBS set, as shown in Figure 3.13. The difference was only evident and significant when $CoCl_2$ and Na_2SO_3 were added to the IMDM+IMDM combination containing the B27 supplement. Surprisingly, the expression of ZO-1 was drastically decreased with B27 when compared not only to the IMDM+IMDM combination but also to the $CoCl_2$ and Na_2SO_3 combinations with FBS, as shown in Figure 3.11. We could conclude that B27 is incompatible with hypoxia-inducers and FBS is more compatible with MSCs cultures.

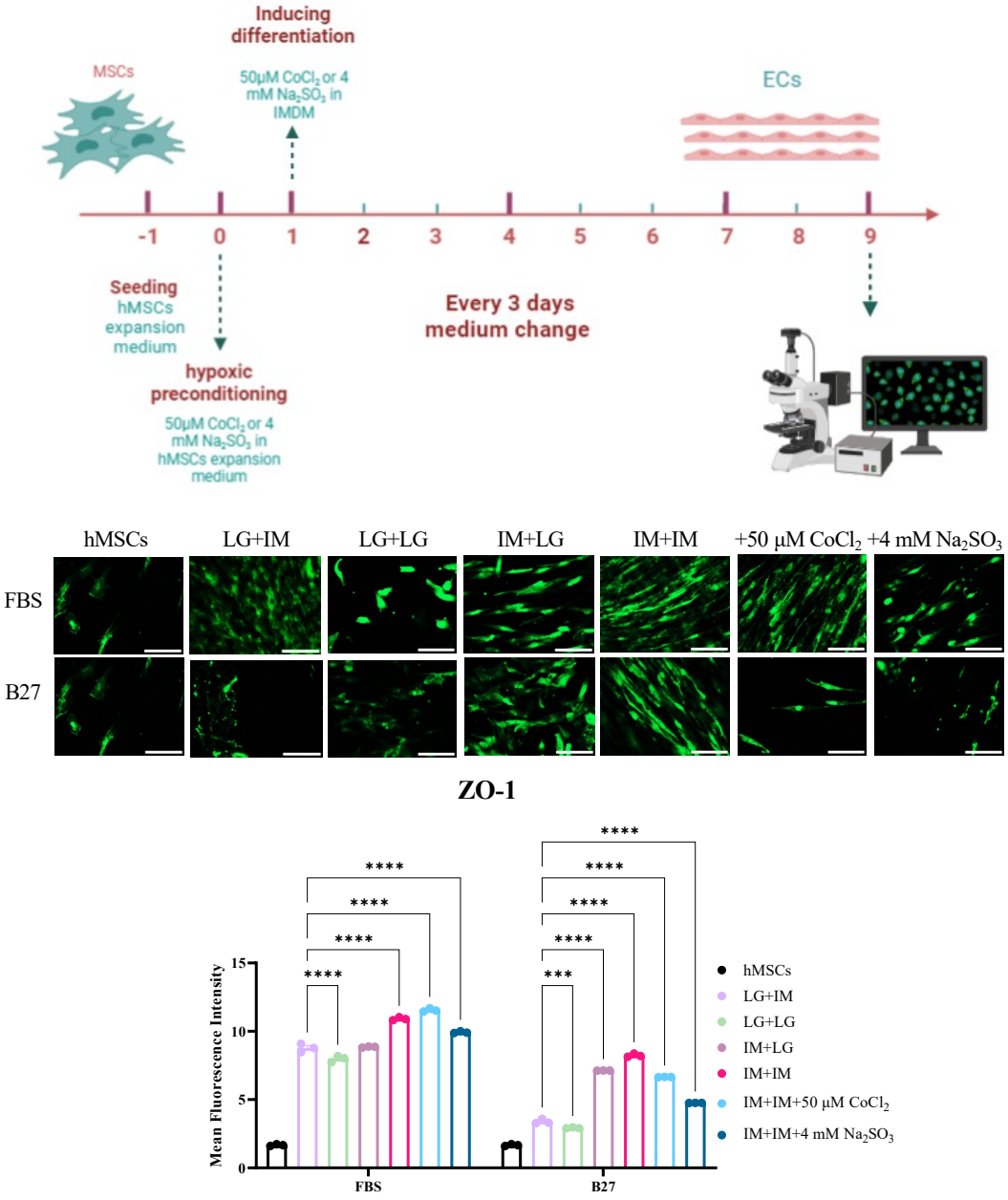

Figure 3.11. Different combinations between LG-DMEM and IMDM basal media for expansion and differentiation of BM-MSCs. BM-MSCs cultured in LG-DMDM or IMDM differentiation media supplemented with either FBS or B27 as $2x10^3$ cells/well of 48 well plates for nine days. The figure shows ZO-1 (a) expressions on D9 of differentiation. Expressions were tested by immunofluorescence staining, and the fluorescence intensity was quantified by Fiji software. Two-way ANOVA showed significance at $P< 0.05$. All the scale bars are 200 µm.

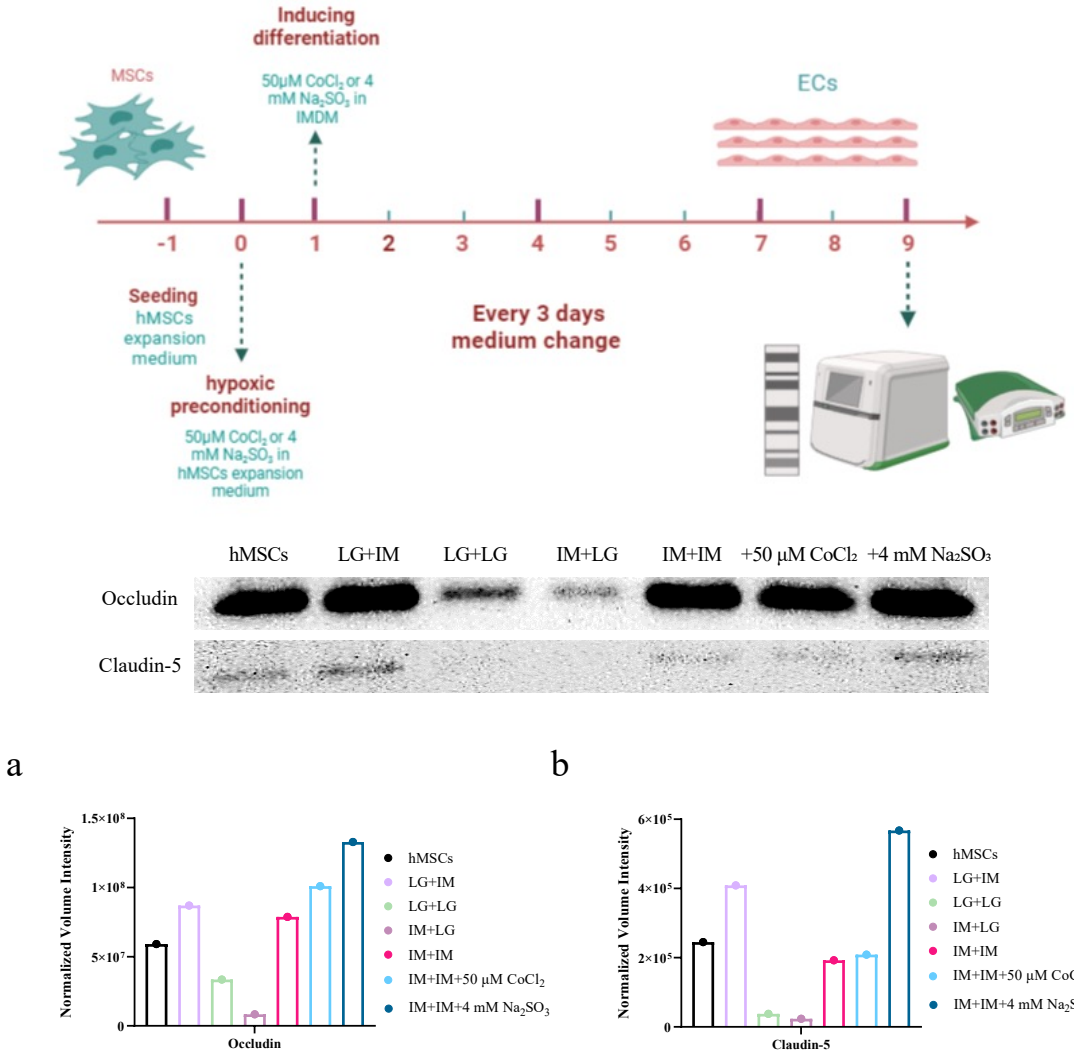

Figure 3.12. Different mixing between LG-DMEM and IMDM basal media for expansion and differentiation of BM-MSCs. BM-MSCs cultured in LG-DMDM or IMDM differentiation media supplemented with FBS as $2x10^3$ cells/well of 48 well plates for nine days. The figure shows (a) Occludin and (b) Claudin-5 expressions. Expressions were tested by Western Blot, and the Chemiluminescent volume intensity was quantified by Image Lab Software. One-way ANOVA showed significance at $P< 0.05$.

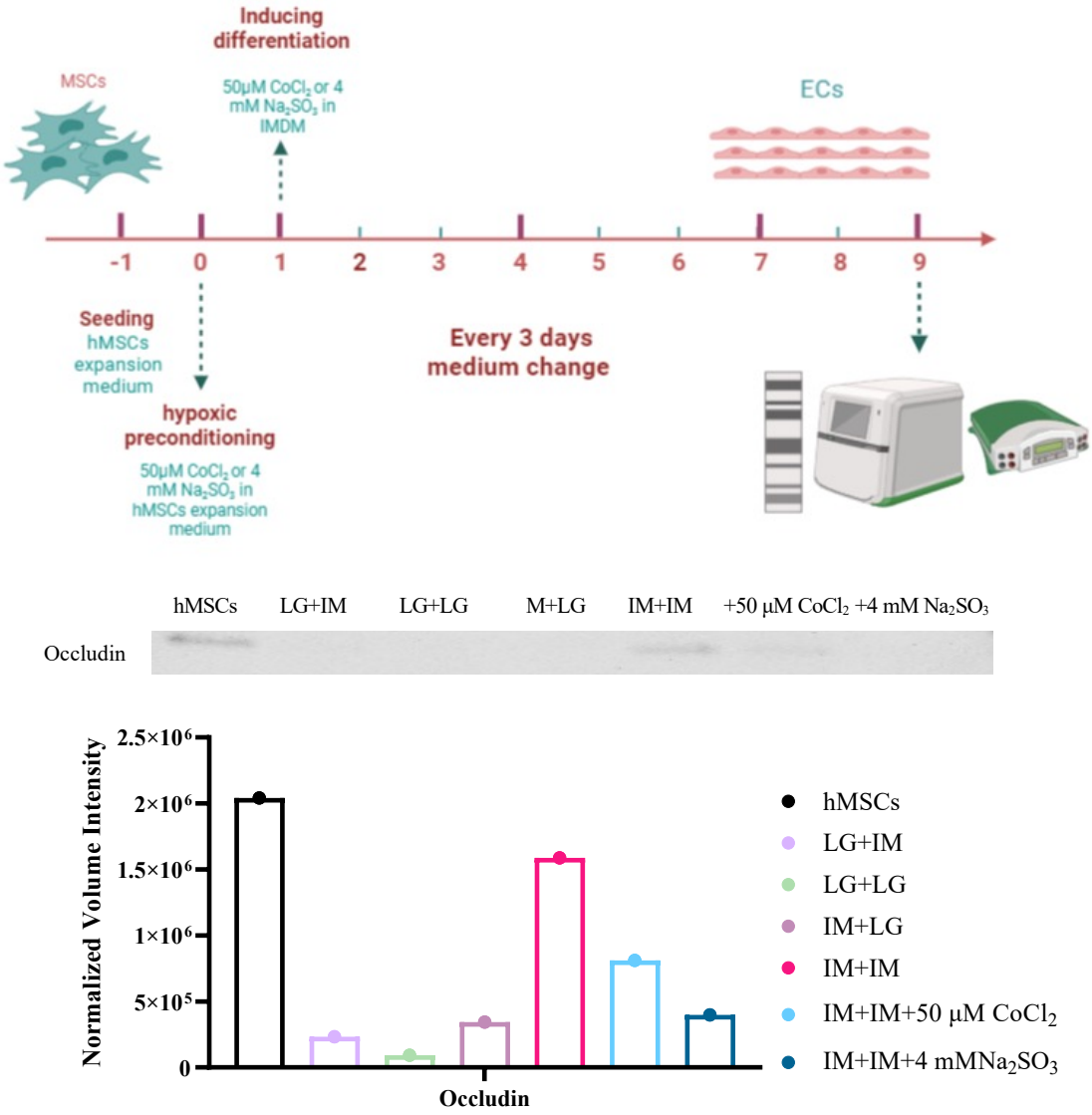

Figure 3.13. Different mixing between LG-DMEM and IMDM basal media for expansion and differentiation of BM-MSCs. BM-MSCs cultured in LG-DMDM or IMDM differentiation media supplemented with B27 as $2x10^3$ cells/well of 48 well plates for nine days. The figure shows Occludin expressions. Expressions were tested by Western Blot, and the Chemiluminescent volume intensity was quantified by Image Lab Software. One-way ANOVA showed significance at $P< 0.05$.

3.3.3. Subculture of the Differentiated Cells Showed Differences in Their Attachment to the Non-Coated Plates

We then tried to subculture the differentiated cells into new uncoated plates. We were surprised when the cells differentiated in LG-DMEM couldn't attach, whereas the cells differentiated in IMDM attached easily to the plates, as shown in Figure 3.14. Also, the sub-cultured cells were tested for CD-31 expression, and $CoCl_2$ and Na_2SO_3 were significantly expressing it over the IMDM without any hypoxia regulators, as shown in Figure 3.15.

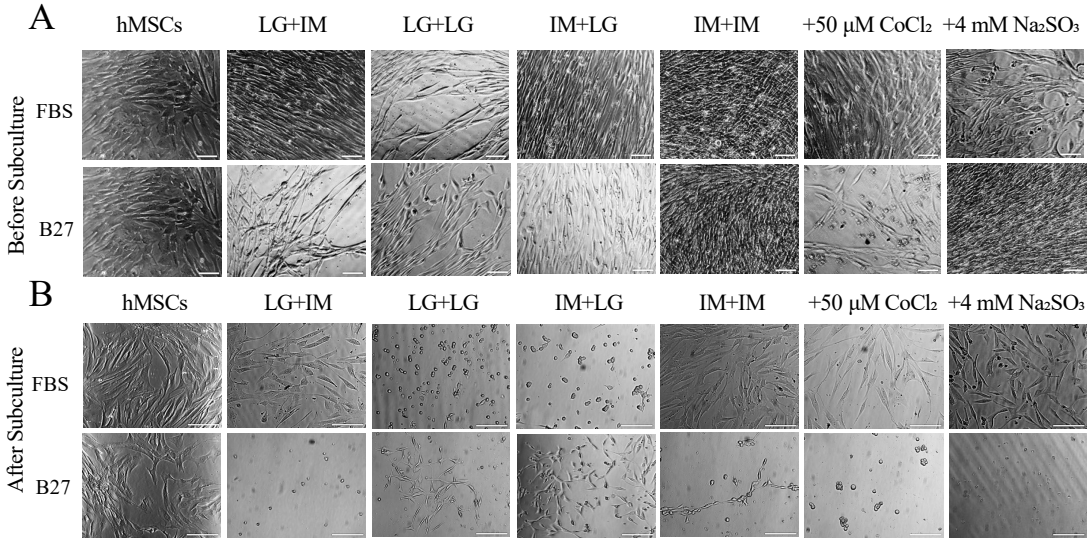

Figure 3.14. Effect of subculturing the cells on uncoated plates. BM-MSCs cultured in LG-DMDM or IMDM differentiation media as $2x10^3$ cells/well of 48 well plates for nine days. The figure shows bright-field images of the cells (a) on D9 of differentiation and (b) after four days in the same medium on uncoated plates. All the scale bars for 'before subculture' images are 100 µm. All the scale bars for 'after subculture' images are 200 µm.

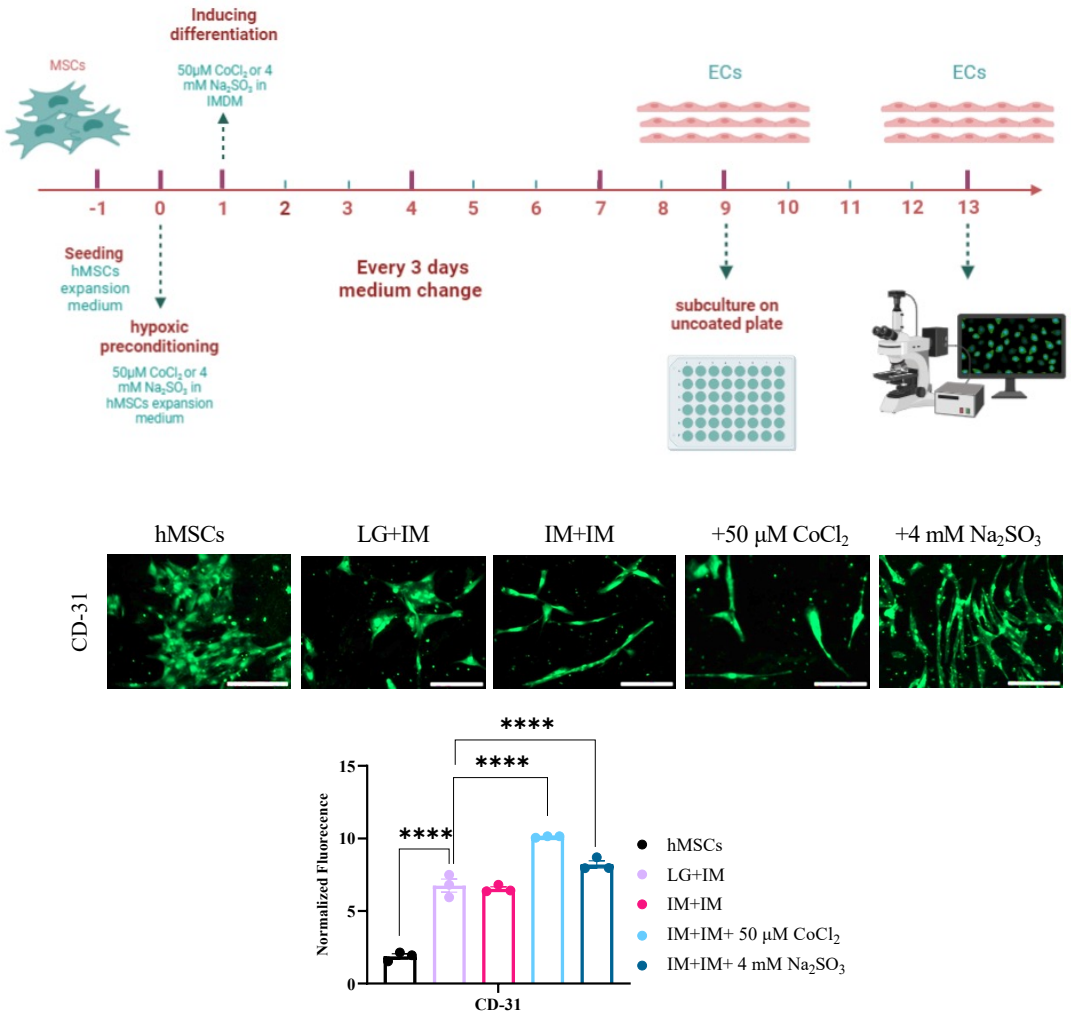

Figure 3.15. Effect of subculture on the differentiated cells from BM-MSCs. BM-MSCs were cultured in LG-DMDM or IMDM differentiation media as $2x10^3$ cells/well of 48 well plates for nine days and then subcultured on non-coated plates. The figure shows CD-31 expressions on D4 after subculturing. Expressions were tested by immunofluorescence staining, and the fluorescence intensity was quantified by Fiji software. One-way ANOVA showed significance at $P< 0.05$. All the scale bars are 200 µm.

3.4. Tube Formation Proved Endothelial Functionality

The primary characteristic of functioning ECs is often thought to be angiogenesis. Numerous studies have shown that ECs may create vascular networks in a dish (Tancharoen et al., 2017). According to an angiogenesis experiment on Matrigel, uninduced MSCs gathered and did not create a capillary network. The construction of vessel-like constructs on Matrigel was significantly increased after the differentiation of MSCs into BLECs, as shown in Figure 3.16a. Moreover, the total lengths of the tubes formed on Matrigel were calculated by ImageJ software. IMDM and LG-DMEM differentiation media proved to induce the differentiation of MSCs into functional ECs, as shown in Figure 3.16b. Additionally, Immunostaining results of the formed tubes showed expression of CD-31 and Occludin, as shown in Figure 3.16c. As a result, the greatly increased quantity of BLECs upon induction seems more promising for medicinal neovascularization and BBB modeling.

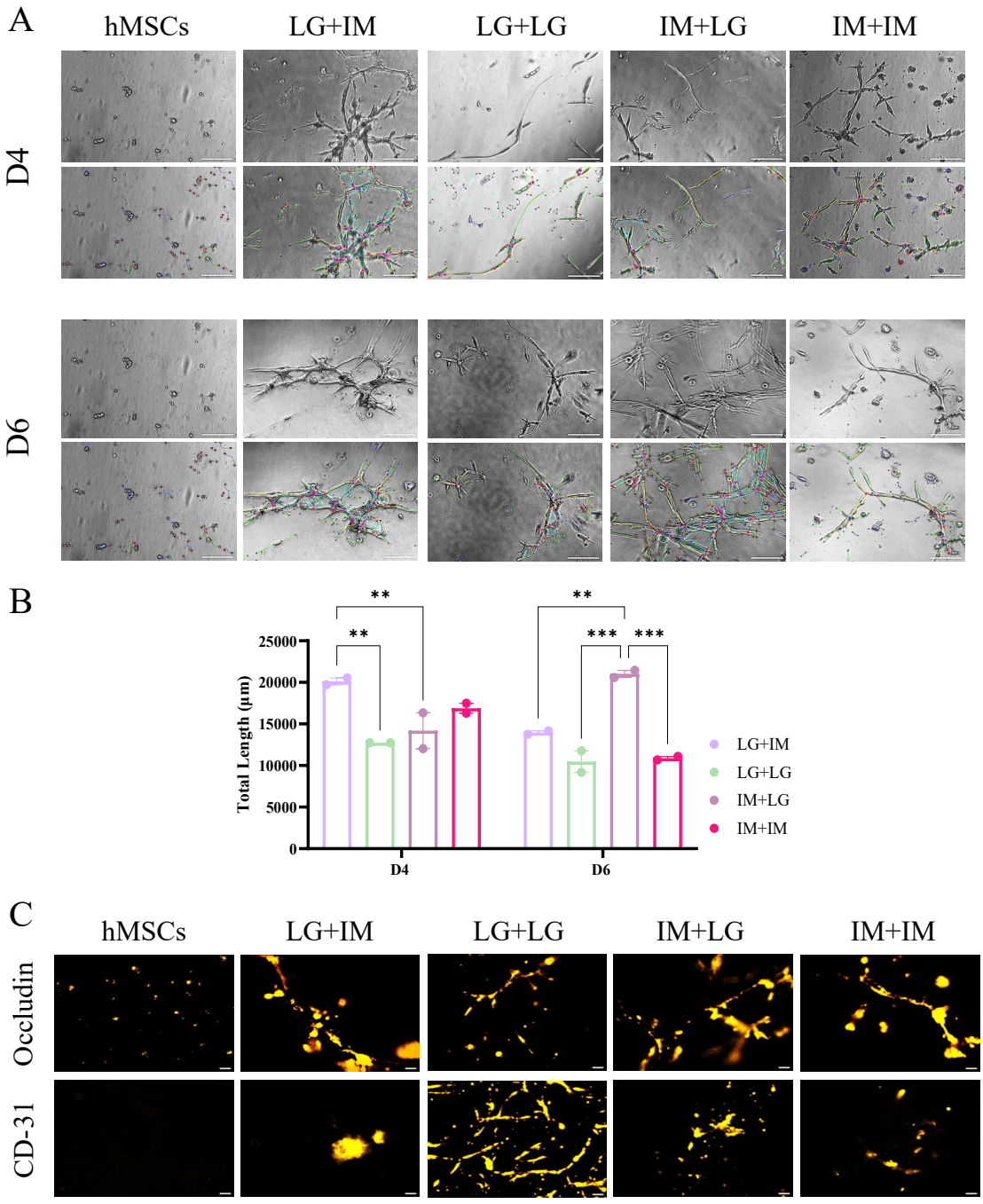

Figure 3.16. Tube Formation Assay. BM-MSCs cultured in LG-DMDM or IMDM differentiation media as $2x10^3$ cells/well of 48 well plates for nine days. The figure shows (a) phase contrast images of the formed tubes after three days and five days on Matrigel, normalized total length. (b) CD-31 and Occludin expressions on the formed tubes. Expressions tested by immunofluorescence staining. Two-way ANOVA showed significance at $P< 0.05$. All the scale bars for the bright-field images are 200 µm. All the scale bars for the fluorescent images are 500 µm.

4. CONCLUSION AND FUTURE PROSPECTIVES

In conclusion, the current work showed that MSCs may effectively develop into BLECs and create *in vitro* vessel-like constructs when cultivated in an environment that induces endothelial growth. The utilization of completely specified media to enhance the BLEC differentiation, animal-derived serum supplement (FBS) rather than synthetic serum supplement (B27), hypoxia regulators ($CoCl_2$ and Na_2SO_3), and RA significantly enhances the BBB phenotype of BLECs. However, this research emphasizes how sensitive BLECs produced from BM-MSCs are to the extracellular environment's makeup and the composition of the basal media utilized to develop and grow BBB endothelial cells. Therefore, we defined the basal media (IMDM), which have publicly available compositions, and supplemented the medium with defined concentrations of RA, $CoCl_2$, Na_2SO_3, and FBS. Cells differentiated using IMDM media were positive for Occludin and Claudin-5, ZO-1, and CD-31, indicating that this media affects our ability to produce Brain endothelial cells. These developments may make MSCs-derived BLECs more accessible to researchers, resulting in high-fidelity human *in vitro* BBB models for a variety of applications.

However, given the extremely initial aspect of our findings, they would need to be thoroughly verified in a wide range of MSC types. Also, more functional tests for the produced BLECs need to be conducted, like TEER measurement, efflux transporter activity assay, and sodium fluorescein permeability assay. Moreover, conducting more experiments to test the reason for the unattachment to the non-coated plates and, finally, test the effect of combining RA with one of the hypoxia regulators. As this manuscript is centered on the differentiation of BM-MSCs into BLECs for *in vitro* differentiation, we have not continued these investigations here.

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APPENDIX

Figure 3.3a

		D7				D14			
		hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM
D7	hMSCs								
	EGM-2	****							
	Endopan	****	****						
	IMDM	****	****	ns					
D14	hMSCs	ns	****	****	****				
	EGM-2	****	****	****	****	****			
	Endopan	***	****	****	****	***	****		
	IMDM	****	****	****	****	****	****	*	

Figure 3.3b

		D7				D14			
		hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM
D7	hMSCs								
	EGM-2	****							
	Endopan	*	***						
	IMDM	****	ns	****					
D14	hMSCs	ns	****	*	****				
	EGM-2	*	***	ns	****	*			
	Endopan	ns	****	ns	****	ns	ns		
	IMDM	**	**	ns	****	**	ns	ns	

Figure 3.4a

		D2				D5				D8				D11				D14			
		hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM
D2	hMSCs																				
	EGM-2	ns																			
	Endopan	ns	ns																		
	IMDM	ns	*	ns																	
D5	hMSCs	ns	ns	ns	ns																
	EGM-2	****	****	****	****	****															
	Endopan	****	****	****	****	****	****														
	IMDM	****	***	****	****	****	****	****													
D8	hMSCs	ns	ns	ns	ns	ns	****	****	****												
	EGM-2	****	****	****	****	****	ns	****	****	****											
	Endopan	****	****	****	****	****	****	ns	****	****	****										
	IMDM	****	****	****	****	****	****	ns	****	****	****	ns									
D11	hMSCs	ns	ns	ns	ns	ns	****	****	****	ns	****	****	****								
	EGM-2	****	****	****	****	****	****	ns	****	****	****	ns	ns	****							
	Endopan	****	****	****	****	****	****	*	*	****	****	**	****	****	ns						
	IMDM	****	****	****	****	****	****	ns	****	****	****	ns	ns	****	**	****					
D14	hMSCs	ns	ns	ns	ns	ns	****	****	****	ns	****	****	****	ns	****	****	****				
	EGM-2	****	****	****	****	****	****	**	ns	****	****	**	****	****	ns	ns	****	****			
	Endopan	****	ns	****	****	****	****	****	ns	****	****	****	****	****	****	***	****	****	***		
	IMDM	****	****	****	****	****	****	ns	****	****	****	ns	ns	****	ns	*	ns	****	**	****	

Figure 3.4b

		D2				D5				D8				D11				D14			
		hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM
D2	hMSCs																				
	EGM-2	ns																			
	Endopan	ns	*																		
	IMDM	ns	ns	ns																	
D5	hMSCs	ns	ns	ns	ns																
	EGM-2	**	****	ns	**	**															
	Endopan	****	****	****	****	****	****														
	IMDM	ns	ns	ns	ns	ns	**	****													
D8	hMSCs	ns	ns	ns	ns	ns	**	****	ns												
	EGM-2	****	****	**	****	****	ns	**	****	****											
	Endopan	****	****	****	****	****	****	ns	****	****	****										
	IMDM	****	****	****	****	****	****	****	****	****	****	ns									
D11	hMSCs	ns	ns	ns	ns	ns	**	****	ns	ns	****	****	****								
	EGM-2	ns	*	ns	ns	ns	ns	****	ns	ns	**	****	****	ns							
	Endopan	****	****	****	****	****	****	ns	****	****	****	ns	**	****	****						
	IMDM	****	****	****	****	****	****	ns	****	****	**	ns	****	****	****	ns					
D14	hMSCs	ns	ns	ns	ns	ns	**	****	ns	ns	****	****	****	ns	ns	****	****				
	EGM-2	****	****	****	****	****	*	ns	****	****	ns	****	****	****	****	***	ns	****			
	Endopan	ns	**	ns	ns	ns	ns	****	ns	ns	*	****	****	ns	ns	****	****	ns	***		
	IMDM	****	****	****	****	****	****	ns	****	****	****	ns	**	****	****	ns	ns	****	***	****	

Figure 3.5a

		D2				D5				D8				D11				D14			
		hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM
D2	hMSCs																				
	EGM-2	ns																			
	Endopan	ns	ns																		
	IMDM	ns	ns	ns																	
D5	hMSCs	ns	ns	ns	ns																
	EGM-2	****	****	***	ns	****															
	Endopan	****	****	****	****	****	ns														
	IMDM	****	****	****	****	****	*	ns													
D8	hMSCs	ns	ns	ns	ns	ns	****	****	****												
	EGM-2	****	****	****	***	****	ns	ns	ns	****											
	Endopan	****	****	****	****	****	***	ns	ns	****	ns										
	IMDM	****	****	****	****	****	****	****	****	****	****	****									
D11	hMSCs	ns	ns	ns	ns	ns	****	****	****	ns	****	****	****								
	EGM-2	****	****	****	***	****	ns	ns	ns	****	ns	ns	****	****							
	Endopan	****	****	****	****	****	****	**	*	****	***	ns	****	****	***						
	IMDM	****	****	****	****	****	****	**	**	****	***	ns	****	****	****	ns					
D14	hMSCs	ns	ns	ns	ns	ns	****	****	****	ns	****	****	****	ns	****	****	****				
	EGM-2	****	****	****	****	****	****	ns	ns	****	**	ns	****	****	**	ns	ns	****			
	Endopan	****	****	****	****	****	****	**	**	****	****	ns	****	****	****	ns	ns	****	ns		
	IMDM	****	****	****	****	****	****	****	****	****	****	****	ns	****	****	****	****	****	****	****	

Figure 3.5b

		D2				D5				D8				D11				D14			
		hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM	hMSCs	EGM-2	Endopan	IMDM
D2	hMSCs																				
	EGM-2	****																			
	Endopan	****	ns																		
	IMDM	ns	****	****																	
D5	hMSCs	ns	****	****	ns																
	EGM-2	****	****	****	****	****															
	Endopan	****	**	ns	****	****	***														
	IMDM	****	ns	ns	*	****	****	****													
D8	hMSCs	ns	****	****	ns	ns	****	****	****												
	EGM-2	****	ns	ns	***	****	****	**	ns	****											
	Endopan	****	ns	ns	****	****	****	*	ns	****	ns										
	IMDM	****	****	****	****	****	ns	ns	****	****	****	****									
D11	hMSCs	ns	****	****	ns	ns	****	****	****	ns	****	****	****								
	EGM-2	****	ns	ns	**	****	****	***	ns	****	ns	ns	****	****							
	Endopan	****	****	****	****	****	ns	ns	****	****	****	****	ns	****	****						
	IMDM	****	****	****	****	****	ns	***	****	****	****	****	ns	****	****	ns					
D14	hMSCs	ns	****	****	ns	ns	****	****	****	ns	****	****	****	ns	****	****	****				
	EGM-2	****	ns	ns	****	****	****	ns	ns	****	ns	ns	***	****	ns	****	****	****			
	Endopan	****	ns	ns	**	****	****	***	ns	****	ns	ns	****	****	ns	****	****	****	ns		
	IMDM	****	****	***	****	****	*	ns	****	****	****	***	ns	****	****	ns	*	****	**	****	

Figure 3.6a

		D5					D11					D14				
		hMSCs	EGM-2 /3D	IMDM/3D	EGM-2 /D	IMDM/D	hMSCs	EGM-2 /3D	IMDM/3D	EGM-2 /D	IMDM/D	hMSCs	EGM-2 /3D	IMDM/3D	EGM-2 /D	IMDM/D
D5	hMSCs															
	EGM-2 /3D	****														
	IMDM /3D	****	****													
	EGM-2 /D	****	****	****												
	IMDM /D	****	****	ns	**											
D11	hMSCs	ns	****	****	****	****										
	EGM-2 /3D	****	****	****	ns	****	****									
	IMDM /3D	****	****	****	****	****	****	****								
	EGM-2 /D	****	****	****	ns	**	****	ns	****							
	IMDM /D	****	****	****	****	****	****	****	ns	****						
D14	hMSCs	ns	****	****	****	****	ns	****	****	****	****					
	EGM-2 /3D	****	ns	****	****	****	****	****	****	****	****	****				
	IMDM /3D	****	****	ns	***	ns	****	****	****	***	****	****	****			
	EGM-2 /D	****	****	ns	**	ns	****	****	****	**	****	****	****	ns		
	IMDM /D	****	****	ns	ns	ns	****	**	****	ns	****	****	****	ns	ns	

Figure 3.6b

		D11					D14				
		hMSCs	EGM-2 /3D	IMDM/ 3D	EGM-2 /D	IMDM/ D	hMSCs	EGM-2 /3D	IMDM/ 3D	EGM-2 /D	IMDM/ D
D11	hMSCs										
	EGM-2 /3D	****									
	IMDM /3D	****	***								
	EGM-2 /D	****	ns	****							
	IMDM /D	****	*	ns	***						
D14	hMSCs	ns	****	****	****	****					
	EGM-2 /3D	ns	****	****	***	****	ns				
	IMDM /3D	****	ns	****	ns	***	****	**			
	EGM-2 /D	**	*	****	ns	****	**	ns	ns		
	IMDM /D	****	ns	****	ns	***	****	***	ns	ns	

Figure 3.7

D12 treatment	D6 treatment	hMSCs	IMDM	IMDM+ 1 µM RA for 48h	IMDM+ 3 µM RA for 48h	IMDM+ 10 µM RA for 48h	IMDM+ 1 µM RA for 72h	IMDM+ 3 µM RA for 72h	IMDM+ 10 µM RA for 72h
IMDM+ 10 µM RA for 72h	IMDM+ 10 µM RA for 72h	****	****	****	****	****	****	****	****
IMDM+ 3 µM RA for 72h	IMDM+ 3 µM RA for 72h		ns		****	ns	ns	ns	ns
IMDM+ 1 µM RA for 72h	IMDM+ 1 µM RA for 72h				**	ns	ns	*	ns
IMDM+ 10 µM RA for 48h	IMDM+ 10 µM RA for 48h					ns	ns	ns	ns
IMDM+ 3 µM RA for 48h	IMDM+ 3 µM RA for 48h						ns	ns	ns
IMDM+ 1 µM RA for 48h	IMDM+ 1 µM RA for 48h							ns	ns
IMDM	IMDM								ns
hMSCs	hMSCs								

D12 treatment	hMSCs	IMDM	IMDM+ 1 µM RA for 48h	IMDM+ 3 µM RA for 48h	IMDM+ 10 µM RA for 48h	IMDM+ 1 µM RA for 72h	IMDM+ 3 µM RA for 72h	IMDM+ 10 µM RA for 72h
hMSCs		ns	**	***	***	****	****	****
IMDM	ns		*	**	**	****	****	****
IMDM+ 1 µM RA for 48h	*	*		****	****	****	****	****
IMDM+ 3 µM RA for 48h	**	**	****		****	****	****	****
IMDM+ 10 µM RA for 48h	***	***	****	****		****	****	****
IMDM+ 1 µM RA for 72h	***	***	****	****	****		****	****
IMDM+ 3 µM RA for 72h	****	****	****	****	****	****		****
IMDM+ 10 µM RA for 72h	****	****	****	****	****	****	****	

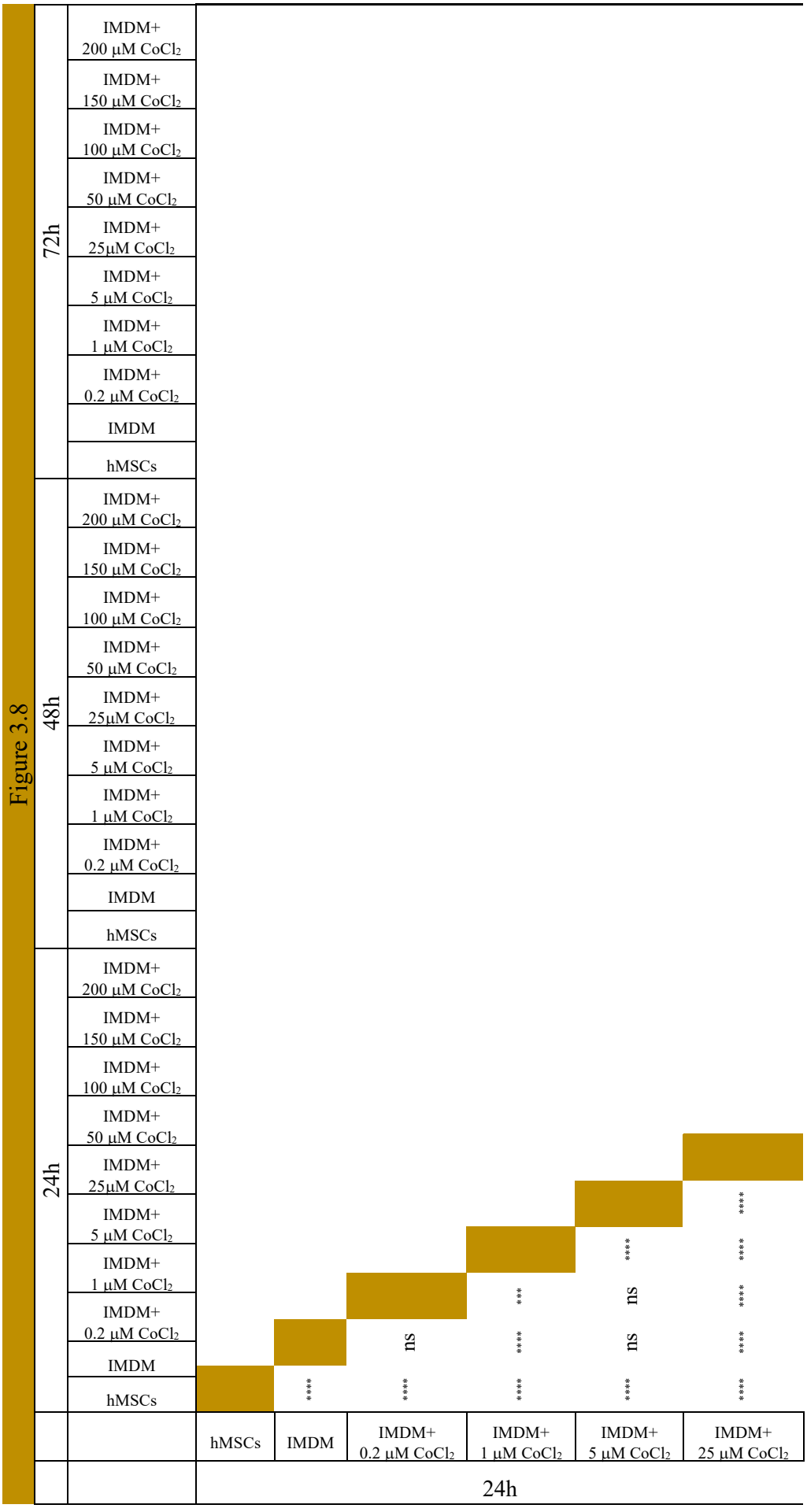
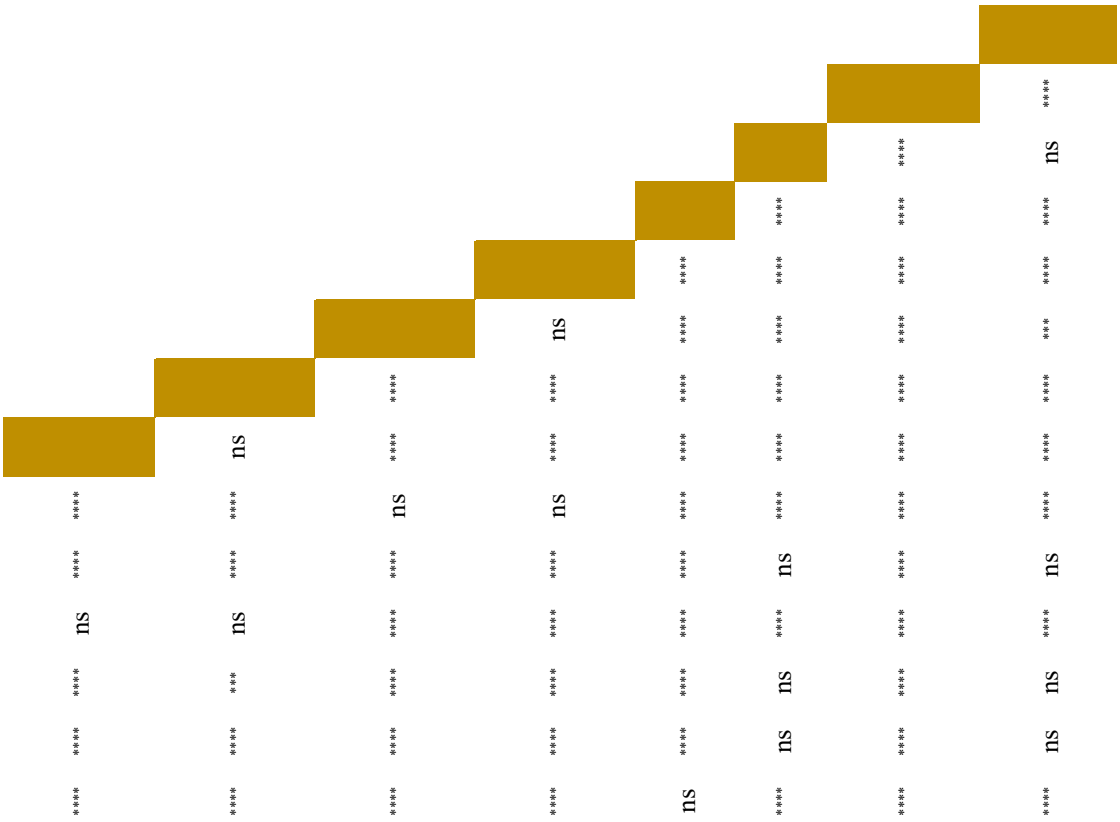


Figure 3.8
72h
48h
24h
IMDM+ 200 µM CoCl2
IMDM+ 150 µM CoCl2
IMDM+ 100 µM CoCl2
IMDM+ 50 µM CoCl2
IMDM+ 25µM CoCl2
IMDM+ 5 µM CoCl2
IMDM+ 1 µM CoCl2
IMDM+ 0.2 µM CoCl2
IMDM
hMSCs
****
ns
***
hMSCs
IMDM
IMDM+ 0.2 µM CoCl2
IMDM+ 1 µM CoCl2
IMDM+ 5 µM CoCl2
IMDM+ 25 µM CoCl2
24h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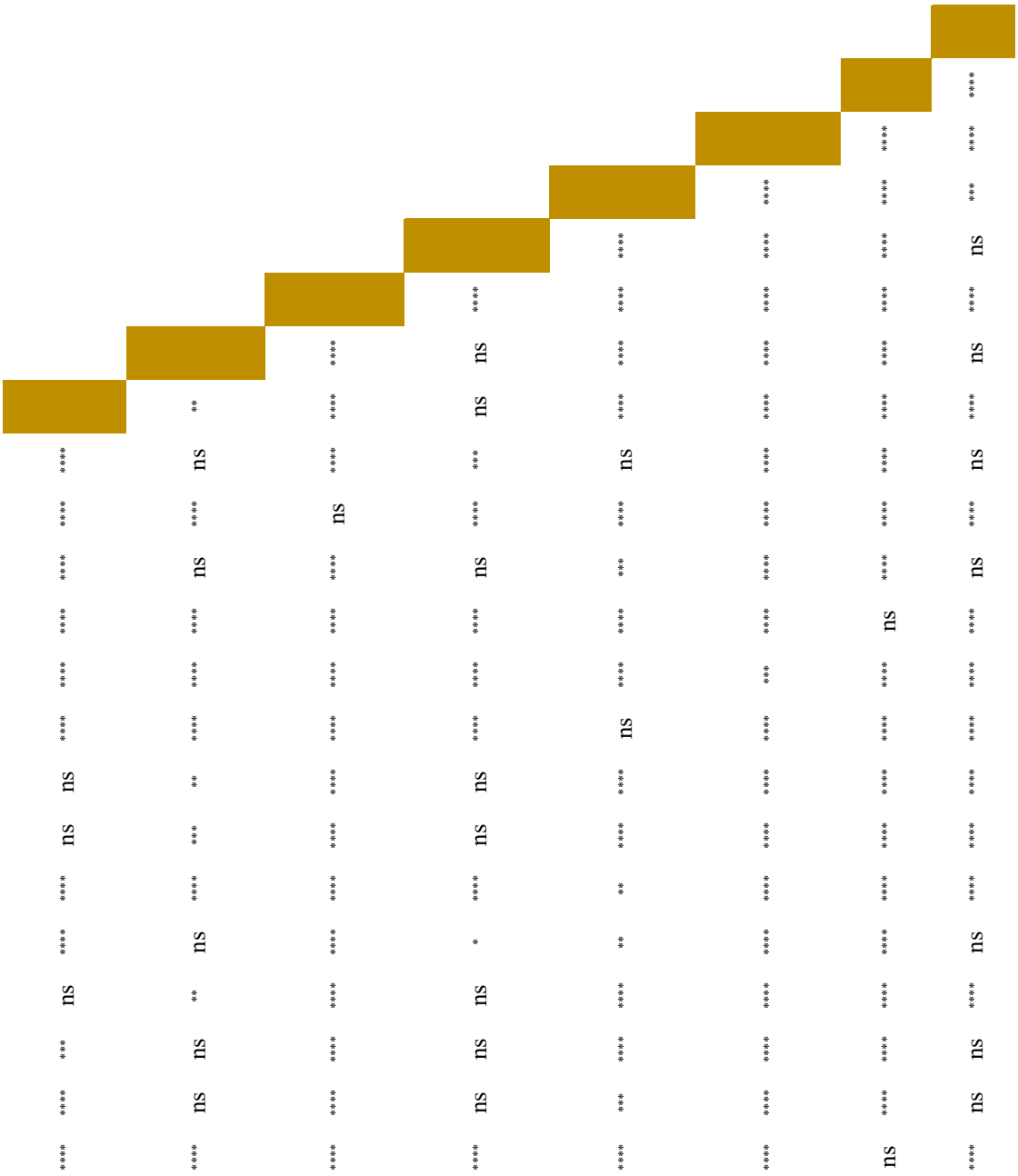
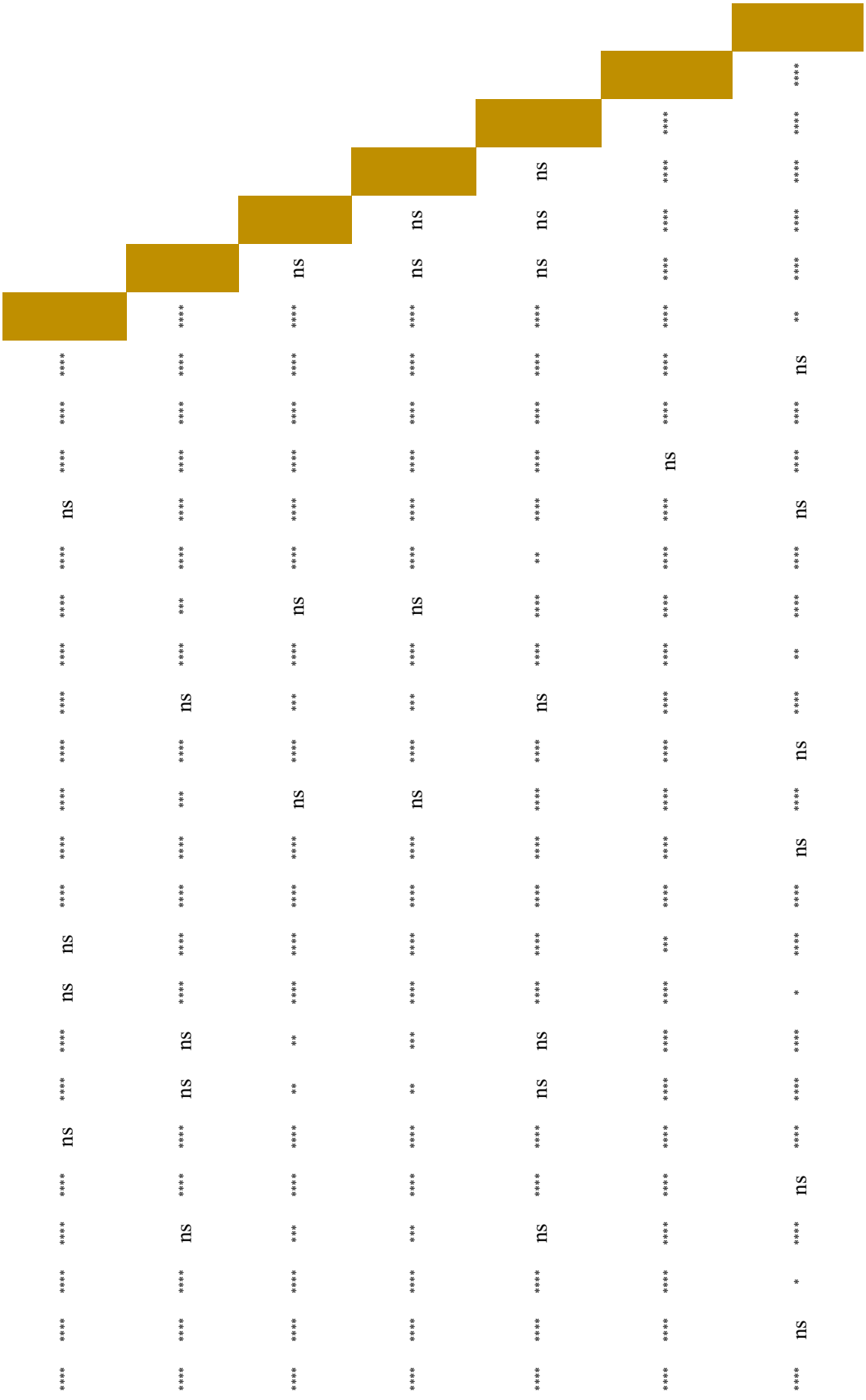
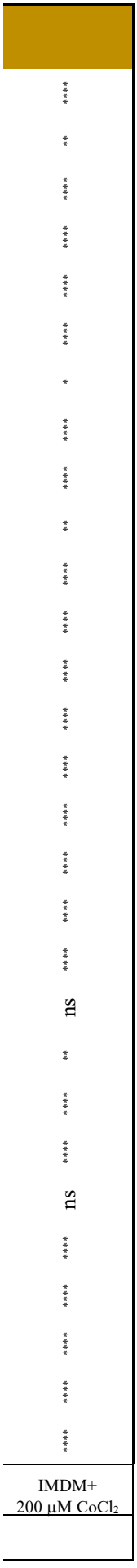
IMDM+ 50 µM CoCl2
IMDM+ 100 µM CoCl2
IMDM+ 150 µM CoCl2
IMDM+ 200 µM CoCl2
hMSCs
IMDM
IMDM+ 0.2 µM CoCl2
IMDM+ 1 µM CoCl2
48h


IMDM+ 5 μM CoCl2
IMDM+ 25 μM CoCl2
IMDM+ 50 μM CoCl2
IMDM+ 100 μM CoCl2
IMDM+ 150 μM CoCl2
IMDM+ 200 μM CoCl2
hMSCs
IMDM
72h


IMDM+ 0.2 µM CoCl2
IMDM+ 1 µM CoCl2
IMDM+ 5 µM CoCl2
IMDM+ 25 µM CoCl2
IMDM+ 50 µM CoCl2
IMDM+ 100 µM CoCl2
IMDM+ 150 µM CoCl2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IMDM+
200 µM CoCl2

Figure 3.11

		FBS							B27						
		hMSCs	LG+ IM	LG+ LG	IM+ LG	IM+ IM	IM+IM+ 50 µM $CoCl_2$	IM+IM+ 4 mM Na_2SO_3	hMSCs	LG+ IM	LG+ LG	IM+ LG	IM+ IM	IM+IM+ 50 µM $CoCl_2$	IM+IM+ 4 mM Na_2SO_3
FBS	hMSCs														
	LG+IM	****													
	LG+LG	****	****												
	IM+LG	****	ns	****											
	IM+IM	****	****	****	****										
	IM+IM+ 50 µM $CoCl_2$	****	****	****	****	***									
	IM+IM+ 4 mM Na_2SO_3	****	****	****	****	****	****								
B27	hMSCs	ns	****	****	****	****	****	****							
	LG+IM	****	****	****	****	****	****	****	****						
	LG+LG	****	****	****	****	****	****	****	****	*					
	IM+LG	****	****	****	****	****	****	****	****	****	****				
	IM+IM	****	**	ns	***	****	****	****	****	****	****	****			
	IM+IM+ 50 µM $CoCl_2$	****	****	****	****	****	****	****	****	****	****	**	****		
	IM+IM+ 4 mM Na_2SO_3	****	****	****	****	****	****	****	****	****	****	****	****	****	

Figure 3.15						
		hMSCs	LG+IM	IM+IM	IM+IM+ 50 μM $CoCl_2$	IM+IM+ 4 mM Na_2SO_3
FBS	hMSCs					
	LG+IM	****				
	IM+IM	****	ns			
	IM+IM+ 50 μM $CoCl_2$	****	****	****		
	IM+IM+ 4 mM Na_2SO_3	****	*	**	**	