The Role of Pannexin 1 during Human Adipogenesis in vitro

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Abstract

Obesity is the unhealthy accumulation of adipose tissue and affects 10% of the global population. A study reported the loss of mouse pannexin 1 (Panx1), a large channel forming protein, increased adipogenesis in murine adipose derived stem cells (ASCs). Our study was designed to determine if human pannexin 1 (PANX1) inhibits adipogenesis in vitro using human ASCs and induced-mesenchymal stem cells (iMSCs) derived from induced pluripotent stem cells (iPSCs). Pharmacological inhibition of PANX1 using carbenoxolone halted adipogenesis in both the ASCs and iMSCs. When PANX1 was inhibited by probenecid, adipogenesis was impeded in iMSCs, but not in ASCs. PANX1-ablated iPSCs were able to differentiate into iMSCs and further differentiate along the adipogenic lineage. Our findings indicate that PANX1 expression is not essential for iPSC differentiation to iMSCs, nor adipogenesis, and the contrasting responses of iMSCs and ASCs to probenecid suggests that mesenchymal stem cells from different origins have distinct characteristics.
Keywords

Pannexin, channel, pannexin 1 (Panx1), mesenchymal stem cells (MSC), adipose, adipocytes, adipose derived stem cells (ASCs), adipogenesis, induced pluripotent stem cells (iPSCs), induced mesenchymal stem cells (iMSCs)
Lay Summary

Approximately 10% of the global population is obese according to the World Health Organization. Obesity is the unhealthy increase of fat tissue, or adipose tissue, and is linked to comorbidities such as cardiovascular disease and type 2 diabetes. Dietary lipids are stored in adipose tissue within cells called adipocytes. During periods of overeating, adipocytes accommodate the additional lipids by increasing in either size or number. Larger adipocyte size leads to mechanical stress and contributes to obesity-related consequences. It has been speculated that by promoting adipocyte production, or adipogenesis, the extra lipids could be distributed among more cells, limiting the need to increase cell size and thus possibly avoiding obesity related disorders.

Adipocytes originate from mesenchymal stem cells (MSCs), a cell type capable of becoming cartilage, bone, or fat cells. MSCs can be collected from various sources, including adipose tissue and induced pluripotent stem cells (iPSCs). During adipogenesis, specific genes are expressed to initiate the MSCs’ transformation into functional adipocytes that can store lipids. The loss of pannexin 1 (Panx1) was reported to increase adipogenesis in mouse adipose derived mesenchymal stem cells (ASCs). Pannexin 1 are channel forming proteins that allow molecules to pass through the cell’s membrane. Although human ASCs express PANX1, it is unclear whether PANX1 influences human adipogenesis.

In our study, human ASCs and iPSC-derived MSCs (iMSCs) were cultured in adipogenic conditions to promote adipogenesis, as indicated by lipid droplet formation. To study PANX1 channel function, cells undergoing adipogenesis were untreated, or treated with either carbenoxolone (CBX) or probenecid (Prob), which are both drugs that block PANX1 channels. CBX inhibited adipogenesis in both the ASCs and iMSCs; however, Prob impeded adipogenesis in only the iMSCs, and not the ASCs. Furthermore, PANX1 was genetically removed from iPSCs, and these cells were able to transition into iMSCs and further undergo adipogenesis in vitro. Overall, we conclude that PANX1 is not required for human adipogenesis in vitro, and the contrasting responses from ASCs and iMSCs to Prob implies that MSCs from different sources have distinct characteristics. These findings suggest that PANX1 may not be an ideal target to regulate human adipogenesis in vitro.
Co-Authorship Statement

Chapter 1

Drs. Eugene Chang and Yangha Kim originally produced the adipogenesis schematic (Figure 1.1) which was published in their study: Chang E & Kim C (2019). Natural Products and Obesity: A Focus on the Regulation of Mitotic Clonal Expansion during Adipogenesis. Molecules 24, 1157. This schematic was modified and included with accordance to the journal’s policy.

The diagram in Figure 1.2 was first published in: Penuela S, Harland L, Simek J & Laird DW (2014). Pannexin channels and their links to human disease. Biochem J 461, 371–381. This diagram was used included with approval as per the journal’s policy.

Chapter 2

Dr. Jacinda Sampson obtained the original patient dermal fibroblasts

The iPSCs used in this study were generated and obtained from the Centre for the Commercialization of Regenerative Medicine (Toronto, ON, Canada).

Dr. Jessica Esseltine was instrumental in reprogramming the fibroblasts to iPSCs and maintaining the iPSCs. She was also responsible for generating the PANX1-ablated iPSCs and PANX1-ablated iMSCs.

Dr. Qing Shao provided direction when performing DNA sequencing to characterize the PANX1-ablated MSCs

Dr. Tao Huang provided the RNA samples of iMSCs during adipogenesis.

Dr. Silvia Penuela provided the materials for some of the immunoblots.

Daniel Nejad, a master’s candidate in Dr. Penuela’s lab, generated the PANX1 overexpressing adherent human embryonic kidney 293 cells.
Anna Kornmuller and Dr. Lauren Flynn collaborated with me and generously provided adipose-derived stem cells, as well as materials and equipment to study adipogenesis in vitro.
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List of Abbreviations

2D – 2 dimensional

3D – 3 dimensional

Abd – Abdominal

AD293 – Adherent human embryonic kidney 293 cells

APC – Allophycocyanin

Arg – Arginine

ASC – Adipose-derived stem cells

ADIPQ – Human gene for adiponectin

ADIPOQ – Human adiponectin protein

ANOVA – Analysis of variance

aP2 – Adipocyte fatty-acid-binding protein 2

ATP – Adenosine triphosphate

AUP – Animal Use Protocol

β1/2/3-AR – Beta 1/2/3 adrenergic receptor

BAT – Brown adipose tissue

BMI – Body Mass Index

BM-MSCs – Bone marrow-derived mesenchymal stem cells

bp – Base pair

BSA – Bovine serum albumin
Ca++ – Calcium

Cas9 – Caspase 9

CBX – Carbenoxolone

CD34/45/73/90/105 – Cluster of differentiation 34/45/73/90/105

cDNA – complimentary DNA

cGMP – cyclic guanosine monophosphate

CEBPα/β/δ – CCAAT enhancer binding protein-alpha/beta/delta

CK14 – Cytokeratin 14

CRISPR – Clustered regularly interspaced short palindromic repeats

CO₂ – Carbon Dioxide

Cx – Connexin

DHAP – Dihydroxyacetone phosphate

DMEM: Ham’s F12 – Dulbecco’s modified Eagle’s medium: Ham’s F12

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

FACS – Fluorescence-activated cell sorter

FBS – Fetal bovine serum

FFA – Free Fatty Acids

FITC – Fluorescein isothiocyanate

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GFP – Green fluorescent protein
Gly 0/1/2 – Glycosylated pannexin species 0/1/2
GPDH – Glycerol-3-phosphate dehydrogenase
gRNA – Guide ribonucleic acid
HFD – High fat diet
HSD11B1 – 11β-hydroxysteroid dehydrogenase type 1
HSMM – Human skeletal muscle myoblast
IBMX – 3-isobutyl-1-methylxanthine
iPSC – Induced pluripotent stem cells
iMSC – Induced pluripotent stem cell-derived mesenchymal stem cells
kDa – Kilodalton
L123fsX144 – Frame shift mutation beginning at leucine amino acid position 123 with an early stop codon at amino acid position 144
LMO3 – LIM only domain 3
LPL – Human gene for Lipoprotein lipase
LPL – Human lipoprotein lipase protein
MEF – Mouse embryonic fibroblast
MMP14 – Matrix metallopeptidase 14
mRNA – Messenger RNA
N254 – Asparagine at amino acid position 254
NaCl – Sodium chloride

NADH – Nicotinamide adenine dinucleotide + hydrogen

NaF – Sodium fluoride

P0/1/2 – Phosphorylation states of Connexins

Prim1 – Primer 1

Prim2 – Primer 2

PAM – Protospacer adjacent motif

PANX1 – Human gene for Pannexin 1

Panx1 – Mouse gene for Pannexin 1

Panx1-KO – Mouse Pannexin 1-Knockout

PANX1 – Human Pannexin 1 protein

Panx1 – Mouse Pannexin 1 protein

PE – Phycoerythrin

Phe – Phenylalanine

PPARγ 1/2 – Peroxisome proliferator-activated receptor-gamma 1/2

Pref-1 – Preadipocyte factor-1

Prob – Probenecid

qPCR – Quantitative polymerase chain reaction

Panx1-REK – Pannexin 1 overexpressing rat epidermal keratinocytes

REK – Rat epidermal keratinocytes
RT-PCR – Reverse transcription polymerase chain reaction

SAT – Subcutaneous adipose tissue

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of the means

SVF – Stromal vascular fraction

T3 – Triiodothyronine

TG – Triglycerides

TNFα – Tumour necrosis factor alpha

TR – Thyroid receptor

Trp – Tryptophan

VAT – Visceral adipose tissue

W127_F130del – deletion from tryptophan amino acid position 127 to phenylephrine amino acid position 130

WAT – White adipose tissue

WREB - Western Human Research Ethics Board

WT – Wildtype
Chapter 1

1 Introduction

1.1 Adipose Tissue

Adipose tissue is the largest endocrine organ in the body, and serves multiple functions such as storing energy, maintaining metabolic hemostasis, healing wounds, and regulating inflammation (Cohen & Spiegelman, 2016). Adipose tissue consists of a variety of cells, but the predominant ones are adipocytes (Desruisseaux et al., 2007). Adipocyte levels in the body can range from 5% of the body mass in athletes to 60% of body mass in obese individuals (Fleck, 1983; Ortega et al., 2009). Adipocytes can also be divided into two tissue types: brown adipose tissue (BAT) and white adipose tissue (WAT) (Fitzgerald et al., 2018). BAT adipocytes are between 15 μm to 50 μm with an ovoid shape and are filled with mitochondria, giving the cells their brown colored appearance (Fitzgerald et al., 2018). BAT is prominently found in infants, and has a high mitochondrial density which is key for thermoregulation through lipid oxidation (Choe et al., 2016). As the infant grows, the BAT is replaced with WAT, the primary focus of our study (Choe et al., 2016). A majority of WAT is located within two depots: visceral fat (VAT; within the abdominal cavity) and subcutaneous fat (SAT; beneath the skin) (Ghaben & Scherer, 2019). WAT adipocytes have a spherical shape and a diameter that can range from 10 μm to 120 μm, and is primarily responsible for insulation and energy storage (Choe et al., 2016). They possess a single large unilocular lipid droplet that pushes the nucleus and other organelles towards the periphery (Fitzgerald et al., 2018). The accumulation of lipids, which is responsible for the white color of WAT, serve an important function as energy reserves for the body (Fitzgerald et al., 2018).

1.1.1 Functions of Adipocytes

During postprandial periods, WAT adipocytes use a process called lipogenesis to convert excess energy (in the form of glycerol and free fatty acids (FFA)) into triglycerides (TG), which are stored in lipid droplets for future use (Cohen & Spiegelman, 2016). Upon fasting, adipocytes hydrolyze the stored TG back to glycerol and FFA via lipolysis, which is then
released into the circulation for delivery to other tissue (Frayn et al., 2006; Cohen & Spiegelman, 2016). The functions of both fat storage and release of FFA are controlled by hormones in the fed and fasted state (Cohen & Spiegelman, 2016). Insulin is the main regulator of adipocyte fat content, and enhances FFA uptake and TG synthesis in adipocytes (Cohen & Spiegelman, 2016). Adipocytes also serve an endocrine function through the production and secretion of several cytokines, also known as adipokines (Halberg et al., 2008). These cytokines include adiponectin (an insulin sensitizing hormone), tumor necrosis factor alpha (TNFα; a proinflammatory signal), and leptin (nutritional intake regulator) (Coelho et al., 2013). Adipocytes therefore maintain the physiological functions of many organs and tissues, including the liver, pancreas, skeletal muscles, kidneys, endothelium, and the immune system (Halberg et al., 2008; Coelho et al., 2013). Other functions of adipocytes include insulating the body, cushioning parts of the body exposed to high levels of mechanical stress (e.g. the heels of the hands and feet), as well as protect delicate organs from damage (e.g. orbital fat cushions the eyes and supports the extraocular muscles to perform eye movements) (Rosen & Spiegelman, 2014; Wester, 2014). Adipocytes are also important to facilitate wound healing, where adipocytes can direct fibroblasts to heal acute burns and chronic non-healing ulcers (Schmidt & Horsley, 2013; Ghaben & Scherer, 2019).

1.1.2 Dysregulated Adipocytes

Dysregulated adipose tissue has been linked to adipose-related diseases such as obesity, type 2 diabetes, and lipodystrophy (Garg, 2006). Obesity is a chronic and complex disease marked by changes in adipocyte secretory function and chronic low-grade inflammation (Hajer et al., 2008). The unhealthy increase in adipose tissue in obese patients has been shown to increase the risk of comorbidities, such as cardiovascular disease, type 2 diabetes, and cancer (Tao & Lagergren, 2013; Hruby & Hu, 2015). Despite these consequences, there has been an increase in obesity rates, with one in three Canadians being overweight or obese (Hruby & Hu, 2015). Obesity is determined by a patient’s body mass index (BMI), calculated as weight (in kilograms) over height (in meters), where a BMI over 25 is regarded as overweight and a BMI over 30 is considered obese (Gesta et al., 2007). In obese patients, adipocytes must adjust to accommodate the excess dietary lipids by either
undergoing hypertrophy (increase in cell size) or hyperplasia via adipogenesis (increase in cell number) (Ghaben & Scherer, 2019). During the early stages of obesity, adipocytes address the increase in lipids by undergoing both hypertrophy and hyperplasia (Gesta et al., 2007). In the later stages however, adipocytes often undergo hypertrophy primarily, and the larger cells have been shown to endure more mechanical stress as well as face hypoxia due to their size (Gesta et al., 2007; Ghaben & Scherer, 2019). This results in lower insulin sensitivity and a steady decline in metabolic health (Ghaben & Scherer, 2019). Enlarged adipocytes release more FFA, where the excess FFA can bind to macrophages and initiate the release of TNFα, a pro-inflammatory signal (Choe et al., 2016; Engin, 2017). The release of TNFα reduces adipocyte insulin sensitivity by inhibiting genes for insulin signaling and adipocyte differentiation (Cawthorn et al., 2007; Coelho et al., 2013). It has been proposed that increasing hyperplasia via adipogenesis would decrease the size of adipocytes, and therefore reduce the risks of negative health consequences (Ghaben & Scherer, 2019).

As mentioned, type 2 diabetes is a health consequence of obesity. Over 2 million people in Canada have type 2 diabetes, a disease where the production or response to insulin is impaired (www150.statcan.gc.ca; Yang et al., 2012). Diabetic complications include neuropathy, nephropathy, retinopathy, cardiovascular disease, stroke, impaired wound healing, and peripheral artery disease, all contributing to the significant mortality of type 2 diabetic patients (Papatheodorou et al., 2018; Ghaben & Scherer, 2019). A recent study showed that 61% of the women with types 2 diabetes were overweight or obese (Hajer et al., 2008). Another study reported that patients with type 2 diabetes possessed enlarged, insulin resistant adipocytes when compared to non-diabetic patients (Yang et al., 2012). It is believed that, like obesity, increasing adipogenesis can help to reduce adipocyte cell size and alleviate the symptoms of diabetes (Ghaben & Scherer, 2019).

Paradoxically, individuals that lack adipose tissue share similar symptoms as those that have an excess (Garg, 2006). Lipodystrophy is a pathological state of adipose deficiency, resulting in abnormal energy storage and irregular adipokine production (Garg, 2006). Due to the lack of adipose tissue, excess lipids accumulate in other areas, such as the liver, muscles, pancreas, and kidneys, causing lipid toxicity, or lipotoxicity (Castro et al., 2014).
These ectopic fat deposits can result in non-alcoholic fatty liver disease, as well as hepatic insulin resistance and skeletal muscle insulin resistance which both eventually lead to the development of type 2 diabetes (Garg, 2006; Hafner & Dani, 2014). By promoting adipocyte production in lipodystrophic patients we can alleviate the negative symptoms related to this disease.

Understanding the production and maintenance of adipocytes is important, since dysregulation of adipocytes can result in severe metabolic disorders. By studying factors that influence adipocyte production, we can identify targets to help regulate it for treating these adipose-related diseases. For instance, increasing adipogenesis in obese patients can help maintain smaller adipocyte sizes and reduce the hypoxic effects which can lead to obesity-related health consequences. Similarly, lipodystrophic patients who lack adipocytes can benefit from promoting adipogenesis, so that lipids can be properly stored in adipocytes rather than causing lipotoxicity by accumulating in other tissues. In this study, we investigate the production of human adipocytes and how it is regulated, in hopes that our findings can further the understanding of adipogenesis and contribute to the treatment of adipose-related diseases.

1.1.3 Adipogenesis

Adipogenesis, the process of adipocyte production, must be carefully regulated to maintain an equilibrium between sustaining energy homeostasis and avoiding the risks for obesity-linked diseases. This differentiation process occurs frequently to compensate for cell death, with approximately 10% of adipocytes being renewed annually (Spalding et al., 2008). Adipocytes originate from mesenchymal stem cells (MSCs) which undergo adipogenesis through a cascade of transcription factors, mostly centered around peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT enhancer binding protein alpha (CEBPα) (Lowe et al., 2011). The process can be divided into two steps: the commitment step (MSC to preadipocyte) and the differentiation step (preadipocyte to mature adipocyte) (Ghaben & Scherer, 2019). During the commitment step, MSCs become restricted to the adipogenic lineage, and thus are no longer able to pursue other fates such as the osteogenic and chondrogenic lineages (Farmer, 2006; Cristancho & Lazar, 2011; Cawthorn et al., 2012a). Currently, there are no reliable markers to distinguish MSCs from pre-adipocytes (Gesta
The differentiation step can be further broken down into four stages: growth arrest, early differentiation (mitotic clonal expansion), intermediate differentiation (post-mitotic growth arrest), and terminal differentiation (Figure 1.1) (Gregoire et al., 1998; Chang & Kim, 2019). Growth arrest is required for adipogenesis, where proadipogenic transcription factors PPARγ and CEBPα inhibit the Wnt signaling pathway to exit the cell cycle (Umek et al., 1991; Altık et al., 1997; Gregoire et al., 1998; Lowe et al., 2011). The growth arrested preadipocytes synchronically re-enter the cell cycle (referred to as mitotic clonal expansion), where CEBPα binds to DNA to initiate the transcription of adipogenic factors (Umek et al., 1991; Tang et al., 2003), a vital step for adipogenesis. Intermediate differentiation is orchestrated by interactions between PPARγ and CEBPα, where both of these transcription factors induce the transcription of mature adipogenic genes, such as adiponectin, as well as the formation of lipid droplets (Wu et al., 1999; Lefterova et al., 2008). Terminal differentiation is indicated by the presence of a unilocular lipid droplet, the activity of adipogenic proteins such as glycerol-3-phosphate dehydrogenase (GPDH; participates in lipid biosynthesis and glycerol production), and the secretion of adiponectin (ADIPOQ; hormone that regulates energy substrates and body composition) (Park et al., 2009; Meyer et al., 2013). It is quite apparent from the differentiation process that PPARγ is the master regulator of adipogenesis (Lowe et al., 2011). PPARγ has two isoforms, of which PPARγ2 is most notable in adipogenesis (Gesta et al., 2007). PPARγ facilitates adipogenesis with the help of the CEBP proteins, especially CEBPα, where 90% of PPARγ DNA binding sites also bind to CEBPα (Ghaben & Scherer, 2019). Together, PPARγ and CEBPα bind to DNA to initiate the expression of adipogenic proteins such as lipoprotein lipase (LPL), ADIPOQ, perilipin and GPDH (Lowe et al., 2011).
Figure 1.1
**Figure 1.1:** Stages of Adipocyte Differentiation. A schematic of the differentiation steps where preadipocytes differentiate into mature adipocytes. This step can be divided into four stages: growth arrest, early differentiation (mitotic clonal expansion), intermediate differentiation (post-mitotic growth arrest), and terminal differentiation. aP2 (adipocyte fatty-acid-binding protein 2); CEBP (CCAAT enhancer binding protein); FAS (fatty-acid synthase); LPL (lipoprotein lipase); PPAR (peroxisome proliferator-activated receptor); Pref-1 (preadipocyte factor-1). Modified figure used with permission from Chang E & Kim C (2019). Natural Products and Obesity: A Focus on the Regulation of Mitotic Clonal Expansion during Adipogenesis. *Molecules* **24**, 1157.
In two dimensional (2D) *in vitro* cultures, MSCs are cultured in adipogenic media to induce adipogenesis, and develop multilocular lipid droplets indicative of immature adipocytes (Klingelhutz *et al.*, 2018). In our study, we used a previously published adipogenic media for adipose derived stem cells (ASCs) (Flynn, 2010). Each ingredient of this specialized media has been specifically selected to promote adipogenesis. Biotin, also known as vitamin B, increases the rate of FFA incorporation into TG by promoting acetyl carboxylase, which is the first step for long-chain FFA synthesis (Levert *et al.*, 2002). Pantothenate, or vitamin B5, is required to synthesize coenzyme A, which is a key component in FFA synthesis (Wang *et al.*, 2013; Huber & Kluger, 2015). Transferrin is a key iron-binding protein, which stimulates a lipolytic effect through the presence of iron (Rumberger *et al.*, 2004). Hydrocortisone is a glucocorticoid that promotes differentiation by increasing levels of CEBPα and insulin sensitivity, as well as facilitating growth arrest required for terminal differentiation. Insulin stimulates the uptake of amino acids and glucose into the cell, and also initiates cell signaling involved in adipogenesis (Hauner *et al.*, 1987; Gerhold *et al.*, 2002; Garofalo *et al.*, 2003; Hynes *et al.*, 2014). Triiodothyronine (T3) interacts with two primary thyroid receptor (TR) isoforms, TRa1 and its antagonist TRa2, within adipose tissue (Ortega *et al.*, 2010). T3 interacts with TRa1 to induce adipogenesis, while Tra2 inhibits T3 activity (Ortega *et al.*, 2010). 3-isobutyl-1-methylxanthine (IBMX) regulates adipogenesis as a competitive non-selective phosphodiesterase inhibitor, and raises the levels of cAMP and protein kinase A, which signal for the transcriptional activity of PPARγ (Kim *et al.*, 2010). Finally, troglitazone is a PPARγ2 agonist and acts as an insulin sensitizer to enhance adipogenesis (Gerhold *et al.*, 2002; Kim *et al.*, 2010).

### 1.1.4 Factors Affecting Adipogenesis

Donor age, sex, and BMI have been reported to influence the adipogenic potential of MSCs. For example, in one study, ASCs from the patients over the age of 70 were able to undergo adipogenesis more efficiently, as indicated by a significant increase in PPARγ mRNA levels, adiponectin secretion, and lipid droplet formation (Kornicka *et al.*, 2015). However, another study investigated the effects of age on adipogenesis by calculating the percentage of ASCs that were positive for oil red O (Zhu *et al.*, 2009). This study found
that there was no correlation between age and adipogenesis, and therefore the influence of age on adipogenesis remains debatable (Zhu et al., 2009). Another reported factor in adipogenesis is high BMIs. A study showed that BMI was negatively correlated with the number of ASCs per gram of adipose tissue, as well as the ASCs’ capacity to perform adipogenesis (van Harmelen et al., 2003). This may be due to obese patients having a smaller pool of cells capable of adipogenesis compared to healthy patients (van Harmelen et al., 2003). Finally, sex differences have also been reported to affect adipogenesis. A study showed that the Pparγ2 mRNA levels of ASCs harvested from female mice is 2.9 times greater than those harvested from male mice (Ogawa et al., 2004). In the human context, larger SAT is typically found in women when compared to men (Björntorp, 1991). This suggests that the adipogenic differentiation of ASCs is closely related to sex differences.

1.1.5 Adipose Tissue in Mice and Humans

Much of what we know about adipogenesis is based on experiments using mouse models; but there are several obvious and subtle differences in adipose tissue physiology between these species that must be considered. BMI is a highly inheritable characteristic, where genetics is responsible for up to 70% of the variability in humans (Chusyd et al., 2016). Mice strains used in obesity experiments are commonly inbred, so theoretically they are genetically similar (Chusyd et al., 2016). Hence the findings from a single strain may not reflect the genetically polymorphic human population. In adipose tissue specifically, there are anatomical and cellular differences between humans and mice. Adipocytes constitute only a third of adipose tissue in animals, while in humans about 50% of the adipose tissue is composed of adipocytes (Ali et al., 2013). The remaining adipose tissue consists of small blood vessels, nerve tissue, fibroblasts and preadipocytes (Ali et al., 2013). Humans also have two main SAT depots, in the abdominal and gluteofemoral regions, whereas rodents have two main subcutaneous pads, located anteriorly and posteriorly (Chusyd et al., 2016). Humans have two layers of SAT separated by superficial fascia; however, there is no evidence of multiple subcutaneous layers in rodents (Chusyd et al., 2016). Furthermore, rodent SAT is separated from dermal adipose tissue by a smooth muscle layer, whereas in humans the SAT is continuous with adipose tissue (Chusyd et al., 2016). These anatomical
variations in adipose tissue between species are likely due to the differential gene expression profiles of Tbox transcription factor 15 (transcription factor for craniofacial and limb development), secreted frizzled related protein 2 (modulator of Wnt signaling), and glypican 4 (involved in cell division and growth), between the adipose depots of both species (Gesta et al., 2006). At the cellular level, human and murine adipocytes have different protein expression and function. Catecholamine-induced lipolysis is required for the breakdown of stored TG into FFA and glycerol, and is mostly driven by activation of three beta adrenergic receptor (β-AR) subtypes (β1, β2, and β3) (Chusyd et al., 2016). Humans and mice both express β1-AR and β2-AR; however, β3-AR is ubiquitously found in mouse adipose tissue, while being marginally expressed in human adipocytes (Chusyd et al., 2016). Therefore, β3-AR agonists are able to induce lipolysis in murine adipocytes, but are unable to produce the same effect in human adipocytes in vitro or in vivo (Tavemier et al., 1996; Weyer et al., 1998). On the other hand, human adipocytes express α2-adrenergic receptors, which is absent in murine adipocytes (Chusyd et al., 2016). α2-receptors have a higher affinity to catecholamines, and, when activated, inhibit lipolysis (Langin, 2006). This indicates that catecholamines may have a different role in lipolysis depending on the species. Also, natriuretic peptides induce lipolysis through the c-GMP pathway, independent of the catecholamine-β-AR pathway (Sengenès et al., 2002). This phenomenon appears to be primate specific, as it is not observed in rodents (Sengenès et al., 2002). Finally, LIM domain only 3 (LMO3) is believed to play a role in human adipogenesis by responding to the presence of glucocorticoids (a vital component of adipogenic media) (Lindroos et al., 2013). However, LMO3 is not found in mice, indicating that they may be some differences in adipogenic induction between mice and humans (Lindroos et al., 2013). Due to these differences, conclusions based on mouse models should be validated in the human context to determine if the findings apply to humans.

1.2 Mesenchymal Stem Cells

To study adipogenesis, we need to understand the adipocyte precursor, MSCs. In general, stem cells are defined by two unique characteristics: they are capable of self-renewal, and can differentiate to other cell types in adult organisms (De Wert & Mummery, 2003).
stem cells differentiate into another cell type, the gene expression profile changes and specific gene markers are up regulated or down regulated (Zhao et al., 2012). MSCs are a class of stem cells that can differentiate into a limited number of cell types, including adipocytes, chondrocytes, fibroblasts, and osteoblasts (Strioga et al., 2012). MSCs serve a role in growth, immunosuppression, and tissue repair (Shao et al., 2015). Due to these characteristics, there is a clinical interest in MSCs as a cellular therapy to treat many conditions, including tissue injuries or degenerative diseases (Wei et al., 2013). In some clinical trials, MSCs have been isolated, differentiated in vitro, and implanted into patients to repair tissues (Ohgushi et al., 2005; Wei et al., 2013). It is therefore important to gain a clear understanding of MSCs during in vitro differentiation, to determine their potential for clinical use in humans.

According to the International Society for Cellular Therapy, there are three criteria to define MSCs. First, the MSCs must be capable of adhering to plastic (Dominici et al., 2006). MSCs are required to have ≥95% expression of CD73 (ecto-5'-nucleotidase), CD90 (Thy-1), and CD105 (endoglin), and have ≤2% expression of CD34 (hematopoietic progenitor antigen) and CD45 (leukocyte common antigen) (Dominici et al., 2006). Lastly, MSCs must be able to differentiate into adipocytes, chondrocytes, and osteoblasts (Dominici et al., 2006). Adipocytes differentiated from MSCs have been reported to express adipocyte markers such as PPARγ, ADIPOQ, LPL, and GPDH (Gimble et al., 2008; Yu et al., 2017; Mohamed-Ahmed et al., 2018). These adipocytes form intracellular lipid droplets which can be detected using oil red O, as well as express functional GPDH used to synthesize glycogen (Sheyn et al., 2016; Yu et al., 2017). Based on these criteria and previous studies, cells can be identified as MSCs and their differentiation into adipocytes can be studied in detail.

MSCs were first isolated from the bone marrow, but since then, MSCs have be harvested from a variety of tissues, including adipose tissue, peripheral blood, placenta, and umbilical cord blood (Wei et al., 2013). Interestingly, tissue origin appears to influence the differentiation ability of the MSCs. MSCs have an enhanced ability to differentiate into the tissue they were isolated from (Gimble et al., 2008; Mohamed-Ahmed et al., 2018). For example, bone marrow derived MSCs (BM-MSCs) have earlier and higher levels of
osteoblast markers compared to adipose-derived stem cells (ASCs), while on the other hand ASCs expressed higher transcript levels of adipocyte markers and greater lipid droplet formation compared to BM-MSCs (Mohamed-Ahmed et al., 2018). Therefore, ASCs’ enhanced ability to differentiate into adipocytes make them an ideal model to study adipogenesis.

1.2.1 Adipose Derived Stem Cells

ASCs are MSCs isolated from adipose tissue. Within adipose tissue, there is a small population of cells referred to as the stromal vascular fraction (SVF) (Lowe et al., 2011). The SVF is composed of endothelial cells, fibroblasts, smooth muscle cells, hematopoietic cells, and, of course, ASCs (Lowe et al., 2011). In terms of immunophenotype, ASCs are 90% identical to BM-MSCs, with a minor difference in CD34 expression (Strioga et al., 2012). ASCs express CD34 when freshly isolated, a phenomena which decreases over time, while all MSCs isolated from other sources do not express CD34 at all (Strioga et al., 2012). In terms of culture, ASCs demonstrate lower senescence and higher proliferation capacities over a longer period of time compared to BM-MSCs (Frese et al., 2016). However, a study reported a significantly lower growth rate for ASCs isolated from patients over the age of 70 when compared to other age groups (Kornicka et al., 2015). Overall, ASCs have shown strong regenerative properties through cell restoration and anti-inflammation (Frese et al., 2016). Due to their adipogenic potential, ASCs are ideal for enhancing skin wound repair by improving re-epithelialization, reducing scar formation, and increasing blood density (Hassan et al., 2014).

With the increasing use of MSCs in clinical trials, it has become apparent that isogenic MSCs are ideal to avoid immunological rejection. Ideally, MSCs can be isolated from the patient’s adipose tissue and reintroduced to tissue injury sites to facilitate repair. Compared to bone marrow, adipose tissue is a preferable source of MSCs due to the ease and low risks associated with liposuction surgeries, with approximately 1.4 million liposuctions being performed globally per year (Gimble et al., 2007; Schlarb, 2018). Furthermore, adipose tissue has a higher yield of MSCs per gram (5%) compared to bone marrow (0.001-0.01%) (Strioga et al., 2012). Taking all these factors into consideration, ASCs have become an ideal candidate for clinical applications of MSCs. To date, there are 270 active
clinical trials investigating the use of ASCs to treat disorders, including soft tissue regeneration, skeletal tissue repair, microbial infections, and immune disorders (www.clinicaltrials.gov; Frese et al., 2016).

1.2.2 Induced Pluripotent Stem Cell Derived Mesenchymal Stem Cells

Regardless of tissue origin, MSCs have a finite self-renewal capacity, and will eventually give rise to daughter cells that have lost their differentiation potential; severely limiting the propagation of MSCs (Wagner et al., 2008). To address this, induced pluripotent stem cells (iPSCs) have become an alternate source of MSCs (Hynes et al., 2016). iPSCs are somatic cells that have been genetically reprogrammed through the expression of the pluripotent factors (octamer-binding transcription factor 4, sex determining region Y-box 2, cellular Myelocytomatosis, and Kruppel like factor 4), and can be propagated indefinitely (Takahashi et al., 2007). iPSCs are considered primed pluripotent cells, meaning they can differentiate into any cell type in the body except the placenta, but are more inclined to differentiate toward a specific lineage due to growth factors (Kilens et al., 2018). By changing the cellular environment, iPSCs can be both reverted to a naïve-like state, in which they are less primed to follow a specific cell-fate, and reconverted back to their primed state, by returning the cells to their original environment (Kilens et al., 2018). The iPSCs can also be readily differentiated into MSCs, creating an essentially unlimited source of MSCs (Lian et al., 2010; Sheyn et al., 2016). Furthermore, MSCs derived from iPSCs have been shown to be capable of adipocyte differentiation as revealed via oil red O staining (Lian et al., 2010; Sheyn et al., 2016).

1.3 Pannexin 1

Pannexins are a family of channel forming glycoproteins discovered in the year 2000, and are characterized by their four transmembrane domains which have two extracellular loops and one intracellular loop, with the N-terminus and C-terminus facing the cytoplasm (Panchina et al., 2000; Penuela et al., 2013). There are three members in the pannexin family: pannexin 1, pannexin 2, and pannexin 3 (Penuela et al., 2013). Pannexin 1 (~45 kDa) is ubiquitously expressed throughout the human body, pannexin 2 (~73 kDa) is predominantly found in the brain, and pannexin 3 (~48 kDa) is primarily expressed in the bone, cartilage, and skin (Baranova et al., 2004; Penuela et al., 2014). Of all the pannexins,
Pannexin 1 has been the most extensively studied. The pannexin 1 gene is located on chromosome 11, and consists of five exons (Baranova et al., 2004). At least two isoforms have been identified, with alternative splicing of the fifth exon, resulting in differences in the C terminal; however, no functional or trafficking differences have been documented between the isoforms (Baranova et al., 2004; Penuela et al., 2014).

Pannexins were first discovered due to their sequence homology to the invertebrate gap junction proteins, innexins, and were named after the Latin words pan (meaning all) and nexus (meaning connection) (Panchina et al., 2000). While innexins are commonly found paired as gap junctions between cells, pannexins are usually found functioning as a single membrane channel. Despite their similar topology, there is no homology between pannexins and the vertebrate gap junction proteins connexins (Cxs) (Penuela et al., 2013). It is also notable that, compared to Cxs, pannexin 1 has a much longer half-life (Penuela et al., 2007). In a study where cells were treated with brefeldin A (BFA), an inhibitor of protein secretion, pannexin 1 clearance was only noticed after 32 hours of BFA treatment (Penuela et al., 2007). On the other hand, it took only 3 to 6 hours to observe clearance of Cx43 (Penuela et al., 2007). These differences show that, while pannexins and Cxs are both channel forming proteins expressed in vertebrates, their distinct homologies should be considered when analyzing their biological properties.

Once translated, pannexin 1 proteins must undergo glycosylation for proper trafficking to the cell membrane. Studies using site-directed mutagenesis revealed that N-glycosylation occurs in pannexin 1 at N254 (Boassa et al., 2007). There are three glycosylated species of pannexin 1: the non-glycosylated species (Gly 0), high mannose species (Gly 1) and the complex glycosylated species (Gly 2) (Penuela et al., 2013). The Gly 0 and Gly 1 species are predominantly found at the endoplasmic reticulum, while the Gly 2 species is predominantly found at the plasma membrane surface (Penuela et al., 2009). Furthermore, the non-glycosylated pannexin 1 proteins have similar channel function as their glycosylated counterpart, suggesting that glycosylation is required for the proper trafficking of the pannexin 1 to the plasma membrane (Penuela et al., 2009).
1.3.1 Pannexin 1 Function

Six pannexin 1 proteins oligomerize into a channel that allows the passage of small signaling molecules, up to 1 kDa in size, such as ATP and Ca\(^{++}\) (Figure 1.2) (Vanden Abeele et al., 2006; Penuela et al., 2013). Studies have shown that pannexin 1 channels can be opened via mechanical stimulation, intracellular calcium, extracellular potassium, ATP, membrane depolarization, or caspase cleavage (Locovei et al., 2006; Chekeni et al., 2010). Structural evidence indicates that the outer pore is composed of the first transmembrane domain and the first extracellular loop, while the C-terminus is believed to act as a plug to reversibly close the channel (Chekeni et al., 2010; Penuela et al., 2014). Permanent cleavage of the C-terminus by caspase 3 or 7 will cause the channel to be irreversibly opened (Chekeni et al., 2010). This feature has been shown to play a key role in releasing "find me" signals required for apoptotic cell clearance (Chekeni et al., 2010). Pannexin 1 also participates in paracrine signaling as an ATP release channel, facilitating serotonin release in presynaptic cells during tasting and calcium signaling in hippocampal pyramidal neurons during epilepsy (Thompson et al., 2008; Vandenbeuch et al., 2015). Pannexin 1 has also been suggested to form endoplasmic reticulum Ca\(^{++}\) permeable channels in the human prostate adenocarcinoma cell line (LNCaP), which may play a role in intracellular signaling (Vanden Abeele et al., 2006).

Pannexin 1 can be pharmacologically inhibited with carbenoxolone and probenecid (Silverman et al., 2009). Carbenoxolone is an approved treatment for gastroesophageal reflux disease, and has been shown to have an inhibiting effect on pannexins at lower concentrations (<100 μM), as well as other channel forming proteins, such as connexins, at higher concentration (>200 μM) (Pinder et al., 1976; Silverman et al., 2009). Probenecid is a common gout remedy that inhibits organic anion transporters (Silverman et al., 2008). Moreover, previous studies have demonstrated that 1 mM probenecid inhibits human pannexin 1 (PANX1) channel function in Xenopus laevis oocytes that over express PANX1, and this same concentration has been used to inhibit PANX1 channel function.
Figure 1.2 Diagram of Pannexin 1 Channel Gating
**Figure 1.2: Diagram of Pannexin 1 Channel Gating.** Pannexin 1 molecules oligomerized to form a single membrane channel that allows the passage of molecule smaller than 1 kDa (A). The glycosylation required for the proper trafficking of the protein is coloured in red, while the actin which interact with pannexin 1 proteins is shown in yellow. The C-terminus can block the pore reversibly (B, C) or irreversibly open the channel (D). Figure used with permission from Penuela S, Harland L, Simek J & Laird DW (2014). Pannexin channels and their links to human disease. Biochem J 461, 371–381.
during human skeletal myoblast differentiation (Silverman et al., 2008; Langlois et al., 2014). Since both small molecules are lipophilic, they can cross the cell membrane and inhibit pannexin 1 channel function regardless of where pannexin 1 is located (Masereeuw et al., 2000; Benfenati et al., 2009). As a result, these drugs are frequently used to study pannexin 1 function in a variety of cell types (Silverman et al., 2009; Langlois et al., 2014; Bhaskaracharya et al., 2014).

In addition to its channel function, pannexin 1 interacts with other binding partners to facilitate cell signaling. One of these proteins is actin, which interacts with the C terminus of pannexin 1 to facilitate proper trafficking and stability in the membrane (Bhalla-Gehi et al., 2010). Pannexin 1 also interacts with the purinergic channels, P2X and P2Y, to facilitate cellular activation, apoptosis, and stress signals (Velasquez & Eugenin, 2014). α1D-adrenergic receptors for phenylephrine are also binding partners of pannexin 1, and together induce vasoconstriction of resistant arteries (Penuela et al., 2014). Finally, pannexin 1 participates in the inflammasome complex, along with P2X7 and caspase 1, within neurons and astrocytes (Silverman et al., 2009). Given this information, it is important to consider the interaction between pannexin 1 and other proteins when studying pannexin 1 function.

1.3.2 Pannexin 1 during Cellular Differentiation

Pannexin 1 has been reported to influence differentiation within rat epidermal keratinocytes (REKs), human skeletal muscle myoblasts (HSMM), and murine ASCs (Celetti et al., 2010; Langlois et al., 2014; Lee et al., 2018). To study pannexin 1 in keratinocyte differentiation, murine pannexin 1 (Panx1) was overexpressed in REKs (Panx1-REKs), which was confirmed by increased dye uptake (Celetti et al., 2010). Using a liquid air chamber, these Panx1-REKs were differentiated into an organotypic epidermis, comprised of a 2-3 cell vital layer covered by a cornified layer (Celetti et al., 2010). Cytokeratin 14 (CK14), which is a marker for undifferentiated basal keratinocytes, had increased expression throughout the Panx1-REK organotypic epidermis compared to the non-differentiated Panx1-REKs (Celetti et al., 2010). Furthermore, the organotypic epidermis of the Panx1-REKs had a disorganized epidermal structure, which included a reduction in vital layer thickness (Celetti et al., 2010). Overall, this study concluded that overexpression
of Panx1 dysregulated keratinocyte differentiation, indicating that pannexin 1 may play a role in regulating epidermis renewal (Celetti et al., 2010).

On the other hand, overexpression of PANX1 promoted earlier HSMM differentiation toward skeletal myotubules, as indicated by the expression of terminal differentiation markers, myosin heavy chain marker, and the formation of multi-nucleated myotubules (Langlois et al., 2014). Pharmacological inhibition of PANX1 channel function using carbenoxolone and probenecid impeded differentiation (Langlois et al., 2014). From these findings, PANX1 is speculated to release ATP, which is required during HSMM differentiation (Langlois et al., 2014). In terms of adipogenesis, the loss of Panx1 was shown to increase murine adipogenesis in ASCs, which will be discussed later in greater detail (Lee et al., 2018). Based on these studies, it is believed that pannexin 1 is involved in differentiation, though its role appears to vary depending on the cell types involved and the state of cell differentiation being assessed.

1.4 Pannexin 1 in Adipocytes

PANX1 is ubiquitous throughout the human body, so it was hardly surprising that PANX1 is expressed in adipose tissue, and specifically in ASCs (Baranova et al., 2004). Furthermore, PANX1 expression was reported to be positively correlated with insulin resistance in obese patients, indicating that PANX1 may influence adipocyte function (Adamson et al., 2015). However, the role of PANX1 in adipocytes remains poorly understood. Currently, PANX1 is believed to play a role in glucose metabolism in adipocytes, but the mechanism remains unclear (Adamson et al., 2015). Moreover, there are limited studies addressing the role of PANX1 in regulating adipogenesis and adipose-related diseases.

1.4.1 Pannexin 1 in Adipose Related Diseases

Since its discovery, PANX1 has been linked to multiple human diseases, and therefore it was no shock to find PANX1 related to obesity (Penuela et al., 2014; Adamson et al., 2015; Lee et al., 2018). Obesity is the accumulation of excess adipose tissue that leads to impaired adipocyte function, and is characterized as a chronic low-grade inflammation (Hajer et al., 2008; Castro et al., 2014). In adipocytes, PANX1 was reported to activate inflammasomes
and increase the release of interleukin 1 beta, a pro-inflammatory cytokine, which in obese patients may contribute to the obesity-related inflammation (Silverman et al., 2009). Furthermore, adipose tissue secretes adiponectin, which acts as an anti-inflammatory signal by inhibiting PANX1 channels (Li et al., 2018). However, during obesity, adipose tissue has a reduction in adiponectin secretion, resulting in inflammation (Li et al., 2018). In addition, obesity contributes to the development of insulin resistance and type 2 diabetes (Castro et al., 2014; Hruby & Hu, 2015). It has been reported that the level of PANX1 expression in adipose tissue of obese patients positively correlates with the degree of insulin resistance (Adamson et al., 2015). A study using adipose-specific Panx1-knockout mice reported that Panx1 channels are activated by insulin, and are necessary for glucose uptake in adipocytes (Adamson et al., 2015). It appears that pannexin 1 is regulated by insulin, but the mechanism remains uncertain. Overall, the ongoing trend indicates that a reduction in PANX1 is advantageous in preventing disease onset or progression (Penuela et al., 2014).

1.4.2 Pannexin 1 in Mice

Through the use of Panxl knockout mice, an understanding of the key roles for Panx1 in vivo and in vitro have greatly advanced. Panx1 is expressed in murine WAT, and when wild type mice were placed on a high fat diet (HFD; 60% by kcal) there was an increase in Panx1 in epidermal WAT (Pillon et al., 2014). To further study Panx1, global Panx1-knockout (Panx1-KO) mice have been generated (Anselmi et al., 2008; Chekeni et al., 2010; Seminario-Vidal et al., 2011; Santiago et al., 2011). On a normal diet, Panx1-KO mice had significantly greater total fat mass and lower lean fat mass (total body weight minus the weight due to the fat mass) when compared to wild type (WT) mice (Lee et al., 2018). When placed on a HFD, there was no difference between the Panx1-KO and WT mice in either weight gain or obesity blood markers, but there was an increase in glucose and insulin levels in the Panx1-KO mice (Lee et al., 2018). The similarity in weight gain may be due to the higher activity level observed in the Panx1-KO mice, with higher ambulatory activity and reduced sleep duration (Lee et al., 2018). Furthermore, skin tissue samples were collected from the fat pads of the Panx1-KO mice on the normal and HFD (Lee et al., 2018). Hematoxylin and eosin staining revealed that the absence of Panx1
increased adipocyte size and reduced the number of adipocytes in subcutaneous adipose tissue, which is typical of obese patients (Lee et al., 2018). In terms of differentiation, Panx1 has been reported to inhibit murine adipogenesis (Lee et al., 2018). ASCs isolated from a Panx1-KO mouse had increased levels of adipocyte markers, including increased oil red O content, GPDH activity, and secretion of adiponectin and leptin (Lee et al., 2018). Since PANX1 is also expressed in human ASCs, there is a possibility that PANX1 inhibits human adipogenesis, however further studies are required.

1.5 Rationale and Hypothesis

Pannexin 1 has been reported to play a role in murine adipogenesis and adipocyte function, and is clearly expressed in human MSCs (Guerrero et al., 2018; Lee et al., 2018). However, it is unclear whether PANX1 plays a role in human adipogenesis, and therefore we aimed to investigate the role of PANX1 during human MSC differentiation into adipocytes. Since MSC origin has an influence on differentiation, we used MSCs derived from iPSCs (further referred to in this report as iMSCs) and ASCs (MSCs collected from human adipose tissue) to investigate the role of PANX1 during adipogenesis. We hypothesized that PANX1 regulates human MSC directed in vitro adipogenesis.

1.6 Objectives

The specific aims of this project were to:

1.6.1 Determine PANX1 mRNA and protein levels as iMSCs and ASCs differentiate along the adipogenic lineage.

1.6.2 Examine the effects of pharmacological inhibition of PANX1 channel function and PANX1-ablation on iMSCs during adipogenesis.

1.6.3 Investigate the effects of pharmacological inhibition of PANX1 channel function on ASC differentiation along the adipogenic lineage.
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Chapter 2

2 The Role of Pannexin 1 during Human Adipogenesis in vitro

Pannexin 1, a channel forming protein, has been reported to play a role in murine adipogenesis and fat accumulation. However, it is not known whether human pannexin 1 (PANX1) plays a role in adipogenesis. Here we study for the first time the role of PANX1 during adipogenesis in vitro using two sources of human mesenchymal stem cells (MSCs): adipose derived stem cells (ASCs), and induced MSCs (iMSCs) which were derived from induced pluripotent stem cells (iPSCs). Using a clustered regularly interspaced short palindromic repeat associated caspase 9 (CRISPR/Cas9) gene ablations strategy, we found that PANX1 was not necessary to differentiate iPSCs into iMSCs, suggesting that PANX1 does not play a role in this early state of stem cell differentiation. Immunoblotting revealed that PANX1 was expressed in both the ASCs and iMSCs; however, PANX1 levels did not change during in vitro adipogenesis, which was induced using a previously published adipogenic media and the StemPro Adipogenesis Differentiation Kit respectively. Both the ASCs and iMSCs were treated with a PANX1-channel blocker, either carbenoxolone or probenecid, during adipogenic induction. Carbenoxolone was able to inhibit later stages of adipogenesis in both the iMSCs and the ASCs, as indicated by the lack of lipid droplet formation and glycerol-3-phosphate dehydrogenase (GPDH) activity, as well as reduced levels of adipogenic mRNA markers, LPL (encoding for lipoprotein lipase) and ADIPOQ (encoding for adiponectin). Determined by the same adipogenic parameters, probenecid was only able to halt later stages of adipogenesis in the iMSCs, and not able to halt it in the ASCs. The different responses from the two cell types to probenecid indicates that MSCs from different sources have distinct characteristics. Furthermore, PANX1-ablated iPSCs, generated using CRISPR/Cas9, were capable of transitioning into iMSCs based on surface marker expression. Furthermore, PANX1-ablated iMSCs expressed similar levels of lipid droplet formation, glycerol-3-phosphate dehydrogenase (GPDH) activity, and adipogenic marker mRNA levels to control iMSCs when induced along the adipogenic lineage. Overall, PANX1 appears to be dispensable for in vitro human adipogenesis, despite being expressed in both ASCs and iMSCs.
2.1 Introduction

According to the World Health Organization, about 10% of the global population is estimated to be obese (World Health Organization, 2014). Obesity is characterized by an accumulation of excess adipose tissue, which results in adipose dysfunction (Hajer et al., 2008; Castro et al., 2014). This leads to a chronic low-grade inflammation that can develop into insulin resistance and type 2 diabetes (Tao & Lagergren, 2013; Hruby & Hu, 2015). Adipose tissue is predominantly made of adipocytes, which perform endocrine functions, tissue repair, protection against mechanical stress, and, most importantly, lipid storage (Cohen & Spiegelman, 2016). During excessive consumption, adipocytes accommodate for the overflow of lipids by expanding in one of two ways: hypertrophy (increase in cell size) or hyperplasia (increase in the number of cells) (Ghaben & Scherer, 2019). Studies have shown that, during obesity, adipocytes undergo hypertrophy to store the excess lipids (Gesta et al., 2007). The larger cell sizes result in mechanical stress, which causes hypoxia and induces inflammation (Coelho et al., 2013; Ghaben & Scherer, 2019). It is believed that promoting hyperplasia in adipocytes would help to distribute the excess lipids among more cells, with the benefit of also maintaining smaller adipocyte cell sizes.

Adipocytes originate from a multipotent stem cell called mesenchymal stem cells (MSCs) (Lowe et al., 2011). MSCs can differentiate into adipocytes, chondrocytes, fibroblasts, and osteoblasts (Strioga et al., 2012). MSCs can be isolated from multiple sources, including bone marrow and adipose tissue (Wei et al., 2013). Previous studies have shown that MSCs have a preference to differentiate into cells from their tissue of origin rather than other cell types (Gimble et al., 2008; Mohamed-Ahmed et al., 2018). For instance, bone marrow MSCs (BM-MSCs) have been shown to prefer differentiation into chondrocytes and osteoblasts, but have a lesser capacity to differentiate into adipocytes (Mohamed-Ahmed et al., 2018). This is true in reverse for adipose derived stem cells (ASCs) (Mohamed-Ahmed et al., 2018). Nevertheless, both these MSCs have a finite replication capacity, and at high passages (~8-12) these cells enter senescence (Wagner et al., 2008). Hence, MSCs must be harvested on a frequent basis to maintain a consistent supply. A novel approach to creating an infinite supply of MSCs is to differentiate induced pluripotent stem cells (iPSCs) into MSCs (Hynes et al., 2014). iPSCs are somatic cells that have been
reprogrammed to become pluripotent stem cells capable of infinite renewal, and therefore iPSCs can be an endless source of MSCs (Hynes et al., 2016). Also, MSCs derived from iPSCs (iMSCs) are able to differentiate into adipocytes, chondrocytes, and osteoblasts, though with a preference towards chondrocytes and osteoblasts (Lian et al., 2010; Sheyn et al., 2016).

Adipogenesis is the differentiation process of MSCs entering the adipogenic lineage to become adipocytes (Lowe et al., 2011). This process can be divided into two stages: commitment and differentiation (Ghaben & Scherer, 2019). During the commitment stage, MSCs transition into pre-adipocytes and become committed towards the adipogenic lineage (Farmer, 2006; Cristancho & Lazar, 2011; Cawthorn et al., 2012b). Throughout the differentiation stage, preadipocytes undergo a transcriptional cascade that leads to the upregulation of proadipogenic factors, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT enhancer binding protein alpha (CEBPα) (Lowe et al., 2011). Both these transcription factors induce the expression of adipogenic genes necessary for the performance of adipogenic functions such as lipid storage, insulin sensitivity, and adipokine secretion (Lowe et al., 2011).

Recently, pannexin 1 has been reported to play a role during differentiation (Celetti et al., 2010; Langlois et al., 2014; Lee et al., 2018). Pannexins are a family of channel forming proteins that have four transmembrane domains, with the N-terminus and C-terminus facing the cytosol (Penuela et al., 2013). Pannexin 1 is glycosylated and trafficked to the cell surface, where it forms a large pore channel which allows the passage of molecules up to 1 kDa in size, an example being ATP (Penuela et al., 2013). In rat epidermal keratinocytes an overexpression of pannexin 1 resulted in keratinocyte dysregulation, which resulted in impaired epidermal architectures (Celetti et al., 2010). However, human pannexin 1 (PANX1) was shown to promote human skeletal myoblast differentiation towards skeletal muscle cells (Langlois et al., 2014). From these studies, it appears that pannexin 1 can promote or inhibit differentiation according to the cell type. Pannexin 1 has also been reported to inhibit murine adipogenesis (Lee et al., 2018). Murine ASCs isolated from pannexin 1-knockout (Panx1-KO) mice had increased adipogenesis, as indicated by the increase in lipid droplet formation, glycerol-3-phosphate dehydrogenase (GPDH)
activity, and secretion of adipokines, leptin, and adiponectin (Lee *et al.*, 2018). So far, PANX1 has also been detected in human ASCs; however, it is unknown whether PANX1 plays a role in human adipogenesis.

Here, for the first time, we determined if PANX1 influences human adipogenesis *in vitro* by both pharmacologically inhibiting PANX1 channel function and genetically ablating PANX1. We investigated the role of PANX1 in two human MSC models: ASCs and iMSCs. Based on the findings in the murine model, we hypothesized that PANX1 does play a role in human adipogenesis *in vitro*. In this study, we found that the MSC models had different responses to probenecid, suggesting that MSCs from different sources have distinct characteristics. Furthermore, it appears that PANX1 was not necessary during human adipogenesis, indicating that PANX1 may not be an ideal target to regulate adipogenesis for treating adipose-related diseases.

2.2 Materials and Methods

2.2.1 iPSC and MSC Culture and Differentiation

Human dermal fibroblasts and the cells derived from them were included on an approved and continuing University of Western Ontario, Human Research Ethics Board protocol (#104190). Previously, human dermal fibroblasts from a 30-year old female were reprogrammed into iPSCs by the Centre for the Commercialization of Regenerative Medicine (CCRM; Toronto, ON, Canada) (Esseltine *et al.*, 2017). The iPSCs were cultured in TeSR™-E8™ Media (StemCell Technologies; Vancouver, BC, Canada; #05990) on Gel-Trex-coated dishes (ThermoFisher Scientific; Waltham, MA, USA; #A1413302) and passaged using Gentle Cell Dissociation buffer (ThermoFisher Scientific; Waltham, MA, USA; #13151014) before reaching 80% confluence. For transfection, the iPSCs were reverted to a naïve-like state by culturing the iPSCs in RSet Media (StemCell Technologies; Vancouver, BC, Canada; #05970) on 0.1% gelatin and mouse embryonic fibroblasts (MEFs) at 37°C in a hypoxic incubator (5% CO2 balanced with nitrogen). Naïve-like iPSCs were split using TrypLE (ThermoFisher Scientific; Waltham, MA, USA; #12605010) and reverted to primed iPSCs by returning the naïve-like cells to TeSR™-E8™ Media on Gel-Trex-coated dishes.
The iPSCs were further differentiated into bona fide iMSCs using the STEMDiff™ Mesenchymal Progenitor Kit (StemCell Technologies; Vancouver, BC, Canada; #05240). The iMSCs were maintained in MesenCult™ MSC Basal Medium (StemCell Technologies; Vancouver, BC, Canada; #05401) on 0.1% gelatin-coated plates (StemCell Technologies; Vancouver, BC, Canada; #07903) and split using the ACF Enzymatic Dissociation Solution (StemCell Technologies; Vancouver, BC, Canada; #05427) and ACF Inhibitor Solution (StemCell Technologies; Vancouver, BC, Canada; #05428). Cells were split before reaching 80% confluency to retain multipotent characteristics, and cells under passage 12 were used.

The iMSCs were plated at 10,000 cells/cm² on 0.1% gelatin-coated plates and differentiated into adipocytes using the StemPro Adipogenesis Differentiation kit (ThermoFisher Scientific; Waltham, CA, USA; #A1007001) for 14 days. For experiments investigating the control and PANX1-ablated iMSCs, the iMSCs were induced to differentiate 24 hours after plating, and non-induced cells were collected immediately prior to induction. For the experiments studying the effects of probenecid and carbenoxolone on adipogenesis, iMSCs were induced to differentiate once the cells were 100% confluent (approximately 72 hours after plating), and non-differentiated iMSCs were cultured in parallel with MesenCult™ MSC Basal Medium without adipogenesis induction. iMSCs were induced in either the absence or presence of either 50 μM carbenoxolone (Sigma-Aldrich; Oakville, ON, Canada; # C4790) or 1 mM of probenecid (ThermoFisher Scientific; Waltham, CA, USA; #P36400). Throughout culturing, the iPSCs and iMSCs were incubated with 100 penicillin units/mL and 0.1 mg/mL streptomycin (ThermoFisher Scientific; Waltham, CA, USA; #15140-122). The iPSCs (except for the naïve-like iPSCs) and iMSCs were cultured at 37°C with 5% CO₂ throughout the experiment.

2.2.2 ASC Culture and Differentiation

Human ASCs from abdominal and breast fat tissue obtained from female donors (age 23 to 72 with a body mass index range of 28.5 to 41.2) were isolated and maintained in the laboratory of Dr. Lauren Flynn (under an approved University of Western Ontario, Human Research Ethics Board protocol (105426) to Dr. Lauren Flynn) (Flynn, 2010) and generously provided for these studies. The ASCs were cultured in ASC growth medium:
Dulbecco's Modified Eagle Medium: Ham’s F12 (DMEM: Ham’s F12) with 10% fetal bovine serum (FBS), 100 penicillin units/mL and 0.1 mg/mL streptomycin (Flynn, 2010; Yu et al., 2017; Lee et al., 2018) at 37°C with 5% CO₂ throughout the experiments. Passage 3 ASCs were plated at 50,000 cells/cm² and stimulated to differentiate into adipocytes in the presence of DMEM: Ham’s F12 with 33 μM biotin, 17 μM pantothenate, 10 μg/mL transferrin, 1000 nM hydrocortisone, 66 nM human insulin, 1 nM triiodothyronine, 0.25 mM isobutylmethylxanthine (IBMX), 1 μg/mL troglitazone, 100 penicillin units/mL and 0.1 mg/mL streptomycin for the first three days (Flynn, 2010). For the last four days, induced cells were cultured in DMEM: Ham’s 12 with 33 μM biotin, 17 μM pantothenate, 10 μg/mL transferrin, 1000 nM hydrocortisone, 66 nM human insulin, 1 nM triiodothyronine, 100 penicillin units/mL and 0.1 mg/mL streptomycin (Flynn, 2010). All media components were obtained from Sigma-Aldrich (Oakville; ON, Canada). In some cases, ASCs were differentiated in either the absence or presence of either 50 μM carbenoxolone or 1 mM of probenecid. As a control, ASCs were maintained in ASC growth media without adipogenesis induction during the seven-day differentiation period.

2.2.3 Adherent Human Embryonic Kidney 293 (AD293) Cell Culture and Transfection

AD293 were used as positive PANX1 controls. These cells were obtained from Agilent Technologies (Santa Clara, CA, USA) were cultured in DMEM (ThermoFisher Scientific; Waltham, CA, USA; # 11965-092) supplemented with 5% FBS, 2 mM L-glutamine (ThermoFisher Scientific; Waltham, CA, USA; #25030-081), 100 penicillin units/mL and 0.1 mg/mL streptomycin (ThermoFisher Scientific; Waltham, CA, USA; #15140-122). Cells were split before reaching 90% confluency using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (ThermoFisher Scientific; Waltham, CA, USA; #25200-056). The human PANX1 overexpression AD293 cells, generated by Daniel Nejad, a master’s candidate in Dr. Silvia Penuela’s lab, were used as a positive control. The AD293 cells were grown to 60-70% confluence, then transiently transfected with 1 ug of pUNO1-hPANX1 (InvivoGen; San Diego, CA, USA; #puno1-hpanx1) using Lipofectamine 3000 (ThermoFisher Scientific; Waltham, CA, USA, #L3000015) as previously described (Shao et al., 2016). Protein lysates were collected 72 hours after transfection.
2.2.4 Genetic Ablation of PANX1

PANX1-ablated iPSCs and AD293 cells were generated by Dr. Jessica Esseltine using CRISPR/Cas9. Two pairs of guide RNAs (gRNAs) were designed using the www.sanger.ac.uk website that specifically targeted the PANX1 gene (Table 1). The first pair targeted the first exon of PANX1 and was used to ablate PANX1 function in AD293 cells; however, there was a slightly higher chance for off-target effects using this pair of gRNAs. To address this, the second pair of gRNAs, which has a lower chance of off-target effects, was used to target the third exon of PANX1 to ablate PANX1 function in the naïve-like iPSCs.
Table 2.1 Guide RNAs Used to Ablate PANX1 with Clustered Regularly Interspaced Short Palindromic Repeat Associated Caspase 9

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>AD293</th>
<th>iPSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger Sequence</td>
<td>1087076273</td>
<td>1087081842</td>
</tr>
<tr>
<td>Forward</td>
<td>CACCGTTTCTCGGATTTTCGGTCA</td>
<td>CACCGCTGCAGAAACGCCAGAA CAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>AAACCTAGCAAGAAATCCGAGAAC</td>
<td>AAACCTGTTCTGCGTTTCGCA GC</td>
</tr>
<tr>
<td>Exon Target</td>
<td>Exon 1</td>
<td>Exon 3</td>
</tr>
<tr>
<td>Structure Target</td>
<td>first transmembrane domain</td>
<td>second transmembrane domain and the intracellular loop</td>
</tr>
</tbody>
</table>
The gRNAs were ordered from the UWO OligoFactory (London, ON, Canada) and cloned into a pSpCas9(BB)-2A-GFP plasmid (Addgene; Watertown, MA, USA; # PX458) that encodes for the caspase-9 protein and green fluorescent protein (GFP). AD293 and naïve-like iPSCs were transfected with 2 μg of gRNA PX458 vector at a 1:6 dilution with Mirus TransIT®-LT1 Reagent (Mirus Bio LLC; Madison, WI, USA; #MIR 2300) according to manufacturer’s instructions. Using Flow-Activated Cell Sorting (FACS) with the BD FACSAria III (BD Biosciences; Mississauga, ON, Canada), successfully transfected cells were identified by GFP expression and were individually plated either on tissue-cultured treated plastic (AD293) or on MEFs (naïve-like iPSCs). The naïve-like iPSC clones were returned to the primed iPSC state and differentiated into MSCs. AD293 cells engineered to have functional PANX1 deleted were immunoblotted for the presence of PANX1 and MSCs were subjected to PANX1 DNA gene sequencing to confirm gene editing.

2.2.5 DNA Sequencing

Primers that flanked the CRISPR/Cas9 cut site were designed using PrimerBlast and were predicted to produce a polymerase chain reaction (PCR) product of 579 base pairs (Forward: CGCAGGAGATCTCGATTGGT; reverse: TGCTGCACACAAACTCGTCT). Total RNA was isolated from the MSCs and its derivatives using the QIAshredder (Qiagen; Germantown, MD, USA) and RNeasy Mini Kit (Qiagen; Germantown, MD, USA). Reverse transcription was executed using the Superscript VILO cDNA Synthesis Kit (ThermoFisher Scientific; Waltham, MA, USA) according to manufacturer’s instructions, using 500 ng of RNA. Reactions were incubated in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) for 25°C/10 min, 42°C/60 min, and then 85°C/5 min. The complementary DNA (cDNA) products were amplified using REDTaq Ready Mix (Sigma-Aldrich; Oakville, ON, Canada; #R2523) with a 20 μL reaction volume in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) for 94°C/3 min, and then 25 cycles of 94°C/30 s and 52°C/30 s and 72°C/30 s, followed by 72°C/7 min. PCR products were separated on a 1% agarose gel, and extracted using the QIAquick Gel Extraction Kit (Qiagen; Germantown, MD, USA; #28704). These PCR products were sequenced by the DNA Sequencing Facility at Robarts Research Institute (London, ON).
2.2.6 Characterization of MSCs Using Flow Cytometry

According to the International Society of Cell Therapy, multipotent MSCs must express specific surface markers: ≥95% positive for CD73, CD90 and CD105 and ≤2% positive for CD34 and CD45. The following anti-human antibodies were purchased: CD34 eFluor 450 (1 µg/100 µL; ThermoFisher Scientific; Waltham, CA, USA; #48-0349-42), CD45 APC (0.25 µg/100 µL; ThermoFisher Scientific; Waltham, CA, USA; #17-9459-41), CD73 FITC (0.25 µg/100 µL; ThermoFisher Scientific; Waltham, CA, USA; #11-0739-42), CD90 (4 µL/100 µL; Molecular probes; Waltham, MD USA; #A15761) and CD105 (1 µg/100 µL; Invitrogen; CA, USA; #12-1057-42). MSCs were harvested and incubated as a single cell-suspension for thirty minutes at 4°C in the dark with antibodies diluted in phosphate buffered saline (PBS) supplemented with 5% FBS, according to the manufacturer’s instructions. Stained and unstained samples were immediately analyzed with single colour flow cytometry using the BD FACSCanto™ II system (BD Biosciences; Mississauga, ON, Canada) and the BD FACSDiva™ software (BD Biosciences; Mississauga, ON, Canada) with a minimum of 10,000 events recorded for each sample.

Similarly, isolated ASCs were analyzed for surface markers using single color flow cytometry with the Guava easyCyte 8HT Benchtop flow cytometer (EMD Millipore; Burlington, MA, USA; #0500-4008), following the previously mentioned methods (Yu et al., 2017). In brief, cells were lifted using EDTA-trypsin and incubated for thirty minutes in the dark on ice with anti-human antibodies diluted in PBS supplemented with 5% FBS. The following anti-human antibodies (purchased from ThermoFisher Scientific; Waltham, CA, USA) were used: CD105 PE (1 µg/100 µL; #12-1057-41), CD90 FITC (0.25 µg/100 µL; #15-0909-42), CD73 FITC (0.25 µg/100 µL; #11-0739-41), CD34 APC (0.25 µg/100 µL; #17-0349-41), and CD45 FITC (0.25 µg/100 µL; #11-0459-41). Stained and unstained samples were included in the analysis. ASC characterization via flow cytometry was performed by the members of the Flynn Lab, and only cells that demonstrated hallmark characteristics of stem cells were used in adipogenesis studies.

2.2.7 MSC Growth Curve

To determine if probenecid caused unexpected MSC cell death, control iMSCs were plated at 2,500 cells/cm² on 0.1% gelatin-coated plates and incubated in MesenCult Basal Media.
supplemented with 0 μM, 1 μM, 10 μM, 100 μM, and 1 mM probenecid. Media supplemented with probenecid was replenished every other day for two weeks. Images were acquired using a Leica DMI6000B Inverted Fluorescent Microscope (Leica Microsystems Inc.; Concord, Ontario Canada) to visually assess for any evidence of cell death.

To measure cell growth, control and PANX1-ablated MSCs were plated at 2,500 cells/cm² on 0.1% gelatin-coated plates. Cells were maintained in MesenCult Basal Media with or without 1 mM probenecid for up to fourteen days, with media changes every other day. Cells were stained with Trypan Blue Stain (0.4%) (ThermoFisher Scientific; Waltham, CA, USA; #T10282) and counted every two days using the Countess® Automated Cell Counter (ThermoFisher Scientific; Waltham, CA, USA; #10227).

2.2.8 Quantitative PCR

Reverse transcription was completed using the Superscript VILO cDNA Synthesis Kit (ThermoFisher Scientific; Waltham, MA, USA) according to the manufacturer’s instructions, using 400 ng of RNA. Quantitative assessment of mRNA levels was performed using PowerUp SYBR Green Master Mix (ThermoFisher Scientific; Waltham, MA, USA) according to the manufacturer’s instructions with 10 μL reactions containing 5 nM forward primer, 5 nM reverse primer, and 0.5 μL cDNA. qPCR was performed in either a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) or a CFX384 Real-Time System (Bio-Rad, Hercules, CA, USA) with the following settings: 50°C/2 min, 95°C/2 min, and then 40 cycles going between 95°C/15 s and 58.5°C/30 s. Relative mRNA levels were calculated using the delta-delta CT method.
### Table 2.2 Human Gene-Specific Primers for Quantitative Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse</th>
<th>Forward</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GTAACCCGTTGAACCCCATT</td>
<td>CCATCCAATCGGTAGTAGCG</td>
</tr>
<tr>
<td>PANX1</td>
<td>GGCAAAAACCTCCCATGTCTGC</td>
<td>CAGAAGTCTCTGTGCGGCGAT</td>
</tr>
<tr>
<td>GADPH</td>
<td>CTGGGCTACACTGAGCACC</td>
<td>AAGTGGTGTTGAGGGCAATG</td>
</tr>
<tr>
<td>ADIPOQ</td>
<td>AACATGCCCATTCGCTTTACC</td>
<td>TAGGCAAGTAGTACAGCACA</td>
</tr>
<tr>
<td>LPL</td>
<td>GTCCGTGGCTACCTGTGATT</td>
<td>TGGCACCCAACTCTCATACA</td>
</tr>
<tr>
<td>CEBPα</td>
<td>CAGAGGGACCGGAGTTATGA</td>
<td>TTCACATTGCACAAGGCACT</td>
</tr>
</tbody>
</table>
2.2.9  Immunoblotting

Cell lysates were collected using 1x immunoprecipitation buffer (2% Triton X-100, 330 mM NaCl, 20 mM Tris, 2 mM EDTA, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1% NP-40, pH 7.4) supplemented with a cOmplete™, Mini Protease Inhibitor Cocktail tablet (Millipore Sigma; Burlington, MA, USA; #11836153001 ROCHE), 1:100 sodium fluoride and 1:100 sodium orthovanadate phosphatase inhibitors. Cell lysates from AD293 cells overexpressing PANX1 were collected 72 hours post transfection. The cell lysates were stored at -80°C until they were analyzed and were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% SDS-gel before being transferred to a nitrocellulose membrane using the iBlot® Dry Blotting System (Life Technologies, Waltham, CA, USA). The membrane was blocked with 3% bovine serum albumin (BSA) diluted in PBS Tween for one hour at room temperature, and then incubated overnight on a rocker at 4°C with either 1 ng/μl custom-made rabbit anti-human PANX1 targeting the C-terminus of PANX1 (Shao et al., 2016), 1:1000 rabbit anti-PPARγ (Cell Signaling Technology; Davers, MA, USA; #2435), or 1:5000 rabbit anti-human Cx43 (Sigma-Aldrich; Oakville, ON, Canada; #C6219), followed by one hour incubation on a rocker at room temperature with 1:5000 dilution of goat anti-rabbit Alexa Fluor 680 secondary antibody (ThermoFisher Scientific; Waltham, MA, USA; #A21076). The membrane was also blotted at room temperature with 1:5000 mouse anti-human GAPDH (EMD Millipore; Burlington, MA, USA; #A21076) for one hour, and then 1:5000 dilution of Infrared Fluorescent Dye 800 goat anti-mouse antibody (Rockland; PA, USA; #611-132-122) for one hour. Membrane imaging was performed using the Odyssey Infrared Imaging System (Li-Cor BioSciences; Lincoln, NE, USA).

2.2.10  Confocal Imaging

Immunofluorescence was performed to determine the cellular localization of PANX1, PPARγ and perilipin. Cells were grown on glass coverslips covered in 0.1% gelatin and fixed with either 80% methanol, 20% acetone for fifteen minutes or with 10% neutral buffered formalin for thirty minutes at room temperature. The cells were covered with blocking solution (2% BSA with 0.1% Triton X-100) for one hour, followed by a one-hour incubation at room temperature in one of the following primary antibodies: 2 ng/μl dilution
of custom-made rabbit anti-human PANX1 directed to the C-terminal of the polypeptide (Shao et al., 2016), 1:100 dilution of rabbit anti-human PPARγ (Cell Signaling Technology; Danvers, MA, USA; #2435), or 1:200 dilution of rabbit anti-human perilipin (Cell Signaling Technology; Danvers, MA, USA; #9349). Primary antibody binding was detected with 1:500 dilution of donkey anti-rabbit Alexafluor 488 (Invitrogen; Waltham, CA, USA; #A21206) and/or 1:200 dilution of HCS LipidTOX™ Green neutral lipid stain (ThermoFisher Scientific; Waltham, CA, USA; #H34475). To stain cell nuclei, cells were labelled with 1:1000 dilution of Hoechst (ThermoFisher Scientific; Waltham, CA, USA; #H3570) for five minutes in the dark at room temperature. Coverslips were mounted on glass slides using Airvol (made in house), left to dry for at least eight hours at 4°C, and then imaged with a Zeiss LSM800 Confocal Microscope equipped with Airyscan (Zeiss; Toronto, Ontario, Canada).

Lipid droplet formation was quantified and analyzed using ImageJ (https://imagej.nih.gov/ij/). In brief, three to ten immunofluorescent images (165.2 μm x 165.2 μm) per biological replicate were captured, and the investigators were blinded to the treatment during data analysis to avoid bias. First, the number of differentiated cells per image was recorded as identified by the presence of lipid droplets. Second, the images were split into separate red, blue, and green channels. Lipid (green channel) images were adjusted to a specific threshold (50-255), and converted to binary images, where lipid droplet content (% lipid area in the total image) and average lipid size was measured. Similarly, nuclei (blue channel) images were adjusted to a specified threshold (43-255) and converted to binary images. Particles (nuclei) larger than 50 μm² were counted. The ratio of lipid droplet content per nuclei, average lipid size per nuclei, and the number of cells that differentiated per nuclei was calculated for each image.
Figure 2.1 Experimental Procedure for Lipid Droplet Quantification via ImageJ
Figure 2.1: Experimental Procedure for Lipid Droplet Quantification via ImageJ. Confocal images were split into the green, blue, or red channel. (A) Lipid droplet content and average lipid droplet size were calculated using images from the green channel. (B) Nuclei were counted from blue channel images.
2.2.11 Oil Red O Staining

After seven days of culturing in differentiation medium, ASCs were fixed with 2% paraformaldehyde for 15 minutes at room temperature. Oil red O is a lysochrome (fat soluble dye) diazo dye used to stain and visualize lipids droplets, and Oil Red O stock solution was prepared by dissolving Oil Red O powder (Sigma-Aldrich; Oakville, ON, Canada; #O0625-100G) in 99.9% isopropanol at 3 g/L. The Oil Red O working solution was prepared with a 3:2 ratio of Oil Red O Stock Solution to deionized water and filtered through a Whatman type 2 filter paper (Sigma-Aldrich; Oakville, ON, Canada; #1002-917). The cells were stained for five minutes at room temperature, followed by a hematoxylin counterstain (Sigma-Aldrich; Oakville, ON, Canada; #GHS116-500ML) for two minutes at room temperature (Turner et al., 2012). Images were taken using an EVOS® XL Core imaging system (Fisher Scientific, Ottawa, ON, Canada).

2.2.12 GPDH Activity

A GPDH Activity Kit (Kamiya Biomedical Inc.; Tukwila, WA, USA; Cat. # KT-010) was used to quantify GPDH activity, which was normalized to total protein content as measured by the Bio-Rad protein sample assay (Bio-Rad; Hercules, CA, USA) which included albumin standards (Turner et al., 2012). In brief, the substrate reagent contained dihydroxyacetone phosphate (DHAP) and NADH (a co-enzyme for GPDH). NADH concentration decreased as DHAP was converted into glycerol-3-phosphate by GPDH, which was measured by spectrophotometry at 340 nm absorbance over a period of 10 minutes at room temperature. The change in absorbance (Δ Optical Density/min) was determined from the slope of the kinetic curve. Data is shown in mUnits/mg intracellular protein (mU/mg), where one unit is defined as the GPDH activity necessary to oxidize one μmol of NADH per min.

2.2.13 Statistical Analysis

Three or more biological replicates were analyzed using a two-tailed unpaired Student’s T-test, or a two-way unpaired ANOVA with Tukey’s post hoc test. A p-value less than 0.05 was considered significant. Data from GPDH activity assays and lipid droplet quantification (total lipid content per nuclei, average lipid size per nuclei, and the number
of cells differentiated per nuclei) were square root transformed prior to statistical analysis. Statistical analysis was done using GraphPad Prism 7 (GraphPad Software; CA, USA).

2.3 Results

2.3.1 PANX1 mRNA Levels, but Not Protein Levels, Increased during Adipogenesis

To study PANX1 mRNA levels during adipogenesis, iMSCs were first induced to differentiate along the adipogenic lineage. Formation of lipid droplets revealed that a population of iMSCs had successfully differentiated along the adipogenic lineage after fourteen days (Figure 2.2). Immunolabeling for PANX1 failed to detect PANX1 in either undifferentiated or differentiated iMSCs while PANX1 was readily detected in wild type (WT) AD293 cells, but absent in AD293 cells engineered to ablate the expression of PANX1 protein (PANX1-ablated AD293) (Figure 2.2). After fourteen days of differentiation, induced iMSCs exhibited a significant increase in PANX1 mRNA compared to non-induced iMSCs (p<0.05) (Figure 2.3A). However, there was no significant difference in the levels of the combined Gly0, Gly1 and Gly2 species of PANX1, which represent different states of glycosylation (Figure 2.3B, C).

After seven days of culturing in adipocyte induction media, a population of ASCs differentiated along the adipogenic lineage, indicated by the presence of lipid droplets (Figure 2.4A). PANX1 mRNA levels increased in induced ASCs compared to non-induced ASCs (p<0.05) (Figure 2.4B); however, PANX1 protein levels (combined Gly0, Gly1 and Gly2 species) did not change between non-induced and induced control ASCs (Figure 2.4C, D). Furthermore, iMSCs had a lower level of PANX1 protein expression compared to ASCs, under both non-induced (Figure 2.5A) and induced conditions (Figure 2.5B). This indicates that even though both cell types can undergo adipogenesis, PANX1 protein levels were significantly different (p<0.05).
Figure 2.2
**Figure 2.2: PANX1 Protein Was Not Observed in iMSCs.** Control iMSCs were induced to differentiate to adipocytes for 14 days. PANX1 protein was not detected in non-induced and induced iMSCs. WT AD293 cells expressed PANX1 protein (red) but PANX1 was absent in the PANX1-ablated AD293 cells. Lipid droplets were observed in the induced iMSCs via staining with the HCS LipidTOX™ Green (green), indicating that adipogenesis was occurring. Nuclei were stained blue with Hoechst. Representative images of N=3. Scale bar = 20 μm.
Figure 2.3
Figure 2.3: PANX1 mRNA and Protein Levels during iMSC Adipogenesis. Control iMSCs were induced to differentiate along the adipogenic lineage for 14 days. (A) PANX1 mRNA levels were significantly increased in induced compared to non-induced iMSCs. (B) PANX1 protein levels did not significantly change between the non-induced and induced cells. (C) Representative immunoblot shows PANX1 protein levels between non-induced and induced iMSCs. The three glycosylation states of PANX1 (Gly0, Gly1 and Gly2) are noted. WT AD293 and PANX1-ablated AD293 cells were used as positive and negative controls respectively. Data shown are the mean of three or four independent trials ± SEM. *, p<0.05 (Two-tailed Student’s T-Test). qPCR samples were provided by Tao Huang.
Figure 2.4 PANX1 mRNA and Protein Levels during ASC Adipogenesis
**Figure 2.4: PANX1 mRNA and Protein Levels during ASC Adipogenesis.** ASCs were induced to differentiate along the adipogenic lineage for seven days. (A) Lipid droplets were observed in the induced ASCs via Oil Red O staining; no lipid droplets were noted in the non-induced ASCs. Representative brightfield images taken from four trials. Scale bar = 200 μm (B) PANX1 mRNA levels significantly increased during cell differentiation. (C) There was no significant difference in PANX1 protein levels between the non-induced and induced cells. (D) Representative immunoblot of PANX1 found in non-induced and induced ASCs. Wild type AD293 and PANX1-ablated AD293 cells were used as positive and negative controls, respectively. (E) Table listing the body mass index (BMI), age of each donor and tissue origin (abdominal (abd.) or breast). Data shown are the average protein levels of three independent trials ± SEM. *, p<0.05 (Two-tailed Student’s T-Test).
Figure 2.5

A) PANX1 protein levels (Normalized to GAPDH) for non-induced cell types.

B) PANX1 protein levels (Normalized to GAPDH) for induced cell types.

ASCs (Adipose-Derived Stem Cells) significantly higher PANX1 protein levels compared to iMSCs (Induced Mesenchymal Stem Cells).
Figure 2.5: ASCs Had Significantly Higher PANX1 Protein Levels Compared to iMSCs. iMSCs were differentiated along the adipogenic cell lineage for 14 days with StemPro Adipogenesis Differentiation Media, while ASCs were induced for seven days with ASC Adipocyte induction media. ASCs had significantly higher PANX1 protein levels compared to iMSCs, when normalized to GAPDH, in both the non-induced (A) and induced (B) condition. Data shown are the average protein levels of three to four independent trials ± SEM *, p<0.05; **, p<0.01 (Two-tailed Student’s T-Test).
Characterization of iMSC Clones with PANX1 Ablated

CRISPR/Cas9 was used to genetically modify the PANX1 gene in iPSCs. Two putative PANX1 deleted clones (clone 2 and 4) were differentiated into iMSCs and assessed for the presence of PANX1 via immunoblotting with the anti-human PANX1 antibody. As expected, PANX1 was detected in the positive controls (WT AD293 cells and AD293 cells engineered to overexpress human PANX1) but was not detected in the negative control (PANX1-ablated AD293 cells) (Figure 2.6). Unfortunately, PANX1 protein levels were sufficiently low that it could not be reliably detected in the unedited cells, raising concerns as to whether the PANX1 gene was successfully ablated (Figure 2.6). To address this question, the gene encoding full length PANX1 was sequenced. Based on DNA sequencing analysis, clone 2 had an eight-base pair deletion causing a frameshift mutation (beginning at amino acid position 123), resulting in an early stop codon at amino acid position 144 (Figure 2.7B, C). Residue 144 is found within the intracellular loop of the PANX1 polypeptide, thus preventing any possibility for PANX1 to successfully form a functional channel. Analysis of the DNA sequence indicated that the eight-base pair deletion occurred in at least one of the PANX1 alleles, resulting in a loss of heterozygosity (Figure 2.7E).

Dr. Jessica Esseltine (Memorial University, St. John’s, NFL) was further able to independently confirm that PANX1 was ablated from clone 2 iPSCs of which the iMSCs were derived via immunoblotting (data not shown; personal communication). Due to the ablation, clone 2 was used for the remainder of the experiments and is further referred as PANX1-ablated iMSCs. A second clone, clone 4 had a nine base pair deletion that resulted in a three amino acid deletion (from position 127 to 130, which is located between the second transmembrane domain and the intracellular loop of the PANX1 polypeptide) (Figure 2.7B, D). Clone 4 was not used for any further experiments as it was deemed to express a PANX1 mutant and did not represent cells that were devoid of functional PANX1.

Control and PANX1-ablated iMSCs expressed >95% CD73, CD90, and CD105, as well as <2% of the hematopoietic markers CD34 and CD45; therefore, both cell types can be classified as MSCs according to their surface CD protein expression profile (Figure 2.8). Furthermore, there was no significant difference in the total number of cells between the
Figure 2.6 Representative Western Blot of PANX1 in iMSCs
Figure 2.6: Representative Western Blot of PANX1 in iMSCs. Control (unedited) iMSCs were found by Western blot to have a low level of PANX1, making it difficult to determine if PANX1 was ablated from iMSCs that were edited by CRISPR/Cas9 to ablate the *PANX1* gene. Wild type AD293 cells were used as a positive control, while PANX1-ablated AD293 cells were used as negative controls. GAPDH was used a gel loading control.
Figure 2.7

A) Schematic representation of the CRISPR/Cas9 gene editing process.

B) Table showing the reading frame changes for different templates:

<table>
<thead>
<tr>
<th>Template</th>
<th>Control</th>
<th>Clone 2</th>
<th>Clone 4</th>
</tr>
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</table>

C) Clone 2: Frame shift mutation; L123fsX144

D) Clone 4: deletion of Trp, Arg, and Phe; W127_F130del

E) Clone 2 has a loss of heterozygosity
Figure 2.7: Based on DNA Sequencing, CRISPR/Cas9 Edited Clone 2 iMSCs Resulted in Truncated PANX1 Protein. (A) Experimental schematic demonstrating the location of the CRISPR/Cas9 cut site and primers in the human PANX1 gene. (B) DNA sequencing determined there was an eight base pair deletion in clone 2 and a nine base pair deletion in clone 4. (C, D) Images of control protein sequence from http://www.rcsb.org/ and highlighted areas indicate sites of mutations. (C) The eight base pair deletion in clone 2 resulted in a frameshift mutation with an early stop codon at position 144 (L123fsX144). (D) For clone 4, the nine base pair deletion cause a three amino acid deletion (W127_F130). (E) Analysis of DNA sequencing indicated that clone 2 was heterozygous for the ablation of PANX1. DNA alignment images from SnapGene Viewer. Arginine (Arg); base pair (bp); Primer 1 (Prim1); Primer 2 (Prim2); Protospacer adjacent motif (PAM); Phenylalanine (Phe); Tryptophan (Trp).
Figure 2.8

PANX1 is Not Necessary for Induced Pluripotent Stem Cell (iPSC) Differentiation to iMSC
Figure 2.8: PANX1 is Not Necessary for Induced Pluripotent Stem Cell (iPSC) Differentiation to iMSCs. PANX1-ablated iPSCs were differentiated into iMSCs and analyzed for MSC surface markers. As expected for MSCs, PANX1-ablated and control iMSCs had >95% expression of CD73, CD90 and CD105, as well as <2% expression of CD34 and CD45.
control and PANX1-ablated iMSCs after growing in culture for seven days in MesenCult Media (Figure 2.9B). Thus, any potential differences in adipogenic capacity cannot be attributed to differences in growth rates.

2.3.3 PANX1 is Not Necessary for iMSC Differentiation along the Adipogenic Lineage

To access adipogenic potential of iMSCs with and without PANX1, cells were induced to differentiate in culture. Based on qPCR results, there was no significant difference between control induced iMSCs and PANX1-ablated induced iMSCs in *CEBPα* (early adipocyte marker) or *LPL* (mid adipocyte marker) mRNA levels (Figure 2.10A, B). The trend indicates that the induced iMSCs of both cell types have higher levels of these mRNA markers compared to their non-induced counterpart (Figure 2.10A, B). The late adipocyte marker, *ADIPOQ*, was found at low levels among all the experimental groups (Figure 2.10C) suggesting that these cells never reached full adipocyte status. Furthermore, the GPDH activity between the control and PANX1-ablated induced iMSCs was not significantly different (Figure 2.10D), but induced cells lacking PANX1 expressed significantly higher levels of GPDH compared to their controls.

Control and PANX1-ablated induced iMSCs formed lipid droplets (Figure 2.11A, B), and expressed PPARγ in the nuclei (Figure 2.11A) and perilipin surrounding the lipid bodies (Figure 2.11B) suggesting that both cell populations had successfully differentiated along the adipogenic lineage. It was noted that lipid droplets were observed in only a fraction of the cell population, indicating that some iMSCs had undergone adipogenesis while others had not, demonstrating that the stem cells were heterogeneous (Figure 2.12A). Between the control and PANX1-ablated induced iMSCs, there was no significant difference in the number of differentiated cells (Figure 2.12B), number of lipid droplets (Figure 2.12C), lipid droplet content (Figure 2.12D), or average size of lipid droplets (Figure 2.12E). These results suggest that PANX1 did not alter the fate or extent of iMSC ability to differentiate along the adipogenic lineage.
Figure 2.9

A) Day 7

Control iMSCs

PANX1-ablated iMSCs

B) Days in Mesencult Media

Total Number of Cells

N = 3

Figure 2.9

No significant difference in growth rate between the PANX1-ablated and control iMSCs.
Figure 2.9: No Significant Difference in Growth Rate between the PANX1-Ablated and Control iMSCs. PANX1-ablated and control iMSCs were cultured in Mesencult Basal Media for seven days. (A) Phase contrast images were taken on the seventh day. Images shown are representative of N=3. Scale bar = 200 μm. (B) There was no significant difference in total cell number between PANX1-ablated and control iMSCs over seven days. Data shown are the average of three independent trials run in duplicate ± SEM. (Two-way ANOVA with post-hoc Sidak’s multiple comparisons test).
A) Early Adipocyte Marker *CEBPα*

- **Figure 2.10**

B) Early to Mid Adipocyte Marker *LPL*

C) Mid to Late Adipocyte Marker *ADIPOQ*

D) GPDH Activity
Figure 2.10: PANX1-Ablated and Control Induced iMSCs Have Similar Levels of Adipogenesis-Associated Genes and GPDH Activity. PANX1-ablated and control iMSCs were induced to differentiate along the adipocyte cell lineage for fourteen days. There was no significant difference in the mRNA levels of (A) CEBPa, (B) LPL, or (C) ADIPOQ between both of the induced cell types. (D) No significant difference was found in GPDH activity levels between the induced control and PANX1-ablated iMSCs. Data is shown in mUnits/mg protein (mU/mg), where one unit is defined as the GPDH activity necessary to oxidize one μmol of NADH per min. Values shown are the mean of three independent trials ± SEM *, p<0.05 (Two-way ANOVA with post-hoc Tukey’s test).
Figure 2.11 PANX1 is Not Necessary for iMSC Adipogenesis
Figure 2.11: *PANX1 is Not Necessary for iMSC Adipogenesis*. Confocal micrographs of control iMSCs and PANX1-ablated iMSCs induced to differentiate and labelled for lipid droplets (A, B), PPARγ (A), or perilipin (B) N=2. Nuclei are stained blue with Hoechst. Scale bar = 20 μm.
Figure 2.12 PANX1 Ablation Does Not Affect Lipid Droplet Formation in iMSCs
Figure 2.12: PANX1 Ablation Does Not Affect Lipid Droplet Formation in iMSCs. Control and PANX1-ablated iMSCs were induced to differentiate for fourteen days. (A) Lipid droplets were detected in both induced cells types but were absent in non-induced iMSCs. N=5, n=3-8. Scale bar = 20 μm. Between the induced control and PANX1-ablated iMSCs, there was no significant difference in either the number of cells that differentiated per nuclei (B), the number of lipid droplet per nuclei (C), the lipid droplet content per nuclei (D) or the average size of lipid droplets per nuclei (E). Data shown are the mean of square root transformed values ± SEM. N=5, n=3-8. **, p<0.01 (Two-tailed Student’s T-Test or Two-way ANOVA with Tukey’s post hoc test).
2.3.4 Pharmacological Inhibition of PANX1 Channel Function Inhibits Adipogenesis

In addition to assessing the role of PANX1 by using PANX1 ablated iMSCs, we also took the approach of blocking PANX1 channels using a pharmacological strategy where probenecid or carbenoxolone was used to inhibit channel function. Based on morphological assessments, iMSCs were able to tolerate the presence of 1 mM probenecid for fourteen days (Figure 2.13A). Furthermore, there was no difference in total cell count (relative to day 0) between the untreated iMSCs and 1 mM probenecid-treated iMSCs over twelve days (Figure 2.13B).

Control iMSCs were induced to differentiate along the adipogenic lineage in the presence of either 1 mM probenecid or 50 μM carbenoxolone. Lipid droplets were absent in undifferentiated cells but were found in induced iMSCs but appeared less prevalent and smaller in carbenoxolone-treated cells. (Figure 2.14A). Based on qPCR results, when drug-free cells were induced to differentiate, they expressed significantly higher levels of *CEBPα*, *LPL* and *ADIPQ* mRNA compared to their non-induced iMSCs counterpart (p<0.05) (Figure 2.14B, C, D). While not consistently significant, iMSCs induced to differentiate in the presence of probenecid or carbenoxolone had higher levels of these same genes compared to their corresponding non-induced iMSCs (Figure 2.14B, C, D). *CEBPα* and *LPL* mRNA levels were comparable between the three induced iMSCs experimental groups (Figure 2.14B, C). However, induced carbenoxolone-treated iMSCs had significantly lower *ADIPQ* mRNA levels compared to induced drug-free iMSCs (p<0.05) (Figure 2.14D). Furthermore, while GPDH activity was higher after cells were induced to differentiate, it was found to be statistically lower in cells induced to differentiate in the presence of either probenecid or carbenoxolone compared to untreated (Figure 2.14E). We conclude that PANX1 channel blocking had little effect on the capacity of iMSC to undergo the early stages of adipogenesis but carbenoxolone was more potent at blocking later stages of differentiation.

2.3.5 ASC Differentiation Was Partially Inhibited by Carbenoxolone

We next assessed if blocking PANX1 channel function would affect the ability of ASCs to differentiate along the adipogenic cell lineage. While control and probenecid-treated
Figure 2.13: Probenecid Had No Effect on iMSC Growth.
Figure 2.13: *Probenecid Had No Effect on iMSC Growth.* Control iMSCs were cultured in Mesencult Basal Media in the presence or absence of probenecid for fourteen days. (A) Control iMSCs were cultured in Mesencult Basal Media with 0 µM, 1 µM, 10 µM, 100 µM or 1 mM probenecid for fourteen days. Phase contrast images shown are representative of N=3. Scale bar = 200 µm. (B) No significant differences in total cell count were found between untreated and probenecid-treated iMSCs. Data shown are the average of three independent trial runs in duplicate ± SEM. (One-way ANOVA with post-hoc Tukey’s test).
Figure 2.14
**Figure 2.14:** *Control iMSCs Induced in the Presence of Carbenoxolone Formed Lipid Droplets, but Exhibited Decreased ADIPOQ levels and GPDH.* Control iMSCs induced in the presence of carbenoxolone formed lipid droplets, but exhibited decreased *ADIPOQ* mRNA levels, and GPDH activity. iMSCs were induced to differentiate in the presence or absence of 1 mM probenecid or 50 μM of carbenoxolone. Lipid droplets were detected in all of the induced cells, regardless of the drug treatment. Representative images of N=3. Scale bar = 20 μm (A). There was no significant difference in the mRNA levels of *CEBPα* (B) or *LPL* (C) between all of the induced cells, regardless of treatment. However, induced iMSCs cultured with carbenoxolone had significantly lower *ADIPOQ* mRNA levels compared to control cells (C). Induced cells cultured with either probenecid or carbenoxolone had significantly lower levels of GPDH activity than control cells. (D) GPDH Data is shown in mUnits/mg protein (mU/mg), where one unit is defined as the GPDH activity necessary to oxidize one μmol of NADH per min. All data shown are the mean of three independent trials performed in triplicate ± SEM. *, p<0.05; **, p<0.01 (Two-way ANOVA with post-hoc Tukey’s test). (E) Table showing the body mass index (BMI), age of each donor and tissue origin.
ASCs actively form lipid droplets when induced to differentiate, this process was dramatically inhibited when the cells were induced to differentiate in the presence of carbenoxolone (Figure 2.15A). There was no significant difference in CEBPa (Figure 2.15B) and LPL (Figure 2.15C) mRNA levels between the induced untreated and treated ASCs; however, induced carbenoxolone treated ASCs appeared to have much lower levels of LPL mRNA compared to the other induced cell groups. In addition, induced carbenoxolone treated ASCs has significantly lower levels ADIPOQ mRNA when compared to the induced untreated ASCs (p<0.05) and the trend indicates that induced carbenoxolone treated ASCs have lower ADIPOQ mRNA levels than induced probenecid treated ASCs (Figure 2.15D). Finally, induced probenecid-treated ASCs had significantly higher levels of GPDH activity compared to non-induced probenecid-treated ASCs (p<0.05), which was not observed when cells were treated with carbenoxolone (Figure 2.15E). Overall, the trend indicates that induced carbenoxolone treated ASCs had lower levels of adipocyte markets, indicating that carbenoxolone inhibited adipogenesis in ASCs.

To further assess that carbenoxolone inhibits adipogenesis, we measured the protein levels of PPARγ, a key adipogenesis regulator, via immunoblotting (Rosen & MacDougald, 2006). PPARγ has two isoforms, PPARγ1 and PPARγ2, where PPARγ1 (50 kDa) is found in multiple tissue types while PPARγ2 (57 kDa) upregulation is a biomarker of cells undergoing adipogenesis (Fajas et al., 1997; Ren et al., 2002). The trend indicated that induced carbenoxolone-treated ASCs had lower levels of PPARγ2 compared to the induced untreated and probenecid-treated ASCs (Figure 2.16C). PANX1 protein levels were also analyzed to confirm that probenecid and carbenoxolone were not affecting PANX1 expression in the ASCs. As expected, there was no significant difference in PANX1 (Gly0, Gly1 and Gly2 combined) protein levels between the experimental groups (Figure 2.16D). We did not anticipate any changes in PANX1 protein levels in ASCs between the untreated, probenecid, and carbenoxolone conditions, since the drugs are thought to only inhibit channel function (Pinder et al., 1976; Silverman et al., 2009) and thus were not expected to affect protein levels.
Figure 2.15: Carbenoxolone Inhibits Adipogenesis in ASCs
Figure 2.15: Carbenoxolone Inhibits Adipogenesis in ASCs. ASCs were induced to differentiate for seven days either untreated or treated with probenecid (Prob) or carbenoxolone (CBX). (A) Lipid droplets were detected in the induced control and probenecid ASCs; however, no lipid droplets were found in the induced carbenoxolone ASCs. Representative brightfield images of N=4 n=3. Scale bar = 200 μm. (B-D) There was no significant difference in adipogenic mRNA markers between induced ASCs, regardless of drug treatment; however, the trend indicates that carbenoxolone induced ASCs have lower levels of LPL mRNA compared to the control and probenecid induced ASCs. The carbenoxolone induced ASCs had significantly lower levels of ADIPOQ mRNA compared to control induced ASCs. Data shown are the mean of three independent trials run in triplicate ± SEM. *, p<0.05 (Two-way ANOVA with post-hoc Tukey’s test). (E) There was no significant difference in the GPDH activity among the induced cells. The trend indicated that induced ASCs cultured with carbenoxolone had lower levels of GPDH activity. Induced cells cultured in probenecid had significantly higher GPDH activity compared to non-induced probenecid ASCs (p<0.05). (F) Table showing the body mass index (BMI), age of each donor and tissue origin.
Figure 2.16: Carbenoxolone inhibits ASC adipogenesis, as indicated by the absence of PPARγ2 protein induction. The figure shows Western blot analysis of PPARγ2 and PANX1 in ASCs and AD293 cells treated with CBX and control conditions.
Figure 2.16: *Carbenoxolone Inhibits ASC Adipogenesis, as Indicated by the Absence of* PPARγ2 *Protein.* ASCs were induced to differentiate for seven days in the absence or presence of either probenecid (Prob.) or carbenoxolone (CBX). (A) Representative immunoblot denoting PPARγ2 protein levels in ASCs from donor 1. Wild type AD293 cells and PANX1-ablated AD293 cells were used as negative controls. (B) Representative immunoblot denoting PANX1 protein levels. Wild type AD293 cells and cells engineered to overexpress human PANX1 were used as a positive control, while PANX1-ablated AD293 cells were used as negative controls. There was no significant difference in either the PPARγ2 (C) or PANX1 (D) protein levels between the experimental groups. Data shown are the average protein levels of four independent trials ± SEM. (Two-way ANOVA with post-hoc Tukey’s test). (E) Table showing the body mass index (BMI), age of each donor and tissue origin.
Since carbenoxolone is also capable of inhibiting gap junctional intercellular communication (Pinder et al., 1976) in addition to PANX1 channel function, it is possible that carbenoxolone was inhibiting connexin function in ASCs. Since Cx43 is known to be expressed in adipocytes and pre-adipocytes (Yeganeh et al., 2012), we used immunoblotting to determine if ASCs express Cx43 and whether it was expressed in ASCs induced to differentiate. Immunoblotting indicated that Cx43 was expressed in control and induced ASCs in the presence and absence of probenecid or carbenoxolone (Figure 2.17). This study suggests that carbenoxolone may be acting to impair adipogenesis via its action on Cx43 channels as opposed to its role in inhibiting PANX1 channels.
Figure 2.17 Representative Immunoblotting Demonstrating the Presence of Connexin 43 in ASCs
Figure 2.17: Representative Immunoblotting Demonstrating the Presence of Connexin 43 in ASCs. Representative immunoblots denoting the presence of connexin 43 (Cx43) and its three phosphorylation states (P0, P1, and P2) in ASCs during adipogenesis. ASCs were induced to differentiate over a period of seven days in the absence of presence of either probenecid (Prob.) or carbenoxolone (CBX). Wild type AD293 were used as a positive control. ASCs from donor 2 (Body Mass Index: 41.2; Age: 61; Tissue origin: Abdominal) were used. N=1.
2.4 Discussion

Pannexin 1 channels participate in paracrine signaling to communicate with neighboring cells through the release of molecules into the extracellular space (Che et al., 2014). In fact, pannexin 1 channels are responsible for releasing nucleotides, or ‘find me’ signals, to recruit monocytes to apoptotic cells (Chekeni et al., 2010). MSCs are also well documented for using paracrine signaling to release growth factors and cytokines, to adjacent cells to prevent apoptosis as well as promote proliferation during tissue regeneration (Squillaro et al., 2016a). Pannexin 1 has been reported to both promote skeletal myoblast differentiation as well as dysregulate keratinocyte differentiation, signifying that the role of pannexin 1 during differentiation depends on the cell type (Celetti et al., 2010; Langlois et al., 2014). Relevant to the current study, paracrine signaling has been reported to play a defining role in regulating adipocyte differentiation within adipose tissue depots (Meissburger et al., 2016). However, it is unclear whether PANX1 plays a role in paracrine signaling during adipocyte differentiation in humans. A previous study demonstrated that Panx1-KO mice had increased GPDH activity, lipid droplet formation, and adipocyte size, while also exhibiting a reduced number of adipocytes, thus indicating that Panx1 inhibits adipogenesis in mice (Lee et al., 2018). To determine if this finding also applies in the human context, the role of PANX1 was studied when human iMSCs and ASCs were subjected to adipogenesis under conditions where PANX1 function or levels were altered. In brief, the pharmacological inhibition of PANX1 channels with carbenoxolone hindered adipogenesis in both iMSCs and ASCs; however, PANX1-blocker probenecid was only able to hinder adipogenesis in the iMSCs, but not the ASCs. Furthermore, PANX1 ablation did not affect either iPSC differentiation into bona fide iMSCs, nor further differentiation along the adipogenic lineage.

For both the iMSCs and ASCs, PANX1 mRNA levels increased during adipogenesis, but this increase was not observed at the protein levels. The reason why the increase in PANX1 mRNA was not reflected by a parallel increase in the protein is not clear, but it may reflect that the mRNA levels were unstable and subject to degradation prior to translation. Also, it is noteworthy that PANX1 has a long half-life, thus avoiding the need for active synthesis of new protein to maintain a high level of PANX1 function (Bhalla-Gehi et al., 2010).
Supporting this notion, it has been shown that proteins with slower degradation rates have a weaker correlation with their corresponding mRNA levels (Raj et al., 2006), which may explain the discrepancy between the PANX1 mRNA and protein levels in MSCs during adipogenesis.

Cell surface PANX1 has been consistently reported as an ATP release channel, so if PANX1 plays a role in adipogenesis, it is likely to do so by its channel function and release of critical signaling molecules (Penuela et al., 2007; Chekeni et al., 2010; Vandenbeuch et al., 2015). A common approach used to study PANX1 function during differentiation is to use pharmacological agents to inhibit its channel function (Silverman et al., 2009; Langlois et al., 2014; Bhaskaracharya et al., 2014). Since a specific PANX1 inhibitor has yet to be identified, carbenoxolone and probenecid have been used as the “best available” inhibitors to block PANX1 channel function (Bruzzone et al., 2005; Locovei et al., 2006; Silverman et al., 2008). Carbenoxolone is also a potent inhibitor of connexin channels and probenecid is clinically used to block organic anion channels (Sagar & Larson, 2006; Silverman et al., 2008) yet both drugs are potent at blocking pannexin 1 channels.

Drug-treated iMSCs were not able to differentiate along the adipogenic lineage, in contrast to untreated iMSCs, prompting us to propose that PANX1 channel function is involved in human adipogenesis. To assess the effects of pharmacologically inhibiting PANX1 function during adipogenesis using a second source of MSCs, we blocked PANX1 function in ASCs (MSCs isolated from adipose tissue). The ASCs treated with carbenoxolone did not differentiate; however, the probenecid-treated ASCs continued to differentiate similar to untreated ASCs. Taken together, carbenoxolone consistently inhibited adipogenesis from both stem cell sources, whereas probenecid had varying effects depending on the MSC origin. One possibility that may explain the different findings is the fact that carbenoxolone is a potent inhibitor of Cx43 gap junction channels that are found in MSCs (Sagar & Larson, 2006; Wang et al., 2010). Since Cx43 plays a role in osteoblast differentiation and skeletal myogenesis in vitro (Araya et al., 2005; Lin et al., 2018), there is a possibility that Cx43 plays a critical role in adipogenesis. Besides PANX1 and connexins, carbenoxolone has been reported to inhibit other proteins such as 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1), which facilitates adipogenesis through
interaction with glucocorticoids (a core component in adipogenesis mediums) (Dhanesha et al., 2012; Sano et al., 2012; Ghaben & Scherer, 2019). It is also notable that mice on a HFD treated with carbenoxolone had reduced adipocyte size, a decrease in adipocyte markers, and an overall reduction in fat accumulation (Sano et al., 2012). In another study, HSD11B1-knockout mice were protected against obesity and hyperglycemia (Kotelevtsev et al., 1997). Thus, it is possible that carbenoxolone inhibited HSD11B1 in both the iMSCs and the ASCs, effectively inhibiting adipogenesis as indicated by the absence of adipocyte markers. Due to the non-specificity of carbenoxolone, it was difficult to determine whether PANX1 channel inhibition halted adipogenesis, hence we used another PANX1-blocker drug, probenecid, and also generated PANX1-ablated MSCs to study PANX1 during adipogenesis.

Our study found that adipogenesis was inhibited in probenecid treated iMSCs, but not the ASCs. The discrepancy between the differentiation capacity of probenecid-treated iMSCs and ASCs was remarkable, suggesting that MSCs from different sources may activate different mechanisms to guide their cell fate. Probenecid in human preadipocytes has been shown to promote adipogenesis by activating the non-selective cation channel transient receptor potential vanilloid 2 (TRPV2), which regulates intracellular calcium signaling (Che et al., 2014). Thus, differences in probenecid treatment between the iMSCs and ASCs may be possibly explained by the fact that MSCs from different sources have distinct proteomes. A comparison of proteome expression between BM-MSCs and ASCs revealed that 23% of the proteins were uniquely expressed in either the BM-MSCs or the ASCs, and a further 18% of the proteins were expressed at different levels between the BM-MSCs and ASCs (Noël et al., 2008). In keeping with this notion, we found that ASCs had a significantly higher level of PANX1 protein compared to iMSCS in both the induced and non-induced conditions. It is possible that probenecid is inhibiting PANX1 and/or altering the function of other proteins which are differentially expressed between the two MSC types, resulting in different responses to probenecid during adipogenesis.

While the pharmacological blocking of PANX1 channels was used to show that PANX1 function is dispensable for adipogenesis, we wanted to further assess if other PANX1 properties might exist that are critical in adipogenesis. In addition to being a large pore
channel, PANX1 can also interact with other binding partners to facilitate cell signaling (Silverman et al., 2008; Penuela et al., 2013). Therefore, a PANX1-ablated iPSC cell population was engineered to determine if eliminating PANX1 would affect adipogenesis. PANX1-ablated iMSCs were able to differentiate into iMSCs, and further differentiate along the adipogenic lineage, indicating that the PANX1 protein is not necessary for human adipogenesis in vitro. Moreover, no significant differences were observed in any of the adipocyte markers between the PANX1-ablated and control iMSCs. Thus, neither PANX1 channel function nor the PANX1 protein itself affect adipogenesis.

As revealed in our studies, iMSCs and ASCs exhibited distinct differentiation potential that may engage distinct mechanisms, highlighting the uniqueness of iMSCs and ASCs. Based on the increased expression of adipocyte markers and lipid droplet formations, iMSCs were able to initiate adipogenesis, but were unable to reach later stages of adipogenesis after fourteen days of differentiation. On the other hand, the ASCs were able to proceed further along the adipogenic lineage and achieve later stages of adipogenesis in a shorter period of time (7 days), suggesting that the ASCs have a greater adipogenic potential compared to the iMSCs. This is in line with previous studies that have demonstrated that MSCs from different tissue origins possess unique differentiation capacities, where ASCs have a higher capacity to differentiate into adipocytes in comparison to BM-MSCs (Li et al., 2015; Mohamed-Ahmed et al., 2018). Moreover, recent studies have shown that iMSCs have a lower adipocyte differentiation capacity compared to BM-MSCs, which further supports our findings that the iMSCs have an inferior adipogenic potential in comparison to ASCs (Hynes et al., 2014; Kang et al., 2015). Differences in adipogenic induction media may also contribute to the difference between the adipogenic potential of the human iMSC and ASCs in our study. The iMSCs were induced to differentiate using the StemPro Adipogenesis Differentiation Kit while the ASCs were induced using a previously published media by Flynn (2010). Since the StemPro Adipogenesis Differentiation Kit is proprietary, it is impossible to identify if differences in the adipogenic cocktails may also account for the differences in adipogenesis. However, one difference that can be identified is that ASC media is prepared fresh in contrast to the commercial kit where the stability of the active components may be a factor which, along with different media compositions, may have an impact on the efficiency of stem cell differentiation.
Our finding that PANX1 was dispensable in human MSCs differentiation was somewhat surprising since Panx1-knockout mice have been shown to have enhanced adipogenesis (Lee et al., 2018). In terms of cellular differences, murine and human ASCs have different protein expression profiles. For example, it is known that catecholamine-lipolysis occurs through the activation of β-adrenergic receptor (β-AR) subtypes, β1, β2 and β3 (Chusyd et al., 2016). While, humans and mice both express β1 and β2, but only mice express β3-ARs (Chusyd et al., 2016). Therefore, β3-AR agonists can initiate lipolysis in mice, but this phenomenon is not observed in humans in vitro or in vivo (Tavemier et al., 1996; Weyer et al., 1998). In another example, humans express α2-adrenergic receptors, which are also activated by catecholamines to inhibit lipolysis (Chusyd et al., 2016). Mice do not express α2-adrenergic receptors; therefore, catecholamines may have different effects on adipogenic lipolysis depending on the species (Langin, 2006). With regards to adipogenesis, LIM domain only 3 (LMO3) is a transcription cofactor that acts as a proadipogenic regulator in human ASCs and adipose tissue, but is absent in murine adipogenesis (Lindroos et al., 2013). Glucocorticoids, a core component of adipogenic media, activates LMO3 to interact with HSD11B1 to facilitate adipogenesis. Furthermore, inhibition of LMO3 in human ASCs resulted in decreased adipogenesis and lipid accumulation. However, glucocorticoid treatment in murine ASCs had no effect on LMO3 expression. Although glucocorticoids have been reported to induce adipogenesis in both mice and humans, there are differences in protein expression and regulatory mechanisms between the two species that may result in differences in adipogenesis.

As noted earlier, another possible explanation for the differences between the murine ASCs and the human iMSC results reported here is the source of stem cells being compared as they have been reported to exhibit distinct adipogenic potentials (Kang et al., 2015; Mohamed-Ahmed et al., 2018). Also Lee et al. (2018), studied adipogenesis in male murine ASCs, while we utilized female human ASCs raising the possibility that sex may play a role. In one study, the quantity of ASCs harvested from female mice was 2.9 times greater than that of male mice, and in another study, women were reported to have larger subcutaneous adipose tissue than when compared to men (Björntorp, 1991; Chusyd et al., 2016). In addition to differences between MSC source, species, and sex, we recognize that examining adipogenesis in vitro does not completely recapitulate the microenvironments
that occur \textit{in vivo}. For example, the loss of membrane-bound matrix metalloproteinase-14, which was found to inhibit adipogenesis \textit{in vivo}, had no effect on adipogenesis \textit{in vitro} 2 dimensional cultures (Chun \textit{et al.}, 2006). Thus, caution needs to be exercised as PANX1 may have a role in human adipogenesis which might only be revealed \textit{in vivo}.

Although PANX1 is ubiquitously found throughout the human body (Baranova \textit{et al.}, 2004), the specific role of this channel forming protein is only beginning to be understood. While Panx1 was shown to be negative regulator of adipogenesis in mice, here we used two sources of human mesenchymal stem cells to show that it is dispensable for \textit{in vitro} adipogenesis, even though PANX1 is found in these stem cell populations. As clinical approaches are designed to deal with the obesity epidemic, assuming that our findings translate to the \textit{in vivo} setting, we propose that PANX1 is not a key regulatory of adipogenesis. Finally, our results using a pharmacological inhibitor of gap junction channels suggests that connexins may play a critical role in human adipogenesis, but further investigations are needed to confirm its possible role.
2.5 References


Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R,


Chapter 3

3 General Discussion

Adipose tissue is the largest endocrine organ in the body, and serves multiple functions such as storing energy, maintaining metabolic homeostasis, regulating inflammation, and facilitating wound healing (Gesta et al., 2007; Ghaben & Scherer, 2019). Adipogenesis has been associated with pathologies such as obesity and metabolic syndrome, where dysfunctional adipogenesis can result in severe health risks (Spiegelman & Flier, 1996; Gesta et al., 2007). On the other hand, adipogenesis has been linked to tissue regeneration and vascularization, where mesenchymal stem cells (MSCs) and adipocytes have been used to treat acute burns and chronic non-healing ulcers (Marino et al., 2013; Hassan et al., 2014; Na et al., 2017). Understanding how adipogenesis occurs and what factors influence this process can prove to be useful in treating adipocyte pathologies and improving adipose tissue-based treatments. Based on a previous study, where pannexin 1 was found to inhibit adipogenesis in mice (Lee et al., 2018), pannexin 1 emerged as a potential target in the fight against obesity. In a novel approach, our study investigated the role of human pannexin 1 (PANX1) during in vitro adipogenesis using adipose derived stem cells (ASCs) and induced MSCs (iMSCs) derived from induced pluripotent stem cells (iPSCs). Here, our findings will be integrated with what is currently understood in the field of pannexins, adipogenesis, and MSCs, as well as address potential avenues for future research.

3.1 Possible Role of PANX1 in Late Stages of Adipogenesis

Although PANX1 appears to play a role in adipocyte functions such as insulin sensitivity and inflammation, our findings indicate that PANX1 is not necessary for human adipocyte differentiation in vitro (Silverman et al., 2009; Adamson et al., 2015). Adipogenesis can be broken down into two stages: the commitment stage, where MSCs become preadipocytes, and the differentiation stage, where the preadipocyte differentiate into mature adipocytes (Ghaben & Scherer, 2019). The transition from preadipocyte into mature adipocytes can be further divided into four stages: growth arrest, colonial expansion, early differentiation, and terminal differentiation (Gesta et al., 2007). After growth arrest,
extracellular ATP has no effect as the cells undergo adipogenesis (Omatsu-Kanbe et al., 2006). Therefore PANX1's function as an ATP release channel is not predicted to have much impact during early differentiation. It is possible that PANX1 is necessary for mature adipocyte function, and that the MSCs used in this study did not reach a point along the adipogenic lineage where PANX1 was necessary for adipocyte function.

3.2 MSCs from Different Sources are Not Identical

Due to their therapeutic potential, MSCs have proven to be a promising tool in regenerative medicine and have been the focal point of over 500 clinical trials (clinicaltrial.gov; Squillaro et al., 2016). MSCs were originally discovered in bone marrow, and hence the properties of bone marrow derived MSCs (BM-MSCs) have been studied extensively (Friedenstein et al., 1968; Strioga et al., 2012). Since then, MSCs have been collected from a variety of sources, including adipose tissue and iPSC sources (Wei et al., 2013; Kang et al., 2015). Each type of MSC has its advantages and disadvantages. BM-MSCs are isolated from bone marrow through an invasive surgery with high risk of infection (Mohamed-Ahmed et al., 2018). BM-MSCs prefer to differentiate towards osteogenic and chondrogenic lineages, and are less inclined to undergo adipogenesis (Mohamed-Ahmed et al., 2018). ASCs, on the other hand, have a greater preference for adipogenic lineages, rather than osteogenesis or chondrogenesis, making them ideal candidates for adipose-related surgeries (Mohamed-Ahmed et al., 2018). Furthermore, ASCs are preferred over BM-MSCs, since adipose tissue is routinely collected during fat reduction surgeries (Kim & Heo, 2014). There is also a higher yield of ASCs per gram of fat when compared to bone marrow tissue (Hass et al., 2011). To put this into perspective, only 0.001 to 0.01% of the total cells in a sample of bone marrow are MSCs, where as in an equivalent amount of adipose tissue, about 5% of the cells are MSCs (Hass et al., 2011). Finally, iMSCs are ideal because iPSCs are capable of infinite renewal, and can offer an unlimited supply of MSCs (Hafner & Dani, 2014; Kang et al., 2015). However, iPSCs are genetically reprogrammed somatic stem cells, and these genetic modification may have an impact on the MSC characteristics (Hynes et al., 2014; Kang et al., 2015). Previous studies have shown that iMSCs share a similar potential for osteogenesis and chondrogenesis as BM-MSCs; however, iMSCs have a lower adipogenic potential when compared to BM-MSCs (Kang
et al., 2015). A possible explanation for the variabilities between the MSCs from different tissue sources is that the tissue-specific cellular microenvironments may have a profound and lingering influence on the cells’ adipogenic characteristics after isolation (Lowe et al., 2011). In the future, detailed characterization of MSCs from different sources are necessary to distinguish the effects of tissue origin on MSC potential.

3.3 Limitations and Future Directions

Our study is the first to focus on the role of PANX1 in human adipogenesis in vitro. Here, we demonstrated that PANX1 was not required for differentiation from iPSCs into iMSCs, nor further differentiation along the adipogenic lineage. Furthermore, the differential response to pharmacological inhibitors indicate that iMSCs and ASCs should be treated as somewhat different cell types. In short, our findings suggest that PANX1 is not necessary for human adipogenesis in vitro.

Within our study, we faced limitations such as studying only one source of iMSCs, only utilizing female ASCs from patients with a high body mass index (BMI), high patient to patient variability, and the lack of drugs that can specifically block PANX1. Because of these limitations, open questions remain about the role of PANX1 in human adipogenesis. One of these questions is whether our key findings apply to both male and female cohorts of all ethnic background and ages, as one of the limitations in our study was that all the cells were isolated from female donors. In fact, the cells from the iMSC studies were derived from a single female donor. For the ASC studies, the adipose tissue was donated by overweight or obese females, all with a BMI >25, who were all over the age of 23 (World Health Organization, 2000). Previous studies have reported that age, sex, and BMI can have an impact on adipogenesis (van Harmelen et al., 2003; Ogawa et al., 2004; Karastergiou & Fried, 2017). Furthermore, because of the patients’ age, our findings reflect adult adipogenesis, and so we cannot exclude the possibility that PANX1 plays a role during early childhood adipocyte development. To confirm the generality of our findings, additional samples are required from patients with a broader BMI and age range, as well as from both sexes.
Carbenoxolone and probenecid were used to block PANX1 channel function; however, these drugs are non-specific. Carbenoxolone is a potent inhibitor of gap junctions, and probenecid is clinically used to inhibit organic anion transporters (Pinder et al., 1976; Silverman et al., 2008, 2009). It is unfortunate that better drugs are not available to specifically block PANX1 channels with no off-target effects. Although previous studies have shown that 1 mM probenecid inhibits human PANX1 channels in skeletal myoblast cells and other cell types (Silverman et al., 2008; Langlois et al., 2014), we have no definitive evidence that 1 mM probenecid and 50 μM carbenoxolone inhibits PANX1 in iMSCs and ASCs. Further studies using ATP release assays (Shao et al., 2016) and electrophysiological assessment of PANX1 channel activity (Locovei et al., 2006; Silverman et al., 2008) should be performed to confirm that PANX1 channels in iMSCs and ASCs are indeed being inhibited by probenecid and carbenoxolone.

Carbenoxolone was able to inhibit adipogenesis in both the iMSCs and ASCs. Since it is a non-specific drug, there is a possibility that carbenoxolone inhibited adipogenesis by blocking other proteins (Pinder et al., 1976; Sano et al., 2012). Carbenoxolone is primarily known as a potent gap junction inhibitor, therefore, it is worth investigating whether carbenoxolone is inhibiting adipogenesis by blocking connexin channels. Since connexin 43 (Cx43) is the most abundant connexin expressed in adipose tissue (Burke et al., 2014; Zhu et al., 2016), we can begin with the assumption that carbenoxolone is inhibiting Cx43. Therefore, Cx43-ablated MSCs can be generated and induced to differentiate. If the cells are able to reach later stages of adipogenesis, then carbenoxolone is inhibiting adipogenesis by blocking a protein other than Cx43. It is noteworthy to mention that carbenoxolone inhibits hydroxysteroid 11-beta dehydrogenase 1 (HSD11b1), which can be activated by glucocorticoids to initiate adipogenesis, and so it is another promising target to probe about the mechanism behind carbenoxolone inhibiting adipogenesis in MSCs (Sano et al., 2012). Elucidating the mechanisms that carbenoxolone uses to repress adipogenesis can help to understand what factors are necessary for adipocyte differentiation in humans.

On the other hand, probenecid was only able to inhibit later stages of adipogenesis in the iMSCs, but had no effect on adipogenesis in the ASCs. This was surprising since we believed that probenecid would have the same effect on MSCs, regardless of origin. This
leads to another open question, what is the difference between iMSCs and ASCs that can explain the different responses to probenecid during adipogenesis? It is possible that the different origins and/or genetic reprogramming of iPSCs may influence the proteomes of iMSCs and ASCs, as it has been reported that the iPSCs may retain epigenetic memory from their somatic cell type, which causes differential gene expression and differentiation capacity (Polo et al., 2010). To further study the effects of origin on differentiation capacity, different types of stromal cells, including ASCs, can be reprogrammed into iPSCs, and then further into iMSCs. These iMSC clones, as well as unedited ASCs, can then be induced along the adipogenic lineage, and analyzed to determine if the proteomics and adipogenic capacity differ between these iMSC clones and unedited ASCs. If so, we can conclude whether cell origin or iPSC genetic reprogramming is responsible for the differences in protein levels.

In terms of ASC differentiation, one of the differences between our study and the study by Lee et al. (2018) is the length of adipogenic induction. In the study by Lee et al. (2018), after fourteen days of mouse adipogenic induction, murine pannexin 1 (Panx1) ablated ASCs had increased lipid content, glycerol-3-phosphate dehydrogenase (GPDH) activity, and secretion of adiponectin and leptin, indicating that Panx1-ablated ASCs had increased adipocyte functionality when compared to the wild type ASCs. On the other hand, our study induced human ASCs toward the adipogenic lineage for only seven days, and there were no significant differences in adipogenic markers between the drug-treated ASCs and the untreated ASCs. Although the drugs may be the reason why there was no significant difference, another possible factor is the shorter differentiation period, which may not have offered an opportunity for PANX1 to play its role in adipogenesis. The reason why we did not induce the human cells for fourteen days is because lipid droplet formation causes the ASCs to lift due to the change in cell density, hence these cells become difficult to study. If the human ASCs were effectively cultured for a longer period, it is possible that the differences in adipogenic markers between the drug-treated and control ASCs would have become significant.

It is also important to note that three dimensional (3D) cultures have been developed to study adipogenesis in a more physiologically relevant model (Emont et al., 2015). For
instance, the loss of matrix metallopeptidase 14 inhibited adipogenesis in vivo, and while this finding has been replicated with 3D cultures, it was not observed when the experiment was performed using two dimensional (2D) cultures (Chun et al., 2006; Lowe et al., 2011). Therefore, it is possible that PANX1 plays a role in adipogenesis in vivo, and that this would only be observed in vitro by using 3D cultures. Ideally, using a 3D culture will help to give us a better idea of how PANX1 influences adipogenesis in vivo. However, there are many reported challenges in designing 3D cultures: constructing proper distribution systems for oxygen, carbon dioxide, nutrients, and waste throughout the culture, the tissue-tissue interfaces, as well as controlling for other microenvironmental factors which are observed in vivo (Duval et al., 2017). On top of these challenges, a standard 3D culturing system has yet to be implemented to maintain consistency within the field (Duval et al., 2017). Although the 3D cultures offer a more physiologically relevant model, it requires more time and funding to perfect these systems.

Going forward it might be useful to use clustered regularly interspaced short palindromic repeat associated caspase 9 (CRISPR/Cas9) approaches to develop PANX1-ablated ASCs and determine whether they can differentiate along the adipogenic lineage. However, this would be challenging due to the fact that ASCs are viable as stem cells for only a limited number of passages before they begin to senesce (Yang et al., 2018). Furthermore, it is clinically recommended to use MSCs between passage 3 to 5, therefore it is ideal to study ASCs at passage 5 or lower (Jiang et al., 2017). Alternatively, one might consider using short hairpin RNA approaches to knockdown PANX1 and assess adipogenesis (Locovei et al., 2006). This would generate a second source of PANX1-attenuated MSCs to study adipogenesis, which would provide another avenue to validate our findings.

From this study, we found that, although iMSCs and ASCs meet the minimum requirements to be classified as MSCs, these cells have distinct properties, exemplified in the different responses to probenecid during adipogenesis. In addition, PANX1 does not appear to be necessary for iPSC differentiation into iMSCs, nor further differentiation along the adipogenic lineage. Overall, future studies are required to elucidate the role of PANX1 during adipogenesis in the human context.
3.4 References


Pinder RM, Brogden RN, Sawyer PR, Speight TM, Spencer R & Avery GS (1976).


Appendix 1: Western Human Research Ethics Board (WREB) Approval

Date: 30 January 2019
To: Dale Laird

Project ID: 104190

Study Title: Connexin genes linked to disease
Application Type: HSERB Amendment Form
Review Type: Delegated

Meeting Date / Full Board Reporting Date: 12 Feb 2019
Date Approval Issued: 30 Jun 2019
REB Approval Expiry Date: 07 Feb 2020

Dear Dale Laird,

The Western University Health Sciences Research Ethics Board (HSREB) has reviewed and approved the WREM application form for the amendment, as of the date noted above.

Documents Approved:

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<td>Consent Form</td>
<td>28 Jan 2019</td>
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<tr>
<td>Laird-104190</td>
<td>Protocol</td>
<td>28 Jan 2019</td>
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Documents Acknowledged:

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REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement Ethical Conduct for Research Involving Humans (TCPN 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPPA, 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Patricia Sergeant, Ethics Officer (ext. 839590) on behalf of Dr. Philip Jones, HSREB Vice-Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).
Appendix 2: Animal Use Protocol (AUP)

AUP Number: 2019-009
PI Name: Laird, Dale W
AUP Title: The role of connexin and pannexin channels in health and disease
Approval Date: 05/01/2019

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2019-009:1: entitled "The role of connexin and pannexin channels in health and disease has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:
1. Animals used in this research project will be cared for in alignment with:
   a. Western's Senate MAPPs 7.12, 7.10, and 7.15
   b. University Council on Animal Care Policies and related Animal Care Committee procedures

2. As per UCAC's Animal Use Protocols Policy,
   a. this AUP accurately represents intended animal use;
   b. external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c. any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d. AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
   e. http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3. As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a. be made familiar with and have direct access to this AUP;
   b. complete all required CCAC mandatory training (training@uwo.ca); and
c. be overseen by me to ensure appropriate care and use of animals.

4. As per MAPP 7.15,
   a. Practice will align with approved AUP elements;
   b. Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c. UCAC policies and related ACC procedures will be followed, including but not limited to:
      i. Research Animal Procurement
      ii. Animal Care and Use Records
      iii. Sick Animal Response
      iv. Continuing Care Visits

5. As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

Dr. Timothy Regnault,
Animal Care Committee Chair

The University of Western Ontario
Animal Care Committee / University Council on Animal Care
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Curriculum Vitae

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Education

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Master of Science (MSc)
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Honors Specialization in Physiology and Pharmacology with Science Internship
2012 – 2017

Honors and Awards

Western Graduate Research Scholarship
2017 – 2019

NSERC Canada Graduate Scholarships-Master's Program
2018 – 2019

Dean’s Honour List

Laurene Paterson Estate Scholarship
2013

Western Scholarship of Excellence
2012

Related Work Experience

4th year Research Student
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2016-2017

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Course Coordinator: Dr. Tom Stavraky
Western University
2016 – 2019

Community Involvement
Retiring with Strong Minds
Advertisement Coordinator
London, Ontario
2018 – present
Let’s Talk Stem Cell Volunteer
Let’s Talk Science
London, Ontario
2017 – 2019

Poster Presentations:


