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Panx1 and Panx3 regulate adipocyte development and fat accumulation in vivo

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Abstract

The obesity epidemic is a growing concern due to its various comorbidities and associated risk factors. Pannexins 1 and 3 (Panx1 and Panx3), are members of a family of channel-forming glycoproteins that have been reported to be important in paracrine signaling and development. Panx1 and Panx3 are homologous and are regulated in many different cell types, mediating cell proliferation and differentiation. We have shown that Panx1 and Panx3 are expressed in adipocytes and adipose-derived stromal cells (ASCs) throughout the process of differentiation. Mice globally lacking Panx1 (Panx1 KO) have significantly greater total fat mass compared to wildtype (WT) mice under a normal diet. Comparatively, mice globally lacking Panx3 (Panx3 KO) have significantly less total fat mass compared to WT mice on the same diet. Multipotent ASCs isolated from both Panx1 KO and Panx3 KO mice proliferate less than WT cells. ASCs lacking Panx1 also have increased adipogenic differentiation and fat accumulation capacity compared to WTs. Despite the Panx1 KO mice having greater fat content, when placed on a high fat diet, they exhibit no differences in weight gain or metabolic parameters compared to WT mice. When placed in metabolic cages, Panx1 KO mice display significantly increased total activity, ambulatory activity, and sleep significantly less than WT mice. In contrast, Panx3 KO mice placed on a high fat diet exhibit a slight reduction in weight gain, however show no significant differences when placed in metabolic cages on regular diets. We conclude that both Panx1 and Panx3 are regulated throughout adipocyte proliferation and differentiation at early stages in the adipogenic lineage and can regulate fat accumulation *in vivo*, potentially playing contrasting roles.

Keywords: Pannexins, Panx1, Panx3, obesity, adipocyte, adipose-derived stromal cells, fat accumulation, adipogenesis

Co-Authorship Statement

All cell culture and primary cell experiments were completed by Vanessa R. Lee. Cody Brown and Kevin Robb assisted with the initial isolation of adipose-derived stromal cells and provided training in adipogenic assays in the laboratory of Dr. Lauren Flynn. Cindy Sawyez assisted with the processing of ELISAs from serum samples of high fat diet mice. Mouse work including breeding and animal husbandry was accomplished with the major assistance of Kevin Barr and metabolic caging was completed with the assistance of Dr. Robert Gros.

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List of Abbreviations

ASC	Adipose-derived stromal cell
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass composition
C/EBP-α	CCAAT-enhancer binding protein-α
DMEM	Dulbecco's modified Eagle's medium
GPDH	Glycerol-3-Phosphate Dehydrogenase
Het	Heterozygous
HFD	High fat diet
KO	Knockout
Panx	Pannexin
PPAR-γ	Peroxisome proliferator-activated receptor-γ
SEM	Standard error of the mean
WAT	White adipose tissue
WT	Wildtype

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Chapter 1 - Introduction

1.1 Obesity

The obesity epidemic is the leading cause of global deaths and according to the World Health Organization approximately 10% of the world's population is obese (as of 2014)[1]. Obesity accounts for a multitude of comorbidities such as: cardiovascular disease, type II diabetes, and cancer[2]. Body mass composition (BMI) is the current standard for determining whether an individual falls under classes of: normal (less than 25), overweight (25.1-29.9), or obese (greater than 30) measured by weight over height squared (kg/m^2)[3]. Currently, options to counteract obesity include caloric restriction, exercise and diet changes, pharmacological approaches, and in extreme cases, surgical intervention. At early onset, the comorbidities associated with obesity can be dramatically reduced by a 10% permanent weight reduction[4] and cardiovascular function can be restored in addition to the reversion of type II diabetes[5]. Reversal of obesity is not a simple task, as it requires dramatic lifestyle changes which are difficult with today's high fat and sugar fast food, and sedentary lifestyles. Maintaining weight loss is often difficult, thus pharmacological intervention may come into play using drugs that target adrenergic receptors aimed at suppressing appetite such as: phentermine, diethylpropion, phendimetrazine, benzphetamine[6], or drugs that inhibit lipases causing malabsorption of triglyceride, such as orlistat[6]. The problem with drug treatments is that most are designed for short-term usage (less than 12 weeks) in conjunction with healthy living. Slight weight loss in combination with drug usage tends to be maintained for approximately 1 year, followed by recurrent weight gain and the underlying comorbidities[6]. Therefore, the usage of drugs in the prevention of obesity is not a viable treatment long-term, but has potential for further development. When pharmacological usage becomes insufficient, surgical intervention normally

follows. Bariatric surgery is the most common type of intervention and results in a greater long-term reduction in weight compared to non-surgical options[7]. It also helps to reduce some of the associated comorbidities and reduces mortality[7]. However, being a surgical intervention, in patients with poor health it is not always a viable alternative. As such, there is a need to better understand the mechanisms and manifestation of obesity to generate better therapeutic alternatives.

1.2 Leptin Resistance

Leptin resistance is one of many challenges faced in obesity. Leptin is a 16 kDa adipokine which is released from white adipose tissue[8]. It regulates food intake, energy expenditure, and immune response[9–11]. Leptin signaling is mediated via the leptin receptor (OB-R) in the serum, which is linked to the gp130 subunit found in the IL-6 receptor-complex, a pro-inflammatory cytokine[12]. There have not been many reports of mutations in the leptin gene, however of those that are known, non-functional mutants lead to morbid obesity[13]. At the onset of obesity, leptin resistance is characterized by the reduction of leptin effects on food intake and energy expenditure. Consequently, the amount of leptin produced is increased in order to compensate[14,15]. *In vivo* studies show that mice lacking leptin become obese and exhibit hyperphagia, exhibited by irregular food consumption and hyperlipidemia which is the irregular increase in circulating free fatty acids and low-density lipoproteins[9]. In obese individuals, the number of OB-R is significantly reduced which could indicate reduced leptin sensitivity. Contrastingly, severely underweight individuals show significantly increased OB-R concentration. This would suggest that the OB-R are dynamically regulated dependent on energy balance and leptin serum concentration[14,16,17]. Blood serum leptin and adiponectin (another hormone regulating lipid metabolism) are some of the markers commonly tested in diet-induced obesity studies.

1.3 Insulin Resistance

Insulin is a polypeptide secreted from islet cells in the pancreas in response to glucose[18]. Insulin resistance is a hallmark of obesity and eventually leads to type II diabetes. Obesity is highly linked with adipokine release and inflammation. Leptin and adiponectin, seem to play a regulatory role in reducing triglyceride synthesis and stimulate fatty acid oxidation in the liver and skeletal muscle[19]. As mentioned previously, at the onset of obesity, leptin levels rise in order to compensate for increased lipids and glucose in the blood stream and thus loses the ability to modulate fatty acid oxidation. The result is a compensatory increase in insulin, in an attempt to maintain normal blood glucose levels, however long-term insulin increases results in insulin-resistance[20]. To make matters worse, obesity dysregulates the conversion of fatty acids into carbohydrates for energy utilization[21]. Insulin-resistance associated with obesity that has a long-term progression could result in type II diabetes by β-cell failure. Under normal conditions, pancreatic islet β-cells are activated by glucose, which results in an increase in ATP and the closing of ATP-sensitive K^+ channels. This causes depolarization of the membrane which activates Ca^{2+}-gate channels to exocytose insulin[22]. The basis of β-cell failure or overload, is that when chronically exposed to an excess of both nutrients and fatty acids, it can ultimately lead to β-cell impairment and trigger cell death[23].

In adipose tissue, glucose transport is mediated by the GLUT4 glucose transporter which is stimulated by insulin released by the pancreas[24]. In normal circumstances, insulin stimulates glucose uptake via the GLUT4 receptor and lipolysis occurs releasing free fatty acids into the bloodstream. During insulin resistance, increased circulating fatty acids promote the generation of very low density lipoprotein (VLDL) from hepatocytes[25], and as such, lipoprotein lipase, an enzyme responsible for breaking down triglycerides in lipoproteins becomes inhibited. This causes an increase in circulating free fatty acids which feedbacks and worsen the effect of insulin

resistance[26]. As mentioned above, the obese adipose tissue becomes an endocrine organ secreting inflammatory hormones and factors. Il-6 and TNFα have been shown to dysregulate or block insulin signaling and lipolysis[27]. Finally, one study suggests that adipocytes from diabetic and insulin resistant individuals have impaired GLUT4 translocation and further dysregulation in intracellular insulin receptor signaling[24]. Insulin resistance is not only localized to the adipose tissue, but also found in skeletal muscle[28], liver [27,24], brain[29], kidney[30], bone[31], and many other tissues.

As outlined above, obesity generates a complex collective of metabolically related pathologies which is now a growing problem worldwide. Therefore, there is an immediate need for research in the progression of the disease, but also on the basic mechanisms that regulate adipose tissue formation and fat accumulation.

1.4 Adipose tissue

In newborns, 16% of body weight is attributed to adipose tissue[32]. Fetal lipid metabolism and fat accumulation occurs within the first two-thirds of gestation, where the mother develops hyperphagia, increased appetite, causing an increase in overall fat accumulation[33]. During the last trimester of pregnancy, increased lipolysis occurs and glucose, amino acids, glycerol, and free fatty acids[34,35]cross the placenta and lipogenesis occurs in the fetus[36]. Fetal lipogenesis is fueled mainly by glucose uptake transferred from the placenta and converted into fat[36]. By week 30 of pregnancy, the fetus accumulates large amounts of fat contributing to daily weight gain[37].

The main physiological functions of adipose tissue are insulation, shock absorption, and energy storage[38]. However, in obesity, excessive fat accumulation occurs impeding normal function of tissues and organs. Adipose tissue is composed of adipocytes, which are unique cells that have the capacity to store large amounts of energy in the form of fat. Adipocytes have long lifespans, and approximately 10% of the body's adipocytes are regenerated annually[39]. Adipose

tissue is highly vascularized and adipocytes come in contact with at least one capillary, allowing them to very effectively uptake and release circulating fatty acids[40]. In order to harness energy from adipocytes, the cell membrane is covered in proteins and metabolic enzymes which respond to hormonal stimulation triggering lipolysis, or the breakdown of triglycerides into free fatty acid for fuel[41]. Cyclic AMP (cAMP) stimulates protein kinase A (PKA) found in adipocytes, which plays a vital role in lipolysis[42]. On the other hand, lipogenesis occurs in the mitochondria of adipocytes. During adipocyte differentiation, adipocytes undergo increased mitochondrial expansion along with increased β-oxidation capacity[43]. In order for triglyceride synthesis to occur, fatty acids are activated and form fatty acyl-CoAs, which enter into the mitochondria via carnitine palmitoyl transferase-1 (CPT-1) and undergo β-oxidation[40].

The most common obesity associated adipose tissue, is white adipose tissue (WAT) found in many depots and areas of the body[44]. In obesity, adipocytes behave differently where they are either hyperplasic and increase in cell number, or hypertrophic where they increase in volume[45]. Hypertrophy occurs at the early onset of obesity progression in order for cells to accommodate excess volume, however once filled to capacity, hyperplasia occurs and is more characteristic of severe obesity[46]. Unfortunately, hyperplasia of the adipocytes contributes to the increasing difficulty in losing weight, as these cells cannot be destroyed and continue to be regenerated[47]. It should be noted however that adipose depots located throughout the body such as white adipose tissue (WAT) that can be subcutaneous (hypodermal, inguinal) or visceral (perigonadal, around organs); and brown adipose tissue (BAT) behave and respond differently under normal condition and during onset of obesity, showing differences in fat accumulation and adipocyte regulation[44]. The main function of BAT is heat generation and non-shivering thermogenesis[48]. BAT is most commonly found in newborns and typically disappears early on (about one year after birth)[49]. In

humans it is generally found in the dorsal regions, and in rodents it is found in the inter-scapular regions, thymus, thorax and abdomen[50].

Adipose tissue in obese individuals (mostly WAT) is known to act as an endocrine organ, creating localized and systemic inflammation[51]. There are many different cytokines and transcription factors upregulated or secreted by obese adipose tissue such as: leptin, resistin, adiponectin, IL-6, IL-1β, TNF-α, MCP-1, VEGF, and many others[52]. All of which contribute to cardiovascular disease, fatty liver disease, hypertension, type II diabetes and insulin resistance[53], to name a few. Additionally, as the number of cells increase and cell volumes expand it causes a localized area of hypoxia resulting in adipocyte cell death and subsequent inflammation[51]. This causes an upregulation of VEGF and angiogenesis to accommodate for increased lipid volume and adipocyte number[54]. Due to this localized inflammation, and the cross-talk between adipocytes and inflammatory cells, many different signaling pathways become activated in both adipocytes and macrophages. Hypoxia-inducible factor-1α (HIF-1α) and NF-κβ mediate the inflammatory response found within the adipocytes[55,56]. These factors recruit and activate other inflammatory molecules resulting in induced cell death and lipolysis[52]. Because of the hypoxic environment, adipose tissue releases chemokines or adipokines, which attract inflammatory macrophages[57,58] . This entire process becomes a positive feedback loop where there is hypoxia induced macrophage infiltration, but also infiltration due to apoptosis, leading to chronic inflammation[59]. It has been reported that greater than 90% of infiltrating pro-inflammatory M1 macrophages surround dead adipocytes[60] in visceral adipose tissue in a crown-like fashion and it is hypothesized that these macrophages are responsible for the progression of insulin resistance and various other comorbidities[61]. Therefore, there is a need for a better understanding of the

mechanisms that regulate adipose tissue, and the potential cross talk between adipocytes and immune cells in obesity.

1.5 Adipose-derived Stromal Cells

Adipose-derived stromal cells (ASCs) are multipotent adipocyte precursor cells[62]. They are found in the stromal vascular fraction of adipose tissue, which is composed of a heterogeneous population of mature adipocytes, fibroblasts, immune cells, and ASCs[63]. ASCs can be isolated by various cellular digestion, separation and filtration steps. Human ASCs can be isolated from surgical reductions or lipo-aspirates making them accessible and an abundant resource[64,65]. ASC specific markers include CD90, CD29, CD73, CD44, CD13, and CD105[66]. Despite their heterogeneity in culture, it has been shown that with each subsequent passage (passage 2 or 3) the selected population becomes increasingly homogeneous expressing all multipotent ASC markers due to selective tissue culture plastic cell adherence and growth in fetal bovine serum[67,68].

ASCs are becoming widely used based on their abundance and ease of access, but also because of their multipotent ability to differentiate down many different lineages: adipocyte, chondrocyte, osteoblasts, myocytes, and peripheral neurons[69,70]. ASCs exhibit a fibroblast-like morphology in culture, and can grow in its undifferentiated state on tissue culture plastic[71]. It is well established in the literature that in order for ASCs to differentiate efficiently, they undergo cell cycle arrest between the G_1 and G_0 cell cycle phases, due to cell confluence[72]. At this point, these cells are considered pre-adipocytes as they are in a state of commitment towards the adipogenic lineage. This state causes them to be less proliferative and more readily equipped to convert carbohydrates into lipids. Subsequently, clonal expansion occurs allowing for increased expression[72] of various transcription factors and signaling molecules such as the key regulators:

Peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT-enhancer binding protein-α (C/EBP-α), maintain the differentiative state[73] and growth arrest[74].

1.5.1 Adipocyte Development and Differentiation

The process of adipocyte development is a tightly regulated and complex process involving many hormones and signal transduction pathways. During adipogenesis, adipocyte precursor cells undergo dramatic transformations. Differentiating adipocytes go from fibroblast-like to a spherical morphology causing changes to cytoskeletal elements and the extracellular matrix (ECM)[75]. These changes are essential for the accommodation of large lipid volumes. Adipocyte precursor cells secrete many different factors to promote angiogenesis and the formation of blood vessels such as: vascular endothelial growth factor (VEGF), angiopoietin-like 4, fibroblast growth factor 2, and matrix metalloproteinases (MMPs)[76]. These changes occur in order for preparation of adipose tissue development and expansion[77]. In the early events of differentiation, both actin and tubulin expression are reduced[78], followed by the conversion of collagen gene expression from type I and III to the secretion of type IV collagen in addition to increases in entactin and laminin[79]. Production of soluble and cell-associated chondroitin sulfate proteoglycan-1 is also increased, which causes cells to appear more viscous[80]. Pericellular fibronectin and synthesis of fibronectin are significantly reduced during differentiation[81], followed by a massive reduction in pre-adipocyte factor-1 (pref-1) gene expression[82]. Once fat accumulation has occurred and the cells fill with lipids, organelles and the nuclei are pushed to the edge of the cell membrane making the adipocytes unique and characteristic.

Adipocyte fat accumulation has been highly studied at the transcriptional level. As mentioned above, PPAR-γ and C/EBP-α are two of the most frequently studied transcription factors involved in adipogenesis, and are considered essential. PPAR-γ has been termed the master regulator of adipogenesis and is necessary for differentiation[38]. *In vitro*, addition of

PPAR-γ can successfully differentiate fibroblasts into adipocytes[73]. There are two PPAR-γ isoforms: PPAR-γ1 and PPAR-γ2, which are both induced during adipogenesis but can also be found in tissue types other than adipocytes (colonic epithelium and macrophages)[83]. *In vitro*, when PPAR-γ is inhibited, addition of PPAR-γ2 has the ability to rescue adipogenesis whereas PPAR-γ1 cannot[83]. The absence of PPAR-γ2 in mice results in reduced adipose tissue mass and impaired adipogenesis[84]. PPAR-γ is not only essential for the induction of adipogenesis, it also plays a role in maintaining the differentiated state[85]. Finally, PPAR-γ promote the activation of several other transcription factors which trigger further adipogenic signaling cascades.

One family of the transcription factors activated by PPAR-γ are the CCAAT-enhancer binding proteins, or C/EBPs which include: C/EBP-α, C/EBP-β, C/EBP- γ, C/EBP- δ, and CHOP (which is homologous to the C/EBPs). The activation of C/EBP-β is initiated by PPAR-γ which then leads to the induction of both C/EBP-α and C/EBP- δ. In pre-adipocyte cell lines, C/EBP- β is essential for adipogenesis, however its presence is less highlighted *in vivo*[86]. Mice lacking C/EBP-α show severe hypoglycemia and lack all depots of adipose tissue with the exception of mammary adipose tissue, they also show delayed BAT development[87]. Even though this family of transcription factors have profound effects on adipogenesis, they cannot function in the absence of PPAR-γ[88].

PPAR-γ also activates the Krüppel-like factors (KLF) which have many functions specifically regulating apoptosis, proliferation, and differentiation of adipocytes[89,90] Most reports emphasize the role of PPAR-γ, C/EBPs and KLFs in adipocyte development, as they have been heavily documented. However, there are now over 100 other transcription factors that are known to be expressed in adipocytes and the list continues to grow.

Adipogenesis is highly regulated as mentioned above at the transcriptional level, but also at the enzymatic level. The enzyme Glycerol-3-Phosphate Dehydrogenase (GPDH) is increased by approximately two orders of magnitude from the early stages of adipogenesis until full adipocyte maturity, as early as 24 hours after induction in 3T3-L1 pre-adipocyte cells[91]. GPDH is an enzyme essential in the catalysis of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate, and causes the oxidation of NADH to NAD^+. After oxidation of NADH, glycerol 3-phosphate is de-phosphorylated into glycerol, the starting material of all lipids[72,91]. An assay was developed which quantitatively measures GPDH activity by assessing the oxidation of NADH by absorption at 340nm[92,93]. This assay is beneficial in assessing lipid biosynthesis and is used for further understanding of adipocyte differentiation capacity described in this thesis. GPDH is essential for the differentiation of cells, however does not contribute to fat accumulation. GPDH overexpression in mice containing additional 25 copies of the GPDH transgene showed dysregulated mass of both brown and white fat depots in young mice, where brown fat was much larger and white adipose depots were significantly reduced, suggesting a developmental role for GPDH[94].

At the hormonal level, leptin, adiponectin and many others are released in mature adipocytes[95]. It is primarily insulin however, that is a key factor influencing glucose uptake[96]. Insulin binds to the insulin receptor at the cell surface of the adipocyte, which activates tyrosine kinase activity causing a signaling cascade of phosphorylation[95,96]. Growth hormone has also been shown to have an opposite mode of action and actually reduces glucose uptake in adipose tissue by interfering with insulin signaling, thus decreasing insulin sensitivity[97]. Another hormone of note is acylation stimulating protein (ASP), which mediates triglyceride synthesis. It is produced by adipose tissue and helps to stimulate fat or lipid accumulation in adipocytes[98].

One of the most commonly used *in vitro* models for assessing adipogenesis and differentiation is the 3T3-L1 pre-adipocyte cell line, an immortal cell line committed to the adipocyte lineage[99] that can be induced to differentiate into mature adipocytes. These 3T3-L1 cells as well as mouse primary adipocytes, have recently been reported to express Pannexin 1 (Panx1), a member of the pannexin family of channel forming proteins, which subsequently has been shown to affect glucose uptake and insulin sensitivity[100]. In the current study, we propose that members of this family of channel proteins may be novel regulators of adipogenic regulation in normal development and at the onset of obesity.

1.6 Pannexins

Pannexins (Panx) are a family of large pore channel forming glycoproteins discovered in 2000[101]. There are three members of the Panx family: Panx1, Panx2, and Panx3. Pannexin channels are glycosylated, have four transmembrane domains and both N and C-termini are cytoplasmic[102]. Synthesis of pannexins start in the endoplasmic reticulum, and in most cases will be N-glycosylated to either a high mannose form (Gly 1) in the ER or further processed into its complex form (Gly2) in the Golgi apparatus and trafficked to the cell membrane[102]. Pannexins can also exist in their unglycosylated species (Gly0), usually found intracellularly such as in dermal fibroblasts[103] and in the retina[104]. Panx subunits form channels by oligomerizing into hexamers[105] allowing for the passage of small molecules and ions (<1kDa) including ATP or glutamate[106]. It is possible for heteromeric Panx channels to form, composed of Panx1 and Panx2, also Panx1 and Panx3, but there is no evidence of Panx2 and 3 forming heteromeric channels[107]. Panx1 is the most well characterized protein of this family, and is ubiquitously found in mammalian tissues. Panx2 is the largest channel (~74 kDa) and the least characterized. It was originally thought to exist primarily in the central nervous system, however there has been recent reports of Panx2 expression in many different tissue types across the body[108]. Panx3 is

found mostly in bone[109], cartilage[110], skin[102], the inner ear[111] and testes[112]. In humans, Panx1 and Panx3 share sequence homology (41%)[113], with similar molecular weights (~48 kDa and ~45 kDa)[102]. The large pore pannexin channels participate in cellular communication through the extracellular environment via paracrine and autocrine signaling[114].

1.6.1 Pannexin1

Panx1 channels have been primarily characterized as ATP conduits[106,115], but their large pore size can allow passage of different ions and metabolites. Panx1 channels are typically closed, however there are multiple mechanisms and instances where Panx channels, specifically Panx1, can be stimulated to open. The first being, mechanical stimulation such as shear cell stress or stretching of the cells[102] or increased fluid shear force[116]. Increased extracellular potassium causes the hyperpolarization of the cell, which in turn opens Panx1 channels[117]. Panx1 channels are voltage gated, and under normal conditions have a negative resting potential, however at high voltage they can become opened[118]. Typically associated with apoptosis, cleavage of the C-terminal tail of Panx1 by caspase 3 or 7 can cause constitutive opening of the Panx1 channel[119,115]. It has also been shown that Panx1 can open via increased intracellular calcium[120]. Recently there is evidence that insulin is also an activator of Panx1 channels in pre-adipocytes[100].

Panx1 has been linked to pathological functions in disease[121,122]. In most cases with a few exceptions, the current body of knowledge shows that increased Panx1 expression is linked to disease states in melanoma, ischemia, hypertension, colitis, and diabetes[121]. Evidence for this has been shown in several cancers such as mouse melanoma cells[123], multiple myeloma cells[124], and human glioma cell line U87-MG[125] where Panx1 expression is higher than in normal cells. In ischemia, Panx1 trafficking and glycosylation is upregulated in response to cellular stress which causes ATP to be released from the channel and thereby causes fibroblast activation potentially

leading to early fibrosis[126]. Panx1 has been shown to be involved in blood pressure control, where Panx1 is expressed in vascular smooth muscle cells and can be stimulated by phenylephrine to release ATP and contribute to vascular constriction through purinergic signaling[127]. Panx1 has been shown to mediate enteric neuron cell death during colitis by activation of neuronal P2X7 receptors via ATP release, recruiting caspases, resulting in cell death[128]. It has been proposed that diabetes and its associated inflammation can lead to neurodegeneration in post-ischemic models. Panx1 is activated by glutamate and ATP released by Aβ-treated microglia, resulting in the activation of NMDA/P2X receptors causing neuronal death[129].

1.6.2 Pannexin 1 Expression in Mammalian Tissues

As mentioned above, Panx1 is the most well-characterized member of the family, and has been shown to be expressed in many tissues and organs[102]. Panx1 is expressed in the brain, heart, skin, thymus, skeletal muscle, testis, ovary, placenta, prostate, liver, lung, small intestine, pancreas, spleen, colon, prostate, and in blood cells[113]. Focusing on metabolically related tissues involved in metabolic disease such as adipose tissue[100], liver [113] small intestine[130], and heart [113], it has been reported that Panx1 can also play an inflammatory role in these organs. Under normal physiological conditions, Panx1 is expressed in hepatocytes[131] and Kupffer cells[132]. They seem to have a pathophysiological role in the liver during lipoapoptosis (apoptosis caused by excess fatty acid exposure), where ATP is released in liver cells in large quantity, potentially mediated by Panx1 channel opening linking it to hepatic inflammation[133]. Additionally, it has been postulated that Panx1 is involved in liver steatosis resulting from non-alcoholic fatty liver disease (NAFLD) where fat accumulation exceeds 5% of hepatocytes[134] relying in part on the recruitment of the inflammasome complex shown to be recruited by Panx1[135].

Another area of metabolic relevance is the small intestine, the main site of lipid absorption. Panx1 is expressed in the small intestine[113,130], and its function in normal physiology is not well-understood. However there have been a few reports of Panx1 in Crohn's and colitis, and similar to the liver seems to be associated with inflammation. It was shown that in ulcerative colitis and Crohn's disease, Panx1 mRNA expression was significantly reduced compared to normal human colon, suggesting a role for Panx1 regulation throughout disease progression[130].

Panx1 has been observed in the heart[113], although Panx1 expression in cardiac myocytes is low and is mostly found in its Gly0 or unglycosylated state[136,126]. Similar to the other cases mentioned above, Panx1 expression is upregulated upon ischemic injury and stress[126]. This could suggest that Panx1 may be an early signal molecule leading to fibrosis or arrhythmias in ischemia.

In an ischemia-induced neurodegeneration model, by occluding the middle cerebral artery of Panx1/2 double knockout mice, there was a reduction in infarct size as these mice seemed to be neuroprotected[137]. Thus, indicating that both Panx1 and Panx2 have a function in ischemic brain damage. Additionally, Panx1 KO mice possess mechanisms of neuroprotection against seizures, as it has been shown that Panx1 is activated during epileptiform activity[138]. Based on behavioral analysis, after induction of seizures, Panx1 KO mice have significantly better behavioral outcome compared to WT mice[138]. In a different global (on an alternate Cre-promoter) Panx1 KO model[139], it has been established that the mice exhibit different behaviors compared to WT mice including greater anxiety[140] and greater motility[141]

Therefore in different tissues, Panx1 is clearly regulated and is commonly associated with inflammation or cell stress leading to the progression of disease.

1.6.3 Panx1 in Cell Development

Panx1 is not only linked to disease but is also involved in normal physiology, particularly in cellular regulation, as it is expressed early on in development in many tissues regulating proliferation and differentiation of many cell types[142]. For instance, overexpression of Panx1 in keratinocytes dysregulated keratinocyte differentiation[116]. *In vivo*, Panx1 expression is regulated in murine skin, where Panx1 is mostly found in the vital layers of embryonic and neonatal skin but expression is gradually reduced in aged or adult skin[116].

Panx1 is also regulated in skeletal myoblast proliferation and differentiation, with low Panx1 expression in undifferentiated cells which increases during proliferation and differentiation[143]. Overexpression of Panx1 significantly increases differentiation capacity of skeletal muscle myoblasts, while blocking Panx1 channels inhibits differentiation[143].The development of mammary glands in lactation are also in part regulated by Panx1. During lactation, mice lacking Panx1 have impaired alveolar development with reduced lumen area and significantly reduced cell proliferation[144].

Over the past decade there has been growing interest in the study of Panx1 in the brain and CNS. Panx1 is present in many areas of the brain[113] and is regulated throughout development[145]. The dynamically regulated expression pattern of Panx1 is consistent with other tissues where it is very highly expressed embryonically and declines towards maturity and adulthood[145]. One group reported that Panx1 is expressed in postnatal neural stem and progenitor cells, and by either inhibiting or overexpressing Panx1, they observe reductions or increases in cell proliferation[146].

Taken altogether, this collectively gives a strong argument for the role of Panx1 in proliferation and differentiation of many different cell types, which could feasibly include adipocytes and their progenitor cells.

1.6.4 Panx1 in Adipocytes

Currently there is only one report in the literature suggesting that Panx1 is expressed in adipocytes. Adamson et al., 2015 proposed a mechanism where insulin activates Panx1 channels causing the release of ATP which in turn results in a signaling cascade indirectly allowing the transport of glucose into the adipocytes. By using the pre-adipocyte 3T3-L1 cell line, it was shown that ATP is released from Panx1 channels after insulin or phenylephrine treatment, however not in carbenoxolone treated cells, which pharmacologically blocks Panx1 channels[147]. A key finding was the use of insulin to activate Panx1 channel opening, to release ATP, which indirectly facilitated the uptake of glucose into the adipocytes, possibly by an interaction with P2 receptors and GLUT-4, as proposed by the authors[100]. This group also explored the effects of knocking out Panx1 in mature adipocytes. A Panx1 adipose-specific knockout mouse was generated on an adiponectin doxycycline inducible promoter. Adiponectin is expressed only in terminally differentiated adipocytes, thus the knockout of Panx1 in this model occurred only in mature adipocytes and not precursor cells[148]. Body mass composition, body weight, metabolic activity, overall activity and both glucose and insulin tolerance were measured in the knockout mice fed on a 12 week high fat diet. No significant differences were observed, however there were some trends towards increased circulating blood glucose and increased insulin resistance in the mice, associated with reduced glucose uptake in adipocytes isolated from the knockout mice[100]. Finally, they assessed via gene array the mRNA expression of Panx1 in the adipose tissue of both lean and obese humans, and observed strong correlations of Panx1 expression with morbidly obese patients, along with those with increased fasting blood glucose levels and insulin resistance[100].

1.7 Pannexin 3

Panx3 shares similar sequence homology to Panx1 (41%)[113]. It is also expressed in a variety of different tissues such as: cartilage[110], bone[149], muscle[102], the inner ear[111], and testes[112].

Similar to Panx1, Panx3 regulates cell proliferation and differentiation in many cell types such as: osteoblasts[149], chondrocytes[110], keratinocytes[116], skeletal muscle myoblasts[143], and odontoblast[150]. In osteoblasts, Panx3 is a target for runt-related transcription factor (Runx2), key to bone formation. This group also identified Panx3 in intramembranous bones in mouse embryos, and in chondrocytes[149]. Additionally, Panx3 is regulated throughout chondrocyte differentiation via the regulation ATP and cAMP content[110]. Panx3 has been shown to play an essential role in cartilage and bone development of rodents, as lack of Panx3 dysregulated chondrocyte and osteoblast development[151].

In conjunction with Panx1, Panx3 has been found in keratinocytes, and also in mature skin[116]. Based on its expression throughout maturity, it has been suggested that Panx3 is also involved in keratinocyte differentiation[116]. Finally, Panx3 has been reported to be involved in the differentiation and proliferation of skeletal muscle myoblasts[143]. Panx3 is expressed in adult differentiated muscle tissue, and overexpression causes reduced proliferation, but induces cell differentiation[143].

Despite the fact that Panx3 has not been reported in adipocytes, it is normally co-expressed with Panx1 in many cell types, and is involved in the differentiation of many mesenchymal cells. Therefore Panx3 may also be involved in the proliferation and differentiation of adipocytes similarly and potentially in concert with Panx1.

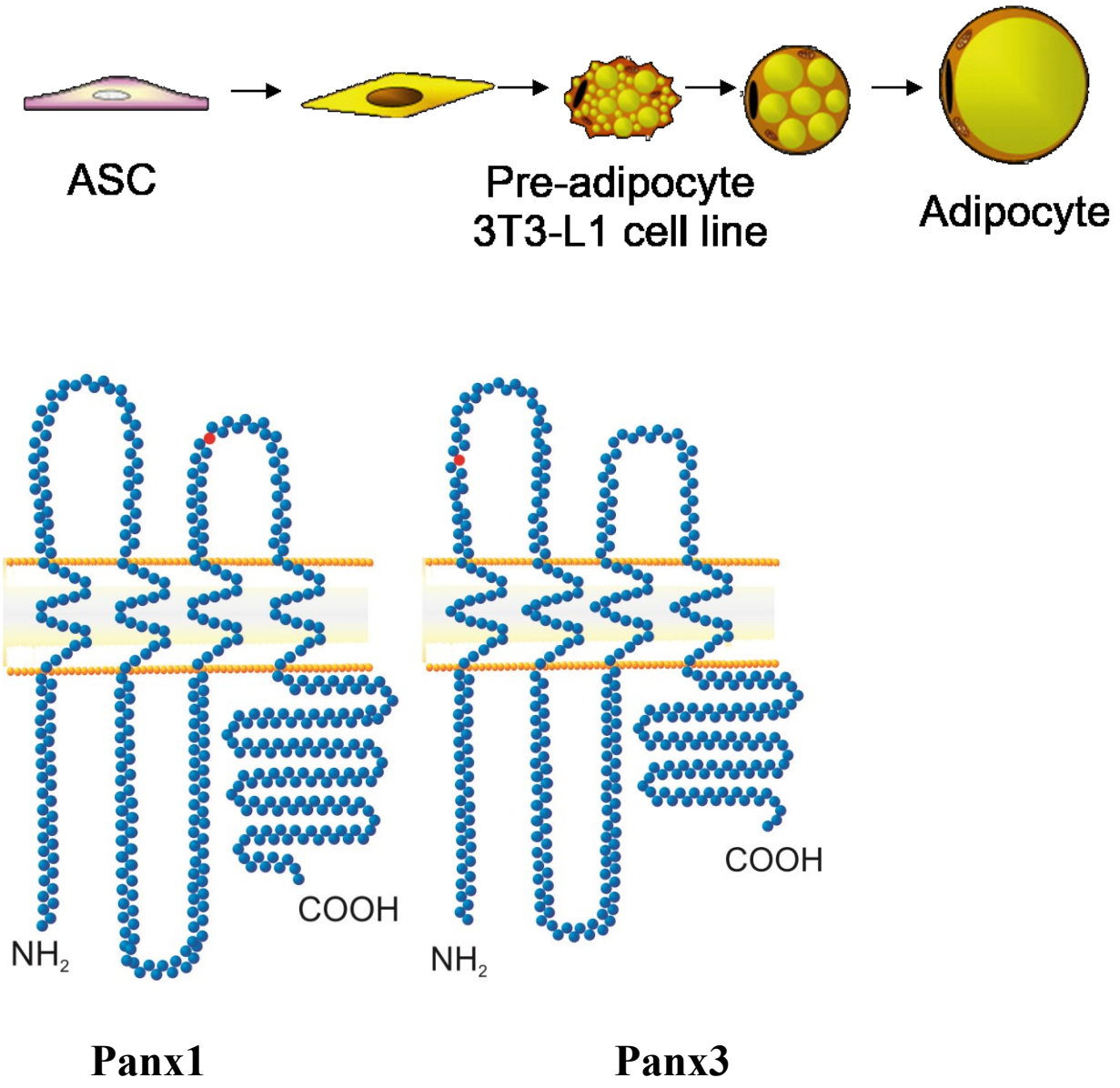

Figure 1.1 Adipocyte lineage with corresponding cell type examples, where Panx1 and Panx3 may play a regulating function in adipogenic development. Proposed tetra-spanning topology of Panx1 and Panx3 with intracellular amino (NH_2) and carboxyl (COOH) termini. ASC: adipose-derived stromal cell. Red amino acid residues: N-glycosylation sites

1.8 Pannexin Knockout Mice and Diet-Induced Obesity Models

In order to study how Panx1 affects development *in vivo*, a variety of different Panx1 global knockout (Panx1 KO) mice have been generated[119,139,137]. The Panx1 KO mouse used in our studies was originally characterized by Genentech, Inc. and published by Qu et al (2011), demonstrating that Panx1 is required for ATP release from apoptotic thymocytes to recruit macrophages[119]. Our group previously characterized and used that KO model to show that Panx1 is important in early skin development specifically at the level of keratinocytes and dermal fibroblasts, and in wound healing[103]. When dorsal skin was wounded, Panx1 was shown to be upregulated at the wound edge and Panx1 KO mice had impaired wound healing[103]. Primary basal keratinocytes from Panx1 KO skin had increased migration capacity, while KO primary dermal fibroblasts showed increased proliferation[103]. Interestingly, Panx1 KO mice showed reduced thickness in dermal and epidermal area but increased hypodermal fat[103], indicating a potential effect of Panx1 in subcutaneous adipose tissue.

Our group generated the first ever Panx3 global KO mouse in addition to a conditional cartilage specific Panx3 KO mouse[152] in order to understand the role of Panx3 in osteoarthritis (OA). Based on this study, mice surgically-induced to develop osteoarthritis showed strong Panx3 expression in OA lesioned knee cartilage[152], and those lacking Panx3 globally or specifically in the cartilage had reduced proteoglycan loss and overall, seemed protected against the development of osteoarthritis[152]. Using this same mouse model, Panx3 has been shown to play an essential role in skeletal long bone development in rodents, as lack of Panx3 results in significantly shorter, and more robust humeri and femora than wild-type controls[153].

Mouse models have been developed to study diet-induced obesity (DIO) *in vivo*, an example being the C57/BL6J mouse, used by The Jackson Laboratory (jax.org) as a model of pre-diabetes (type II) and obesity with high blood glucose and impaired glucose tolerance. The

C57-BL6J mouse strain has been well-characterized to respond effectively to high fat diets by gaining significant amounts of weight compared to mice fed on regular chow diets[154]. They also develop pathologies similar to the human condition: hyperinsulinemia, hyperglycemia, increased fat mass, and hypertension[155]. However, there are many other mouse strains that do not respond equally to a high fat diet[156], so careful consideration to the genetic background of the mice is critical in the experimental design. Commonly used methods of quantifying body mass composition (lean and fat mass) includes echo-MRI[157,158], used here in this study in addition to metabolic cage analysis[159] for parameters of metabolism and activity such as volume of O_2 and CO_2, mobility, sleep, etc. We have chosen the C57/BL6J mouse model for the present study, in a breeding scheme that included the two global pannexin knockout mouse models globally lacking either Panx1 or Panx3 from germline.

1.9 Rationale and Hypothesis

Based on the literature presented, Panx1 is present in adipocytes, but its role in cellular development and obesity is unclear. The development of adipocytes and fat accumulation is highly regulated involving many different transcription factors, hormones, signaling cascades and enzymes. The current body of knowledge strongly supports Pannexins as channel proteins that interact with many signaling networks in both disease and in normal development. It is clear however, that Panx1 and Panx3 play a variety of roles in cellular development spanning many different organs and tissues, and may also function in the adipocyte cell fate and development. Therefore it is hypothesized that Panx1 and Panx3 regulates proliferation and differentiation of adipocytes, resulting in changes in fat accumulation.

1.10 Objectives

i. Assess the expression and localization of Panx1 during the adipogenesis process in WT ASCs and pre-adipocytes

ii. Determine if the lack of Panx1 in ASCs affect cell growth and differentiation by isolating ASC from Panx1 KO mice

iii. Determine the effect of a high fat diet on WT and Panx1 KO mice by testing markers of obesity and assessing metabolism, activity and mobility.

iv. Assess the function of Panx3 in adipocyte development and in the context of a high fat diet using the Panx3 KO mouse

1.11 References

1. World Health Organization (WHO). *Global Status Report on Noncommunicable Diseases 2014*. Geneva, Switzerland; 2014.
2. Tao W, Lagergren J. Clinical management of obese patients with cancer. *Nat Rev Clin Oncol*. 2013;10(9):519-533.
3. Kuczmarski RJ, Flegal KM. Criteria for definition of overweight in transition : background and recommendations for the United States. *Am J Clin Nutr*. 2000;72:1074-1081.
4. Carvajal R, Wadden TA, Tsai AG, Peck K, Moran CH. Managing obesity in primary care practice: A narrative review. *Ann N Y Acad Sci*. 2013;1281(1):191-206.
5. Wing R, Lang W, Wadden T, et al. Benefits of Modest Weight Loss in Improving Cardiovascular Risk Factors in Overweight and Obese Individuals With Type 2 Diabetes. *Diabetes Care*. 2011;34(7):1481-1486.
6. Yanovski SZ, Yanovski JA. Long-term Drug Treatment for Obesity: A Systematic and Clinical Review. *J Am Med Assoc*. 2014;311(1):74-86.
7. Picot J, Jones J, Colquitt JL, et al. The clinical effectiveness and cost- effectiveness of bariatric (weight loss) surgery for obesity : a systematic review and economic evaluation. *Health Technol Assess (Rockv)*. 2009;13(41).
8. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;372:426-432.
9. Forbes S, Bui S, Robinson BR, Hochgeschwender U, Brennan MB. Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. *Proc Natl Acad Sci U S A*. 2001;98(7):4233-4237.
10. Prieur X, Tung YCL, Griffin JL, Farooqi IS, O'Rahilly S, Coll AP. Leptin regulates

peripheral lipid metabolism primarily through central effects on food intake. *Endocrinology*. 2008;149(11):5432-5439.
11. Matarese G, Procaccini C, De Rosa V, Horvath TL, La Cava A. Regulatory T cells in obesity: the leptin connection. *Trends Mol Med*. 2010;16(6):247-256.
12. Tartaglia LA, Dembski M, Weng X, et al. Identification and expression cloning of a leptin receptor, OB-R. *Cell*. 1995;83(7):1263-1271.
13. Clement K, Vaisse C, Lahlou N, et al. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature*. 1998;392(6674):398-401.
14. Reinehr T, Kratzsch J, Kiess W, Andler W. Circulating soluble leptin receptor, leptin, and insulin resistance before and after weight loss in obese children. *Int J Obes (Lond)*. 2005;29(10):1230-1235.
15. Lonnqvist F, Arner P, Nordfors L, Schalling M. Overexpression of the obese (ob) gene in adipose tissue of human obese subjects. *Nat Med*. 1995;1(9):950-953.
16. Stein K, Vasquez-Garibay E, Kratzsch J, Romero-Velarde E, Jahreis G. Influence of nutritional recovery on the leptin axis in severely malnourished children. *J Clin Endocrinol Metab*. 2006;91(3):1021-1026.
17. Zepf FD, Sungurtekin I, Glass F, et al. Differences in zinc status and the leptin axis in anorexic and recovered adolescents and young adults: A pilot study. *Food Nutr Res*. 2012;56:1-8.
18. Bratanova-Tochkova T, Cheng H, Daniel S, et al. Triggering and augmentation mechanismus, granule pool, and biphysic insulin secretion. *Diabetes*. 2002;51:S83-S90.
19. Dodson G, Steiner D. The role of assembly in insulin's biosynthesis. *Curr Opin Struct Biol*. 1998;8:189-194.
20. Könner AC, Brüning JC. Selective insulin and leptin resistance in metabolic disorders. *Cell Metab*. 2012;16(2):144-152.
21. Gerozissis K. Brain insulin and feeding: A bi-directional communication. *Eur J Pharmacol*. 2004;490(1-3):59-70.
22. Newgard CB, McGarry JD. Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annu Rev Biochem*. 1995;64:689-719.
23. Tassone F, Lanfranco F, Gianotti L, et al. Obstructive sleep apnoea syndrome impairs insulin sensitivity independently of anthropometric variables. *Clin Endocrinol (Oxf)*. 2003;59(3):374-379.
24. Smith U. Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance - is insulin resistance initiated in the adipose tissue? *Int J Obes*. 2002;26(7):897-904.
25. Grundy SM. What is the contribution of obesity to the metabolic syndrome? *Endocrinol Metab Clin North Am*. 2004;33(2):267-282, table of contents. http://www.ncbi.nlm.nih.gov/pubmed/15158519. Accessed June 6, 2016.
26. Krauss RM, Siri PW. Metabolic abnormalities: triglyceride and low-density lipoprotein. *Endocrinol Metab Clin North Am*. 2004;33(2):405-415.
27. Devaraj S, Rosenson RS, Jialal I. Metabolic syndrome: an appraisal of the pro-inflammatory and procoagulant status. *Endocrinol Metab Clin North Am*. 2004;33(2):431-453, table of contents.
28. Hunter SJ, Garvey WT. Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system 11In collaboration with The American Physiological Society, Thomas E. Andreoli, MD, Editor.

Am J Med. 1998;105(4):331-345.
29. Gerozissis K. Brain insulin and feeding: a bi-directional communication. *Eur J Pharmacol*. 2004;490(1-3):59-70.
30. Sechi LA, Bartoli E. Molecular mechanisms of insulin resistance in arterial hypertension. *Blood Press Suppl*. 1996;1:47-54.
31. Thomas DM, Udagawa N, Hards DK, et al. Insulin receptor expression in primary and cultured osteoclast-like cells. *Bone*. 1998;23(3):181-186.
32. Noble RC, Shand JH. The placenta: Its role in the relationship between the lipids of mother and foetus. *IRCS Med Sci*. 1981;9:174-177.
33. King JC, Butte NF, Bronstein MN, Kopp LE, Lindquist SA. Energy metabolism during pregnancy: influence of maternal energy status. *Am J Clin Nutr*. 1994;59(2 Suppl):439S - 445S.
34. Lasunción MA, Lorenzo J, Palacin M, Herrera E. Maternal factors modulating nutrient transfer to fetus. *Biol Neonate*. 1987;51(2):86-93.
35. Hay WW. Placental transport of nutrients to the fetus. *Horm Res*. 1994;42(4-5):215-222.
36. Van Aerder JE, Wilke MS, Feldman M, Clandinin MT. *Accretion of Lipid in the Fetus and Newborn*. 2nd ed. (Polin R, Fox W, eds.). Philadelphia: W. B. Saunders Co; 1998.
37. Heim T. Energy and lipid requirements of the fetus and the preterm infant. *J Pediatr Gastroenterol Nutr*. 1983;2 Suppl 1:S16-S41.
38. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol*. 2006;7(12):885-896.
39. Westermark O, Bernard S, Buchholz BA, et al. Dynamics of fat cell turnover in humans. *Nat Lett*. 2008;453:783-787.
40. Rutkowski JM, Stern JH, Scherer PE. The cell biology of fat expansion. *J Cell Biol*. 2015;208(5):501-512.
41. Brasaemle DL, Dolios G, Shapiro L, Wang R. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J Biol Chem*. 2004;279(45):46835-46842.
42. Zechner R, Strauss JG, Haemmerle G, Lass A, Zimmermann R. Lipolysis: pathway under construction. *Curr Opin Lipidol*. 2005;16(3):333-340.
43. Wilson-Fritch L, Nicoloro S, Chouinard MM, et al. Mitochondrial remodelling in adipose tissue associated with obesity and treatment with Rosiglitazone. *J Clin Invest*. 2004;114(9):1281-1289.
44. Lee M-J, Wu Y, Fried SK. Adipose Tissue Heterogeneity: Implication of depot differences in adipose tissue for Obesity Complications. *Mol Asp Med*. 2013;34(1):1-11.
45. Hirsch J, Batchelor B. Adipose tissue cellularity in human obesity. *Clin Endocrinol Metab*. 1976;5(2):299-311.
46. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia*. 2000;43(12):1498-1506.
47. Spalding KL, Arner E, Westermark PO, et al. Dynamics of fat cell turnover in humans. *Nature*. 2008;453(7196):783-787.
48. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev*. 2004;84(1):277-359.
49. Fruhbeck G, Becerril S, Sainz N, Garrastachu P, Garcia-Velloso MJ. BAT: a new target for human obesity? *Cell Press*. 2009;30(8):387-396.

50. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab*. 2007;293(2):E444-E452.
51. Ye J. Adipose Tissue Vascularization: Its Role in Chronic Inflammation. *Curr Diab Rep*. 2011;11(3):203-210.
52. Attie AD, Scherer PE. Adipocyte metabolism and obesity. *J Lipid Res*. 2009;50 Suppl:S395-S399.
53. Wolfe BM, Kvach E, Eckel RH. Obesity, Diabetes, and Cardiovascular Diseases Compendium Treatment of Obesity. *Circ Res*. 2016;118:1844-1856.
54. Miyazawa-Hoshimoto S, Takahashi K, Bujo H, Hashimoto N, Saito Y. Elevated serum vascular endothelial growth factor is associated with visceral fat accumulation in human obese subjects. *Diabetologia*. 2003;46(11):1483-1488.
55. Cancello R, Henegar C, Viguerie N, et al. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes*. 2005;54(8):2277-2286.
56. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab*. 2007;293(4):E1118-E1128.
57. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*. 2003;112(12):1796-1808.
58. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 2003;112(12):1821-1830.
59. Yin J, Gao Z, He Q, Zhou D, Guo Z, Ye J. Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. *Am J Physiol Endocrinol Metab*. 2009;296(2):E333-E342.
60. Cinti S, Mitchell G, Barbatelli G, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res*. 2005;46(11):2347-2355.
61. Murano I, Barbatelli G, Parisani V, et al. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *J Lipid Res*. 2008;49(7):1562-1568.
62. Otto TC, Lane MD. Adipose Development : From Stem Cell to Adipocyte. *Crit Rev Biochem Mol Biol*. 2005;40(4):229-242.
63. Baer PC, Geiger H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int*. 2012;2012:1-11.
64. De Ugarte DA, Morizono K, Elbarbary A, et al. Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs*. 2003;174(3):101-109.
65. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*. 2001;7(2):211-228.
66. Russo V, Yu C, Belliveau P, Hamilton A, Flynn LE. Comparison of Human Adipose-Derived Stem Cells Isolated from Subcutaneous, Omental, and Intrathoracic Adipose Tissue Depots for Regenerative Applications. *Stem Cells Transl Med*. 2014;3(2):206-217.
67. Mitchell JB, McIntosh K, Zvonic S, et al. Immunophenotype of Human Adipose-Derived Cells: Temporal Changes in Stromal-Associated and Stem Cell–Associated Markers. *Stem Cells*. 2006;24(2):376-385.
68. Varma MJO, Breuls RGM, Schouten TE, et al. Phenotypical and Functional

Characterization of Freshly Isolated Adipose Tissue-Derived Stem Cells. *Stem Cell Dev*. 2007;16(1):91-104.
69. Rodrıguez L V, Alfonso Z, Zhang R, Leung J, Wu B, Ignarro LJ. Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. *PNAS*. 2006;103(32):12167-12172.
70. Zuk PA, Zhu M, Ashjian P, et al. Human Adipose Tissue Is a Source of Multipotent Stem Cells. *Mol Biol Cell*. 2002;13(12):4279-4295.
71. Flynn L, Semple JL, Woodhouse KA. Decellularized placental matrices for adipose tissue engineering. *J Biomed Res Part A*. 2006;79(2):359-369.
72. Pairault J, Green H. A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc Natl Acad Sci USA*. 1979;76(10):5138-5142.
73. Tontonoz P, Hu E, Spiegelman BM. Stimulation of Adipogenesis in Fibroblasts by PPARy2, a Lipid-Activated Transcription. *Cell*. 1994;79:1147-1156.
74. Altiok S, Xu M, Spiegelman BM. PPARγ induces cell cycle withdrawal: inhibition of E2F/ DP DNA-binding activity via down-regulation of PP2A. *Genes Dev*. 1998;11:1987-1998.
75. Gregoire FM, Smas CM, Sul HS. Understanding Adipocyte Differentiation. *Physiol Rev*. 1998;78(3):783-809.
76. Berry DC, Stenesen D, Zeve D, Graff JM. The developmental origins of adipose tissue. *Development*. 2016;140(19):3939-3949.
77. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest*. 2007;117(9):2362-2368.
78. Safonova I, Darimont C, Amri EZ, et al. Retinoids are positive effectors of adipose cell differentiation. *Mol Cell Endocrinol*. 1994;104(2):201-211.
79. Ono M, Aratani Y, Kitagawa I, Kitagawa Y. Ascorbic acid phosphate stimulates type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells. *Exp Cell Res*. 1990;187(2):309-314.
80. Calvo JC, Rodbard D, Katki A, Chernick S, Yanagishita M. Differentiation of 3T3-L1 preadipocytes with 3-isobutyl-1-methylxanthine and dexamethasone stimulates cell-associated and soluble chondroitin 4-sulfate proteoglycans. *J Biol Chem*. 1991;266(17):11237-11244.
81. Antras J, Hilliou F, Redziniak G, Pairault J. Decreased biosynthesis of actin and cellular fibronectin during adipose conversion of 3T3-F442A cells. Reorganization of the cytoarchitecture and extracellular matrix fibronectin. *Biol Cell*. 1989;66(3):247-254. http://www.ncbi.nlm.nih.gov/pubmed/2690986. Accessed June 6, 2016.
82. Smas CM, Sul HS. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell*. 1993;73(4):725-734.
83. Ren D, Collingwood TN, Rebar EJ, Wolffe AP, Camp HS. PPAR gamma knockdown by engineered transcription factors : exogenous PPAR gamma 2 but not PPAR gamma 1 reactivates adipogenesis. *Genes Dev*. 2002;16(734):27-32.
84. Zhang J, Fu M, Cui T, et al. Selective disruption of PPARgamma 2 impairs the development of adipose tissue and insulin sensitivity. *Proc Natl Acad Sci U S A*. 2004;101(29):10703-10708.
85. Tamori Y, Masugi J, Nishino N, Kasuga M. Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes.

Diabetes. 2002;51(7):2045-2055.
86. Tang Q-Q, Otto TC, Lane MD. CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proc Natl Acad Sci U S A*. 2003;100(3):850-855.
87. Linhart HG, Ishimura-Oka K, DeMayo F, et al. C/EBPalpha is required for differentiation of white, but not brown, adipose tissue. *Proc Natl Acad Sci U S A*. 2001;98(22):12532-12537.
88. Zuo Y, Qiang L, Farmer SR. Activation of CCAAT/enhancer-binding protein (C/EBP) ?? expression by C/EBP?? during adipogenesis requires a peroxisome proliferator-activated receptor-??-associated repression of HDAC1 at the C/ebp?? gene promoter. *J Biol Chem*. 2006;281(12):7960-7967.
89. Hunter SJ, Garvey WT. Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system. *Am J Med*. 1998;105(4):331-345. http://www.ncbi.nlm.nih.gov/pubmed/9809695. Accessed June 6, 2016.
90. Mori T, Sakaue H, Iguchi H, et al. Role of kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. *J Biol Chem*. 2005;280(13):12867-12875.
91. Kuri-Harcuch W, Wise LS, Green H. Interruption of the adipose conversion of 3T3 cells by biotin deficiency: differentiation without triglyceride accumulation. *Cell*. 1978;14(1):53-59.
92. Kozak LP, Jensen JT. Genetic and Developmental Control of Multiple Forms of L-Glycerol 3-Phosphate Dehydrogenase. *J Biol Chem*. 1974;240(24):7775-7781.
93. Wise LS, Green H. Participation of One Isozyme Cytosolic Glycerophosphate Dehydrogenase in the Adipose Conversion of 3T3 Cells. *J Biol Chem*. 1979;254(2):273-275.
94. Kozak LP, Kozak UC, Clarke GT. Abnormal brown and white fat development in transgenic mice overexpressing glycerol 3-phosphate dehydrogenase. *Genes Dev*. 1991;5(12 A):2256-2264.
95. Lane MD, Flores-Riveros JR, Hresko RC, et al. Insulin-receptor tyrosine kinase and glucose transport. *Diabetes Care*. 1990;13(6):565-575.
96. Nakae J, Park BC, Accili D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a wortmannin-sensitive pathway. *J Biol Chem*. 1999;274(23):15982-15985.
97. Yin D, Clarke SD, Peters JL, Etherton TD. Somatotropin-dependent decrease in fatty acid synthase mRNA abundance in 3T3-F442A adipocytes is the result of a decrease in both gene transcription and mRNA stability. *Biochem J*. 1998;331 (Pt 3:815-820.
98. Sniderman AD, Maslowska M, Cianflone K. Of mice and men (and women) and the acylation-stimulating protein pathway. *Curr Opin Lipidol*. 2000;11(3):291-296.
99. Green H, Kehinde O. An Established Preadipose Cell Line and its Differentiation in Culture II . Factors Affecting the Adipose Conversion. *Cell*. 1975;5(May):19-27.
100. Adamson SE, Meher AK, Chiu Y, et al. Pannexin 1 is required for full activation of insulin-stimulated glucose uptake in adipocytes. *Mol Metab*. 2015;4(9):610-618.
101. Panchin Y, Kelmanson I, Matz M, Lukyanov K, Usman N, Lukyanov S. A ubiquitous family of putative gap junction molecules. *Curr Biol*. 2000;10(13):473-474.
102. Penuela S, Bhalla R, Gong X-Q, et al. Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. *J*

Cell Sci. 2007;120(Pt 21):3772-3783.
103. Penuela S, Kelly JJ, Churko JM, Barr KJ, Berger AC, Laird DW. Panx1 regulates cellular properties of keratinocytes and dermal fibroblasts in skin development and wound healing. *J Invest Dermatol*. 2014;134(7):2026-2035.
104. Dvoriantchikova G, Ivanov D, Panchin Y, Shestopalov VI. Expression of pannexin family of proteins in the retina. *FEBS Lett*. 2006;580:2178-2182.
105. Boassa D, Ambrosi C, Qiu F, Dahl G, Gaietta G, Sosinsky G. Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane. *J Biol Chem*. 2007;282(43):31733-31743.
106. Bao L, Locovei S, Dahl G. Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett*. 2004;572(1-3):65-68.
107. Penuela S, Bhalla R, Nag K, Laird DW. Glycosylation regulates pannexin intermixing and cellular localization. *Mol Biol Cell*. 2009;20(20):4313-4323.
108. Le Vasseur M, Lelowski J, Bechberger JF, Sin W-C, Naus CC. Pannexin 2 protein expression is not restricted to the CNS. *Front Cell Neurosci*. 2014;8(392).
109. Ishikawa M, Iwamoto T, Nakamura T, Doyle A, Fukumoto S, Yamada Y. Pannexin 3 functions as an ER Ca(2+) channel, hemichannel, and gap junction to promote osteoblast differentiation. *J Cell Biol*. 2011;193(7):1257-1274.
110. Iwamoto T, Nakamura T, Doyle A, et al. Pannexin 3 regulates intracellular ATP/cAMP levels and promotes chondrocyte differentiation. *J Biol Chem*. 2010;285(24):18948-18958.
111. Wang X-H, Streeter M, Liu Y-P, Zhoa H-B. Identification and characterization of Pannexin expression in the mammalian cochlea. *J Comp Neurol*. 2010;512(3):336-346.
112. Turmel P, Dufresne J, Hermo L, Smith CE, Penuela S, Laird DW. Characterization of Pannexin1 and Pannexin3 and Their Regulation by Androgens in the Male Reproductive Tract of the Adult Rat. 2011;138:124-138.
113. Baranova A, Ivanov D, Petrash N, et al. The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. *Genomics*. 2004;83(4):706-716.
114. Scemes E, Spray DC, Meda P. Connexins, pannexins, innexins: novel roles of "hemi-channels." *Pflugers Arch*. 2009;457(6):1207-1226.
115. Chekeni FB, Elliott MR, Sandilos JK, et al. Pannexin 1 channels mediate "find–me" signal release and membrane permeability during apoptosis. *Nature*. 2010;467(7317):863-867.
116. Celetti SJ, Cowan KN, Penuela S, Shao Q, Churko J, Laird DW. Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation. *J Cell Sci*. 2010;123(Pt 8):1363-1372.
117. Silverman WR, de Rivero Vaccari JP, Locovei S, et al. The pannexin 1 channel activates the inflammasome in neurons and astrocytes. *J Biol Chem*. 2009;284(27):18143-18151.
118. Bruzzone R, Hormuzdi SG, Barbe MT, Herb A, Monyer H. Pannexins, a family of gap junction proteins expressed in brain. *PNAS*. 2003;100(23):13644-13649.
119. Qu Y, Misaghi S, Newton K, et al. Pannexin-1 is required for ATP release during apoptosis but not for inflammasome activation. *J Immunol*. 2011;186:6553-6561.
120. Kienitz M-C, Bender K, Dermietzel R, Pott L, Zoidl G. Pannexin 1 constitutes the large conductance cation channel of cardiac myocytes. *J Biol Chem*. 2011;286(1):290-298.
121. Penuela S, Harland L, Simek J, Laird DW. Pannexin channels and their links to human disease. *Biochem J*. 2014;461(3):371-381.
122. Jiang JX, Penuela S. Connexin and pannexin channels in cancer. *BMC Cell Biol*.

2016;17(Suppl 1(12).
123. Penuela S, Gyenis L, Ablack A, et al. Loss of Pannexin 1 Attenuates Melanoma Progression by Reversion to a Melanocytic Phenotype. *J Biol Chem*. 2012;287(34):29184-29193.
124. Largo C, Alvarez S, Blesa D, et al. Identification of overexpressed genes in frequently gained/amplified chromosome regions in multiple myeloma. *Haematologica*. 2006;91(2):184-191.
125. Wei L, Yang X, Shi X, Chen Y. Pannexin- 1 silencing inhibits the proliferation of U87-MG cells. *Mol Med Rep*. 2015;11(5):3487-3492.
126. Dolmatova E, Spagnol G, Boassa D, et al. Cardiomyocyte ATP release through pannexin 1 aids in early fibroblast activation. *Am J Physiol Heart Circ Physiol*. 2012;303:H1208-H1218.
127. Billaud M, Lohman AW, Straub AC, et al. Pannexin1 regulates α1-adrenergic receptor-mediated vasoconstriction. *Circ Res*. 2011;109(1):80-85.
128. Gulbransen BD, Bashashati M, Hirota SA, et al. Activation of neuronal P2X7 receptor – pannexin-1 mediates death of enteric neurons during colitis. *Nat Med*. 2012;18(4):600-604.
129. Orellana JA, Froger N, Ezan P, et al. ATP and glutamate released via astroglial connexin43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. *J Neurochem*. 2011;118(5):826-840.
130. Diezmos EF, Sandow SL, Markus I, et al. Expression and localization of pannexin-1 hemichannels in human colon in health and disease. *Neurogastroenterol Motil*. 2013;25(6):e395-e405.
131. Csak T, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty Acid and Endotoxin Activate Inflammasomes in Mouse Hepatocytes that Release Danger Signals to Stimulate Immune Cells. *Hepatology*. 2011;54(1):133-144.
132. Sáez PJ, Shoji KF, Aguirre A, Sáez JC. Regulation of Hemichannels and Gap Junction Channels by Cytokines in Antigen-Presenting Cells. *Mediators Inflamm*. 2014;2014(742734).
133. Xiao F, Waldrop SL, Khimji A, Kilic G. Pannexin1 contributes to pathophysiological ATP release in lipoapoptosis induced by saturated free fatty acids in liver cells. *Am J Physiol Cell Physiol*. 2016;303(10):C1034-C1044.
134. Loomba R, Sanyal AJ. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol*. 2013;10(11):686-690.
135. Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J*. 2006;25(21):5071-5082.
136. Kienitz M, Bender K, Dermietzel R, Pott L, Zoidl G. Pannexin 1 Constitutes the Large Conductance Cation Channel of Cardiac Myocytes. *J Biol Chem*. 2011;286(1):290-298.
137. Bargiotas P, Krenz A, Hormuzdi SG, Ridder DA, Herb A, Barakat W. Pannexins in ischemia-induced neurodegeneration. *PNAS*. 2011;108(51):20772-20777.
138. Santiago MF, Veliskova J, Patel NK, et al. Targeting Pannexin1 Improves Seizure Outcome. *PLoS One*. 2011;6(9):e25178.
139. Dvoriantchikova G, Ivanov D, Barakat D, et al. Genetic Ablation of Pannexin1 Protects Retinal Neurons from Ischemic Injury. *PLoS One*. 2012;7(2):e319991.
140. Dvoriantchikova G, Hanske J, Petrasch-Parwez E, et al. Pannexin1 Stabilizes Synaptic Plasticity and Is Needed for Learning. *PLoS One*. 2012;7(12):e51767.

141. Kurtenbach S, Whyte-Fagundes P, Gelis L, et al. Investigation of olfactory function in a Panx1 knock out mouse model. *Front Cell Neurosci*. 2014;8(266):1-8.
142. Penuela S, Gehi R, Laird DW. The biochemistry and function of pannexin channels. *Biochim Biophys Acta*. 2013;1828(1):15-22.
143. Langlois S, Xiang X, Young K, Cowan BJ, Penuela S, Cowan KN. Pannexin 1 and Pannexin 3 Channels Regulate Skeletal Muscle Myoblast Proliferation and Differentiation. *J Biol Chem*. 2014;289(44):30717-30731.
144. Stewart MKG, Plante I, Penuela S, Laird DW. Loss of Panx1 Impairs Mammary Gland Development at Lactation : Implications for Breast Tumorigenesis. *PLoS One*. 2016;11(4):e0154162.
145. Vogt A, Hormuzdi SG, Monyer H. Pannexin1 and Pannexin2 expression in the developing and mature rat brain. 2005;141:113-120.
146. Wicki-stordeur LE, Dzugalo AD, Swansburg RM, Suits JM, Swayne LA. Pannexin 1 regulates postnatal neural stem and progenitor cell proliferation. *Neural Dev*. 2012;7(11):1-10.
147. Bruzzone R, Barbe MT, Jakob NJ, Monyer H. Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. *J Neurochem*. 2005;92(5):1033-1043.
148. Wang Z V, Deng Y, Wang QA, Sun K, Scherer PE. Identification and Characterization of a Promoter Cassette Conferring Adipocyte-Specific Gene Expression. *Endocrinology*. 2010;151(June):2933-2939.
149. Ishikawa M, Iwamoto T, Fukumoto S, Yamada Y. Pannexin 3 Inhibits Proliferation of Osteoprogenitor Cells by Regulating Wnt and p21 Signaling *. *J Biol Chem*. 2014;289(5):2839-2851.
150. Fu D, Song F, Sun H, Pei D, Wang Y, Lei J. Archives of Oral Biology Expression of Pannexin3 in human odontoblast-like cells and its hemichannel function in mediating ATP release. *Arch Oral Biol*. 2015;60(10):1510-1516.
151. Oh S, Shin J, Baek J, et al. Pannexin 3 is required for normal progression of skeletal development in vertebrates. *FASEB J*. 2015;29:4473-4484.
152. Moon PM, Penuela S, Barr K, et al. Deletion of Panx3 Prevents the Development of Surgically Induced Osteoarthritis. *J Mol Med*. 2016;93(8):845-856.
153. Caskenette D, Penuela S, Lee V, et al. Global deletion of Panx3 produces multiple phenotypic effects in mouse humeri and femora. *J Anat*. 2016;288(5):746-756.
154. Wang C, Liao JK. A Mouse Model of Diet-Induced Obesity and Insulin Resistance. *Methods Mol Biol*. 2012;821:421-433.
155. Collins S, Martin TL, Surwit RS, Robidoux J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: Physiological and molecular characteristics. *Physiol Behav*. 2004;81(2):243-248.
156. Kern M, Knigge A, Heiker J, et al. C57BL/6JRj mice are protected against diet induced obesity (DIO). *Biochem Biophys Res Commun* . 2012;417(2):717-720.
157. Lengacher S, Nehiri-Sitayeb T, Steiner N, et al. Resistance to Diet-Induced Obesity and Associated Metabolic Perturbations in Haploinsufficient Monocarboxylate Transporter 1 Mice. Luque RM, ed. *PLoS One*. 2013;8(12):e82505.
158. Williams LM, Campbell FM, Drew JE, et al. The Development of Diet-Induced Obesity and Glucose Intolerance in C57Bl/6 Mice on a High-Fat Diet Consists of Distinct Phases. Müller M, ed. *PLoS One*. 2014;9(8):e106159.

159. Guzman MS, De Jaeger X, Drangova M, Prado MAM, Gros R, Prado VF. Mice with selective elimination of striatal acetylcholine release are lean, show altered energy homeostasis and changed sleep/wake cycle. *J Neurochem*. 2013;124:658-669.

Chapter 2 –Manuscript

Pannexin 1 regulates early adipocyte development and fat accumulation *in vivo*

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2.1 Abstract

Obesity is a growing worldwide epidemic with various comorbidities and associated risk factors. Pannexin 1 (Panx1) is a channel-forming glycoprotein important in paracrine signaling and cellular development. It is expressed in the cells responsible for fat accumulation, namely adipocytes, as well as adipogenic progenitors throughout the process of adipogenic differentiation. *In vivo*, male mice globally lacking Panx1 (Panx1 KO) have significantly greater total fat mass as compared to wild type (WT) mice under a normal diet. At the cellular level, ASCs isolated from Panx1 KO mice proliferate more slowly, but demonstrate enhanced adipogenic differentiation in terms of intracellular lipid accumulation, glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity, and adipokine secretion, as compared to WT ASCs. In spite of their higher fat content in normal conditions, Panx1 KO mice on a high fat diet exhibited no differences in weight gain and blood markers of obesity as compared to controls. However, metabolic cage data revealed that these global Panx1 KO mice display significantly increased activity levels, higher ambulatory activity, and reduced sleep duration relative to their WT littermates. We conclude that Panx1 plays a key role in adipogenic cell proliferation and differentiation and can regulate fat accumulation *in vivo*.

2.2 Introduction

The obesity epidemic is the leading cause of global deaths and according to the World Health Organization approximately 10% of the world's population is obese (as of 2014)[1]. Obesity accounts for a multitude of comorbidities including cardiovascular disease, type II diabetes, and cancer[2]. Adipocytes are the main cell type responsible for fat accumulation and play a key role in the metabolic complications associated with obesity.

Adipocytes are unique cells that have the capacity to store large amounts of energy and have a long lifespan, where approximately 10% of adipocytes are regenerated annually[3]. Adipocytes are problematic in the context of obesity in that their innate ability to accumulate lipids causes excess adipose tissue, that behaves as an endocrine organ participating in cytokine release and associated inflammatory problems[4]. At the onset of obesity, adipose tissue can release many different transcription factors, inflammatory cytokines, and hormones including: leptin, resistin, adiponectin, IL-6, IL-1β, TNF-α, MCP-1, to name a few[4]. As a collective, many of these factors contribute to the development of chronic inflammation and subsequently cell death and lipolysis[4]. As a result of chronic inflammation and adipocyte cell death, macrophage infiltration occurs and pro-inflammatory M1 macrophages surround dead adipocytes in a crown-like fashion, further exacerbating the inflammatory response[5] and comorbidities[6].

Mature adipocytes originate from the expansion and differentiation of a heterogeneous population of multipotent precursor cells and more committed pre-adipocytes, collectively referred to as adipose-derived stromal cells (ASCs)[7]. Adipogenesis is a highly complex process involving dynamic variations in the expression of numerous intracellular and secreted proteins, as well as dramatic changes in cell morphology[8]. Previous research using both the immortalized 3T3-L1 pre-adipocyte cell line [9] and primary ASC populations[10] has helped to elucidate the mechanisms of adipogenic differentiation. These cell populations can be induced in culture to

differentiate using adipogenic media containing a cocktail of hormones and other factors that stimulate the pathways involved in adipogenesis[11]. Following the induction of differentiation, the cells undergo cell cycle arrest[8,11], which then triggers a signaling cascade that upregulates the transcription factors necessary for adipogenesis, including peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT-enhancer binding protein-α (C/EBP-α)[12]. PPAR-γ and C/EBP-α function in concert to help maintain growth arrest[13] and the differentiative state[14], and through downstream signaling pathways upregulate the expression of adipogenic genes required for intracellular lipid accumulation and differentiation into mature adipocytes[12].

Pannexins (Panx) are a family of three channel-forming glycoproteins (Panx1, Panx2 and Panx3) that form large pore channels at the cell surface and intracellular compartments[15]. Panx1 has been shown to be ubiquitously expressed in most mammalian organs, while Panx2 (central nervous system[16] and other organs[17]), and Panx3 (skin[18], cartilage[19] and bone[20]) are more restricted in their expression. Pannexin subunits oligomerize into hexameric non-selective functional channels[21] that allow for the passage of ions and small molecules (<1kDa) such as ATP or glutamate[22]. The large pore pannexin channels participate in cellular communication in an autocrine fashion, or through the extracellular environment via paracrine signaling[23].

Panx1 is present in metabolically relevant tissues[24] such as liver[16], white adipose tissue[25], and large intestine [26], and has been linked to diseases[27] such as melanoma[28], ischemia[29], hypertension[30], colitis[31], and diabetes[32]. In adult tissues, Panx1 expression or dysregulation is conducive to the onset or progression of different diseases[27]. However, in early stages of development, Panx1 has been reported to regulate functions of cell proliferation and differentiation in many cell types such as: dermal fibroblasts[33], keratinocytes[18], skeletal muscle myoblasts[34], osteoblasts[35], and neural progenitor cells[36]. There is only one report in the literature

suggesting that Panx1 is expressed in mature adipocytes and mediates glucose uptake along with insulin sensitivity[25], but the role of pannexins in early adipogenic development is currently unknown.

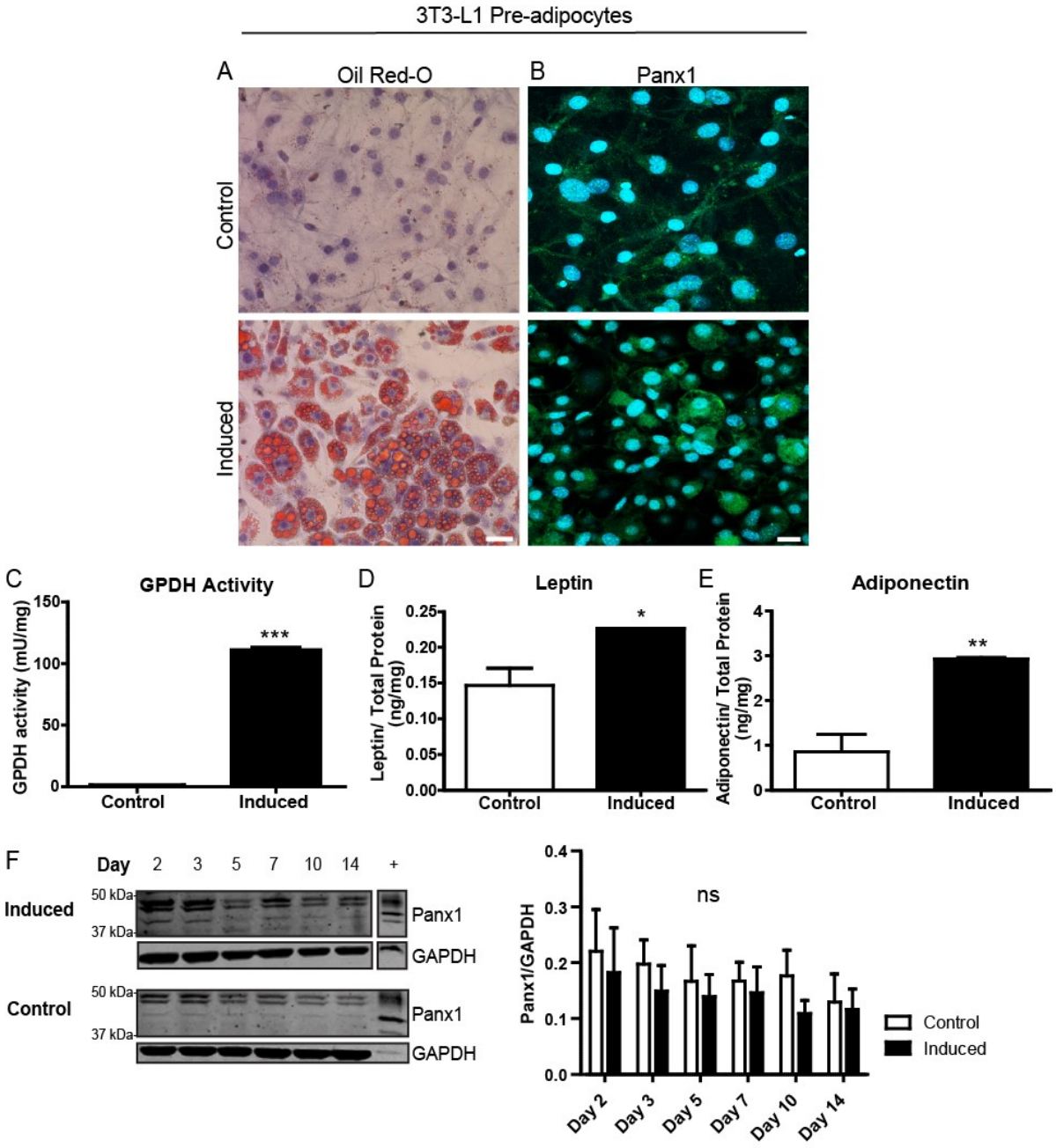
Using a global Panx1 knockout mouse model (Panx1 KO used in this study)[37], we previously contributed to the characterization of this model and reported that the dorsal skin of the Panx1 KO mice had a thinner dermal area and delayed wound healing capabilities, due to the lack of Panx1 in keratinocytes and dermal fibroblasts[33]. Interestingly, we also noticed that there was a significant increase in the subcutaneous fat of the Panx1 KO that was evident at 4 days of age, and persisted into adulthood[33]. Based on this, we hypothesized that Panx1 may regulate adipogenic cell proliferation and differentiation, thus resulting in changes in fat accumulation. In this study, we highlight for the first time the function of Panx1 in early adipocyte development. Panx1 regulates the proliferation and differentiation of ASCs, and the germline deletion of Panx1 results in increased fat mass *in vivo*, underlining a role for Panx1 in fat accumulation and obesity.

2.3 Results

2.3.1 Panx1 is expressed during adipogenic differentiation

Since it has been well established that Panx1 is involved in cell proliferation and differentiation of many different cell types[33–35], we evaluated its potential regulation of adipogenic development and differentiation. It was initially reported by Adamson et al., 2015[25] that Panx1 was expressed in mature mouse adipocytes and the more-committed murine 3T3-L1 pre-adipocyte cell line[25]. In order to assess changes in Panx1 expression during adipogenic differentiation over time, we induced the 3T3-L1 cells to differentiate using adipogenic differentiation media and stained the cells with oil red O to assess intracellular lipid accumulation at varying time points over 14 days (Fig. S1A). We confirmed that there was extensive multilocular lipid accumulation in the cells that had been induced to differentiate (Fig. 1A). When we stained cells by immunofluorescence for Panx1, we observed that Panx1 was expressed mostly intracellularly in the pre-adipocyte cells both before and after differentiation (Fig. 1B). We conducted a lipid biosynthesis assay by measuring glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity; a key enzyme involved in the process of converting carbohydrates to lipids. GPDH activity was significantly upregulated, about 100-fold, after adipogenic induction ($P<0.001$, $n=6$) (Fig. 1C). We also assessed the secretion of the adipokines leptin and adiponectin by ELISA and found upregulation of both markers in the medium of the differentiated 3T3-L1 cells ($P<0.05$, $P<0.01$, $n=6$) (Fig. 1D, E). Knowing that the cells were efficiently differentiating, we compared Panx1 expression throughout 14 days of differentiation in both cells induced to differentiate and non-induced controls maintained in proliferation medium. Panx1 was consistently expressed throughout the differentiation process without significant changes in protein levels (Fig. 1F, $n=6$).

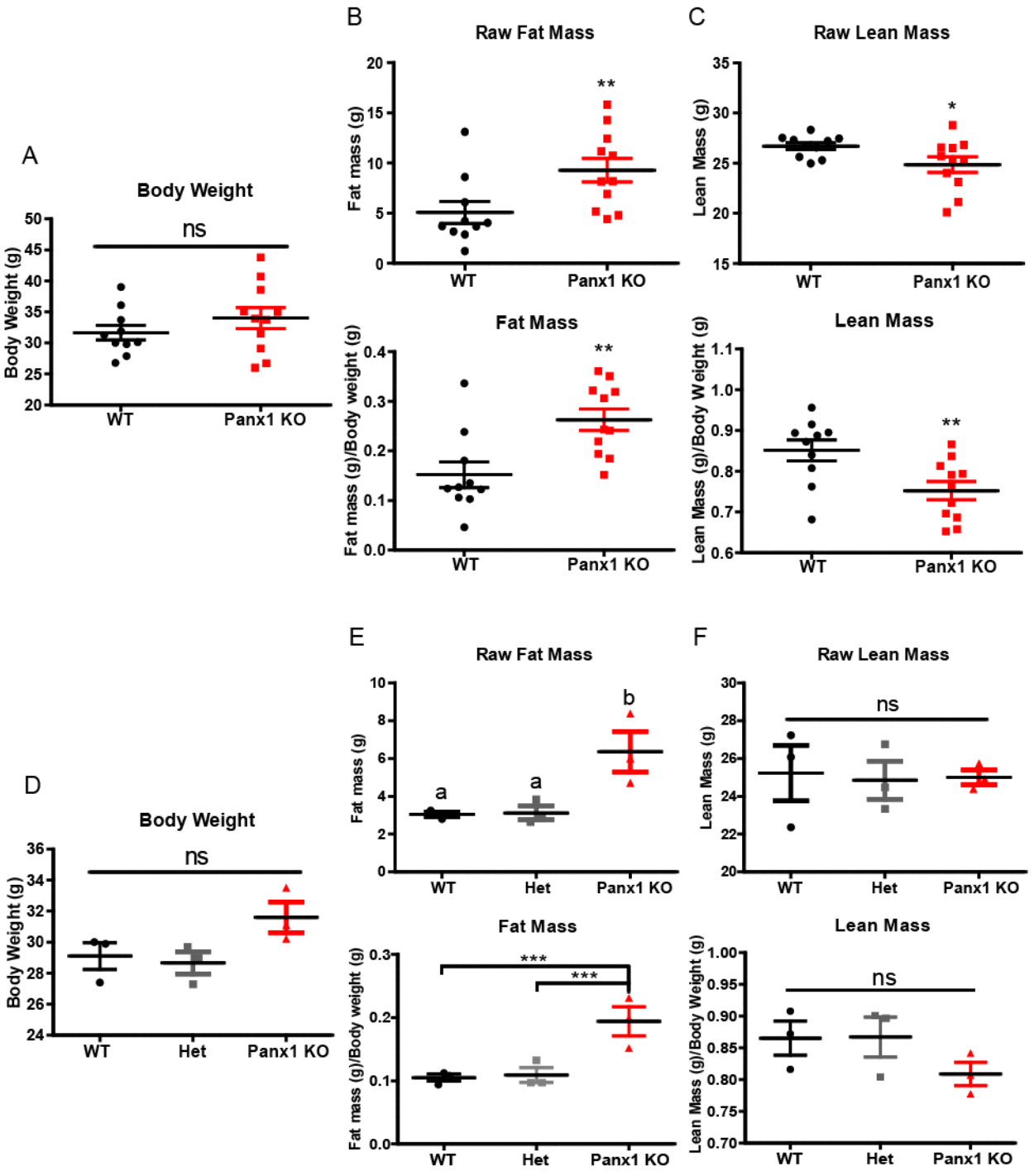
Figure 2.1 Panx1 is expressed during adipogenic differentiation. 3T3-L1 mouse pre-adipocyte cells shown before and after adipogenic induction, 14 days in culture. A. Bright field images of cells stained with oil red O confirm intracellular lipid accumulation (red) characteristic of differentiated multilocular adipocytes, counterstained with hematoxylin (blue). Scale bar= 100 μm. B. Fluorescent micrographs depicting expression of Panx1 (green) and nuclei (blue). Scale bar= 20 μm. C-E, identical microscope parameters were maintained to compare between samples. GPDH activity, leptin and adiponectin ELISAs of 3T3-L1 cells before and after adipogenic induction validating adipocyte differentiation. F. Western blot and respective quantification of Panx1 expression in 3T3-L1 cell lysates over a period of 14 days in induced cells (top) and control cells (bottom). Panx1 is expressed in both control and differentiated cells and is not significantly different over time or throughout differentiation. N=3, n=6, *$P<0.05$, **$P<0.01$, ***$P<0.0001$, means ± s.e.m.


3T3-L1 Pre-adipocytes
A
Oil Red-O
B
Panx1
Control
Induced
C
GPDH Activity
GPDH activity (mU/mg)
150
100
50
0
***
Control
Induced
D
Leptin
Leptin/ Total Protein (ng/mg)
0.25
0.20
0.15
0.10
0.05
0.00
*
Control
Induced
E
Adiponectin
Adiponectin/ Total Protein (ng/mg)
4
3
2
1
0
**
Control
Induced
F
Day 2 3 5 7 10 14 +
Induced
50 kDa
37 kDa
Panx1
GAPDH
Control
50 kDa
37 kDa
Panx1
GAPDH
Panx1/GAPDH
0.4
0.3
0.2
0.1
0.0
ns
Day 2
Day 3
Day 5
Day 7
Day 10
Day 14
Control
Induced

2.3.2 Panx1 KO mice have significantly greater fat mass compared to WT mice.

Since we observed that Panx1 was expressed throughout adipogenic differentiation, we hypothesized that it may also play a role in fat accumulation. With the use of the global Panx1 KO mice, described by Qu et al., 2011[37], we backcrossed the mice to C57BL/6 until a congenic line of mice was generated and assessed their overall body mass composition via echo-MRI. We first compared average body weight between WT and Panx1 KO mice and saw no significant differences (WT N=10, KO N=11) (Fig. 2A). When assessing overall fat mass composition, we observed that Panx1 KO mice had significantly greater fat mass (42% increase) compared to WT mice (WT N=10, KO N=11, $P<0.01$) (Fig. 2B), and significantly decreased overall lean mass ($P<0.01$) (Fig. 2C). To ensure that these differences were not due to genetic variations between mice, we crossed heterozygous mice to generate a small cohort of littermate mice (WT, Heterozygous, and Panx1 KO) and repeated the measurements. We saw no change in body weight (WT N=3, Het N=3, KO N=3) (Fig. 2D), or lean mass between these groups of mice (Fig. 2F). Consistent with the non-littermate group, we saw that the Panx1 KO mice had significantly greater fat mass (45% increase) compared to WT and heterozygous mice ($P<0.0001$) (Fig. 2E). Therefore, the significant increase in overall fat mass was due to the Panx1 deletion, indicating that Panx1 may play a role in fat accumulation.

Figure 2.2 Panx1 KO mice have significantly greater fat mass compared to WT mice. Congenic wildtype (WT) and Panx1 global knockout (KO) mice (12 months-old, A-C), and 6 month-old WT, KO and heterozygous (Het) male littermate mice (D-F) were fed *ad libitum* on normal chow diet and analyzed with echo-MRI to determine overall body mass composition. A, D. There was no significant difference in body weight between groups of mice. B, E. Panx1 KO mice had significantly increased fat mass compared to WT mice normalized to body weight (including raw fat mass). C, F. Congenic KO mice showed a slight decrease in lean mass (both normalized to body weight and raw data), while there was no significant difference in lean mass among littermate mice (F). A-C: WT N=10, Panx1 KO N=11. D-F: WT N=3, Heterozygous N=3, Panx1 KO N=3. *P<0.01, **P<0.01, ***P<0.0001, means ± s.e.m.

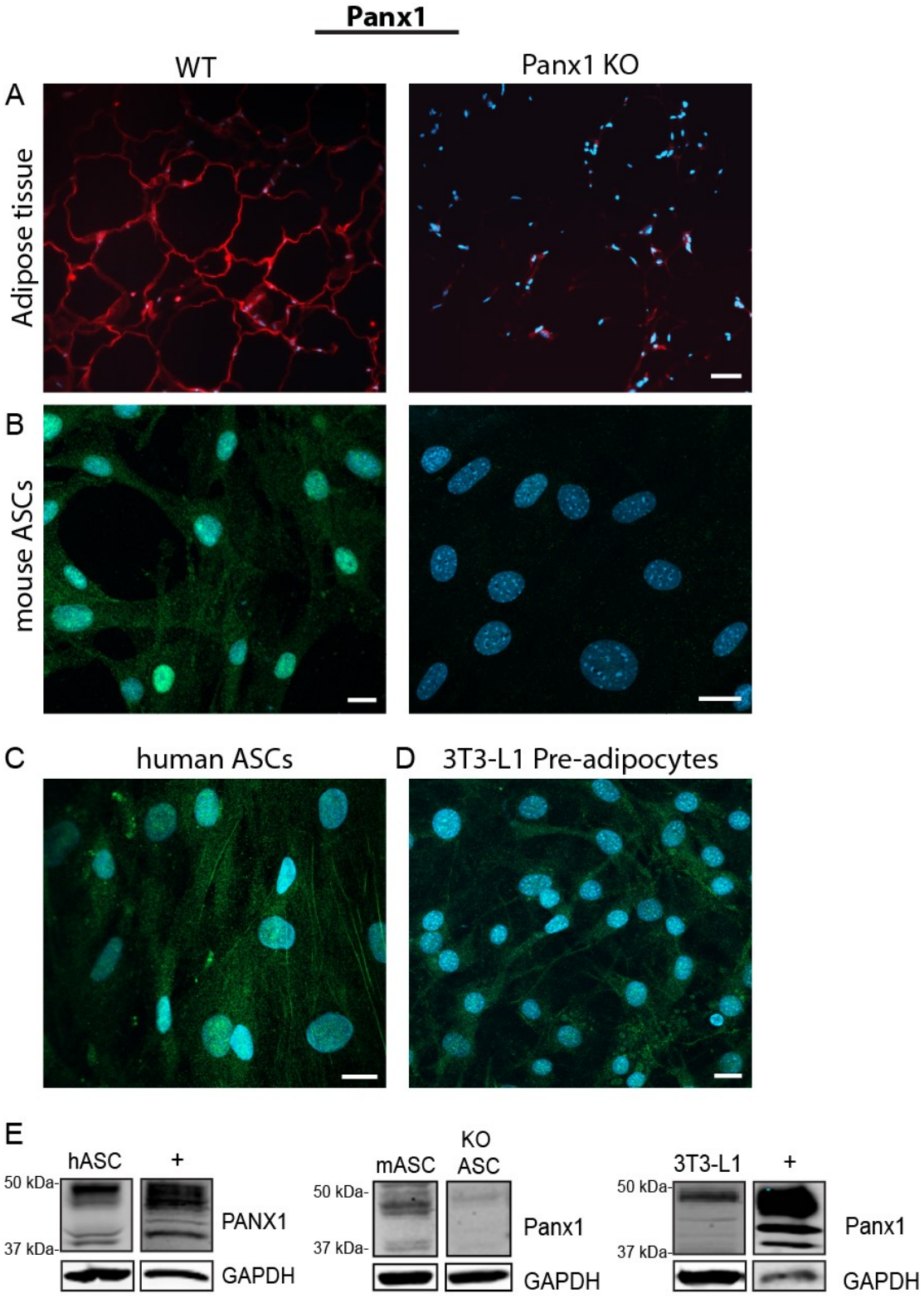

A
Body Weight
ns
Body Weight (g)
WT
Panx1 KO
B
Raw Fat Mass
**
Fat mass (g)
WT
Panx1 KO
C
Raw Lean Mass
*
Lean Mass (g)
WT
Panx1 KO
Fat Mass
**
Fat mass (g)/Body weight (g)
WT
Panx1 KO
Lean Mass
**
Lean Mass (g)/Body Weight (g)
WT
Panx1 KO
D
Body Weight
ns
Body Weight (g)
WT
Het
Panx1 KO
E
Raw Fat Mass
a
a
b
Fat mass (g)
WT
Het
Panx1 KO
F
Raw Lean Mass
ns
Lean Mass (g)
WT
Het
Panx1 KO
Fat Mass
***
***
Fat mass (g)/Body weight (g)
WT
Het
Panx1 KO
Lean Mass
ns
Lean Mass (g)/Body Weight (g)
WT
Het
Panx1 KO

2.3.3 Panx1 is expressed in adipose tissue, adipose-derived stromal cells (ASCs), and pre-adipocytes.

Since we observed an increase in fat accumulation in the Panx1 KO mice, it led us to investigate if Panx1 could be regulating the proliferation and differentiation of the adipogenic cell populations within adipose tissue. Adamson et al., 2015 previously reported that Panx1 is present in perigonadal and perivascular adipose tissue of C57/BL6 mice fed on a normal chow diet, therefore we evaluated if the same was true for other depots of white adipose tissue. We labelled white adipose tissue from WT and Panx1 KO male mice via immunohistochemistry and observed that Panx1 was expressed, however localization of Panx1 could neither be determined at the cell surface nor intracellulary, as cytoplasm of adipocytes are difficult to visualize (Fig. 3A). We also confirmed that the signal was ablated in the Panx1 KO white adipose tissue (Fig. 3A). We placed WT and Panx1 KO mice on a high fat diet (HFD) in order to isolate a greater number of adipose-derived cells for *in vitro* assays. We successfully isolated primary ASCs from WT and Panx1 KO mice. In WT ASCs in culture, we observed that Panx1 was expressed mostly in the intracellular compartment (Fig. 3B) and Panx1 expression was absent in the ASCs isolated from Panx1 KO mice, as expected. To ensure that we were utilizing a translatable *in vivo* model, we also isolated human ASCs derived from breast tissue of two female donors (reduction surgery patient), and immunofluorescence (IF) with anti-human PANX1 antibodies (Fig. 3C), revealed a similar intracellular pattern to that observed with anti-mouse Panx1 antibodies in mouse ASCs and the 3T3-L1 adipocyte cell line (Fig. 3B, D). When protein lysates from these cell cultures were run on a Western blot we saw that human ASCs, WT mouse ASCs and pre-adipocyte 3T3-L1 cells exhibited the characteristic multi-banding pattern of Panx1 due to the different glycosylation species (Fig. 3E). Thus, Panx1 was shown to be expressed in the pre-adipocyte

3T3-L1 cell line, white adipose tissue, mouse and human ASCs, that were used in the present studies.

Figure 2.3 Panx1 is expressed in visceral adipose tissue, mouse and human adipose-derived stromal cells (ASCs), and 3T3-L1 pre-adipocytes. A. Fluorescent micrographs of WT and Panx1 KO adipose tissue from male mice fed on a high fat diet (HFD), depicting Panx1 (red) labeling in the WT and absent in the KO. B. ASCs isolated from adipose tissue of WT and KO mice on HFD and grown in culture, show Panx1 expression (green), that is ablated in the KO cells. C. Human ASCs (hASCs) isolated from female breast adipose tissue express PANX1 (green). D. 3T3-L1 pre-adipocyte cells in culture stained for Panx1 (green) E. Corresponding Western blots of cell lysates from hASCs, WT and Panx1 KO mouse ASCs (mASC), and pre-adipocytes. A375 human melanoma cells used as a PANX1 positive control (+) and 293T cells ectopically expressing mouse Panx1 as (+) control. GADPH used as loading control. Nuclei (blue), scale bars=44 (A), 20 μm (B-D). Identical microscope parameters were maintained to compare between samples.


Panx1
WT
Panx1 KO
A
Adipose tissue
B
mouse ASCs
C
human ASCs
D
3T3-L1 Pre-adipocytes
E
hASC
+
50 kDa-
37 kDa-
PANX1
GAPDH
mASC
KO ASC
50 kDa-
37 kDa-
Panx1
GAPDH
3T3-L1
+
50 kDa-
37 kDa-
Panx1
GAPDH

2.3.4 Lack of Panx1 causes a reduction in ASC proliferation

Since Panx1 is expressed in ASCs (Fig. 3B) and there have been no documented reports of the role of Panx1 in ASCs or in early adipocyte development, we chose to initially assess cell proliferation and viability. We isolated ASCs from the inguinal fat of WT and Panx1 KO male littermate mice on a HFD, expanded the cells in culture, and assessed proliferation at passage 2 by systematic cell counts over 7 days. We observed that Panx1 KO ASCs grew significantly slower, with an approximately 50% reduction in total cell number compared to WT ASCs at days 5 and 7 in culture (N=3, n=3, $P<0.01$, $P<0.001$). (Fig. 4A). This reduction in proliferation was also observed in Panx1 KO ASCs isolated from the epididymal fat depot (N=3, n=3 $P<0.001$) (Fig. S1B). In order to assess whether this reduction in cell number was an effect of reduced cell proliferation and/or increased cell death, we immuno-labeled the cells at day 5 with the proliferation marker, Ki67 and the apoptosis marker, cleaved caspase 3 by immunocytochemistry. Panx1 KO ASCs proliferated significantly less than WT cells, with a ~20% reduction in Ki67 positive cells (N=3, n=15, $P<0.001$) (Fig. 4B). When assessing cleaved caspase 3, we saw no significant difference in expression between WT and Panx1 KO ASCs, indicating there was minimal cell apoptosis in both populations (N=3, n=15) (Fig. 4C). Therefore based on these results, Panx1 KO ASCs proliferate significantly slower than WT ASCs, but there was no effect on cell death. Thus, Panx1 regulates ASC proliferation.

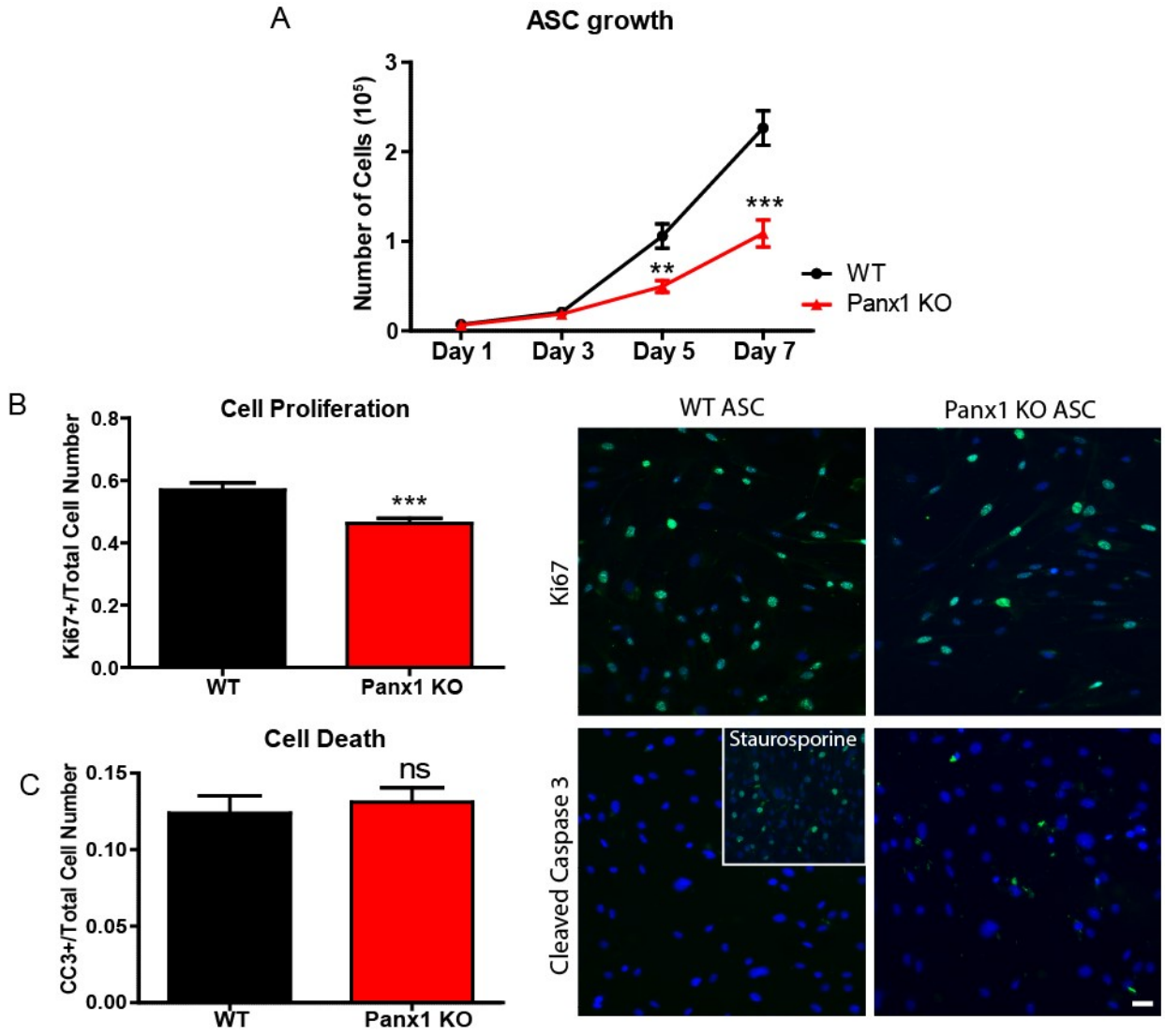


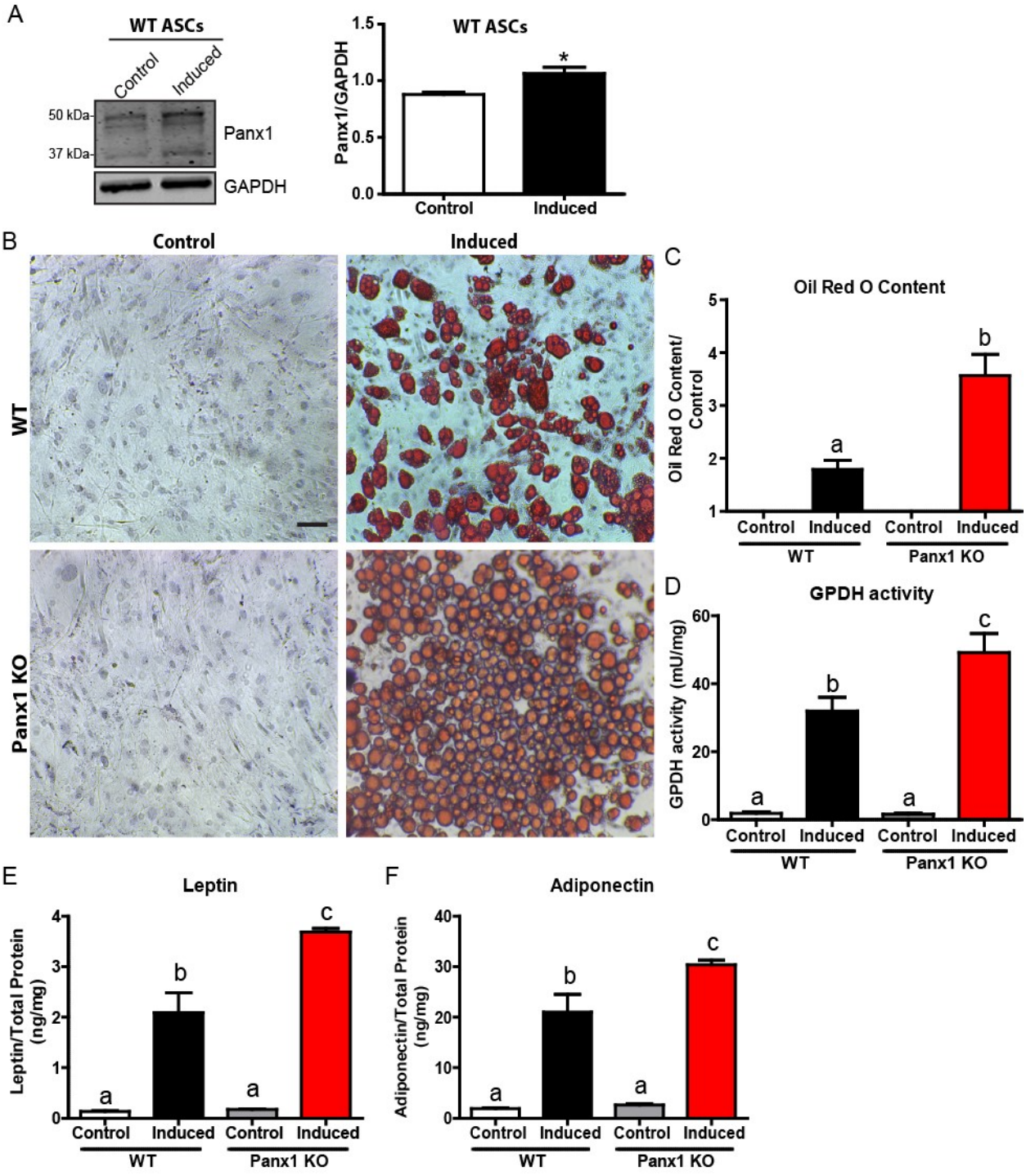
Figure 2.4 Lack of Panx1 causes a reduction in ASC proliferation. A. Panx1 KO ASCs show significantly decreased growth compared to WT ASCs in a growth curve assay over 7 days. B-C. Quantification of cell proliferation (Ki67, green) and cell death (cleaved caspase 3, green), with corresponding fluorescent micrographs from WT and Panx1 KO ASCs at day 5. Positive control (inset) of staurosporine induced WT ASC cell death. Panx1 KO ASCs show significantly reduced cell proliferation, and no difference in cell death compared to WT ASCs. Nuclei (blue). Scale bar= 40um. Identical microscope parameters were maintained to compare between samples. A: N=3, n=3, B-C: N=3. N=15, **P<0.01, ***P<0.001, means ± s.e.m.

2.3.5 Panx1 regulates adipogenic differentiation.

We determined that Panx1 expression was slightly upregulated after 14 days of adipogenic induction (by approximately 18%) in WT ASCs, suggesting that Panx1 may be regulated during adipogenic differentiation (N=3, n=6, $P<0.05$) (Fig. 5A). WT and Panx1 KO ASCs were grown in culture in parallel and induced to differentiate in adipogenic differentiation medium over 14 days. When stained with the lipid marker oil red O, both WT and Panx1 KO ASCs were able to differentiate and develop characteristic round lipid droplets (Fig. 5B). In contrast, there was no lipid accumulation in control cells maintained in proliferation medium. In comparing the induced populations, we observed that the Panx1 KO ASCs had a more homogeneous response, with a higher fraction of cells that were accumulating intracellular lipid and a more mature unilocular morphology, as compared to WT ASCs (Fig. 5B). In fact, the Panx1 KO ASCs differentiated so extensively that the cells were beginning to detach from the plate by 14 days, with a small number of mature adipocytes observed in suspension. To further confirm the enhanced lipid content, we extracted the intracellular oil red O dye from the stained WT and Panx1 KO ASCs and quantified the levels by absorbance spectroscopy. Panx1 KO induced ASCs had significantly increased oil red O content (approximately 45% more) as compared to WT induced ASCs (N=3, n=6, $P<0.01$) (Fig. 5C). To further compare the differentiation response, we measured intracellular GPDH activity and observed that the induced Panx1 KO ASCs had significantly higher adipogenic enzyme activity than the induced WT ASCs by approximately 35% (N=3, n=9 $P<0.05$) (Fig. 5D). As a final measure of terminal differentiation, we assessed leptin and adiponectin expression levels in conditioned media from induced and control Panx1 KO and WT ASCs. These adipokines are regarded as late-stage markers of adipogenic differentiation and therefore provide a measure of the maturity of the differentiating cell populations [38,39]. Consistent with the other markers, we saw that Panx1 KO induced ASCs had significantly increased leptin

(about 40%) and adiponectin (approximately 27%) levels within their conditioned media as compared to WT induced ASCs (N=3, n=9, $P<0.05$) (Fig. 5E, F). Therefore, the lack of Panx1 can dysregulate ASC differentiation, enhancing adipogenic differentiation capacity and intracellular lipid accumulation as compared to WT ASCs.

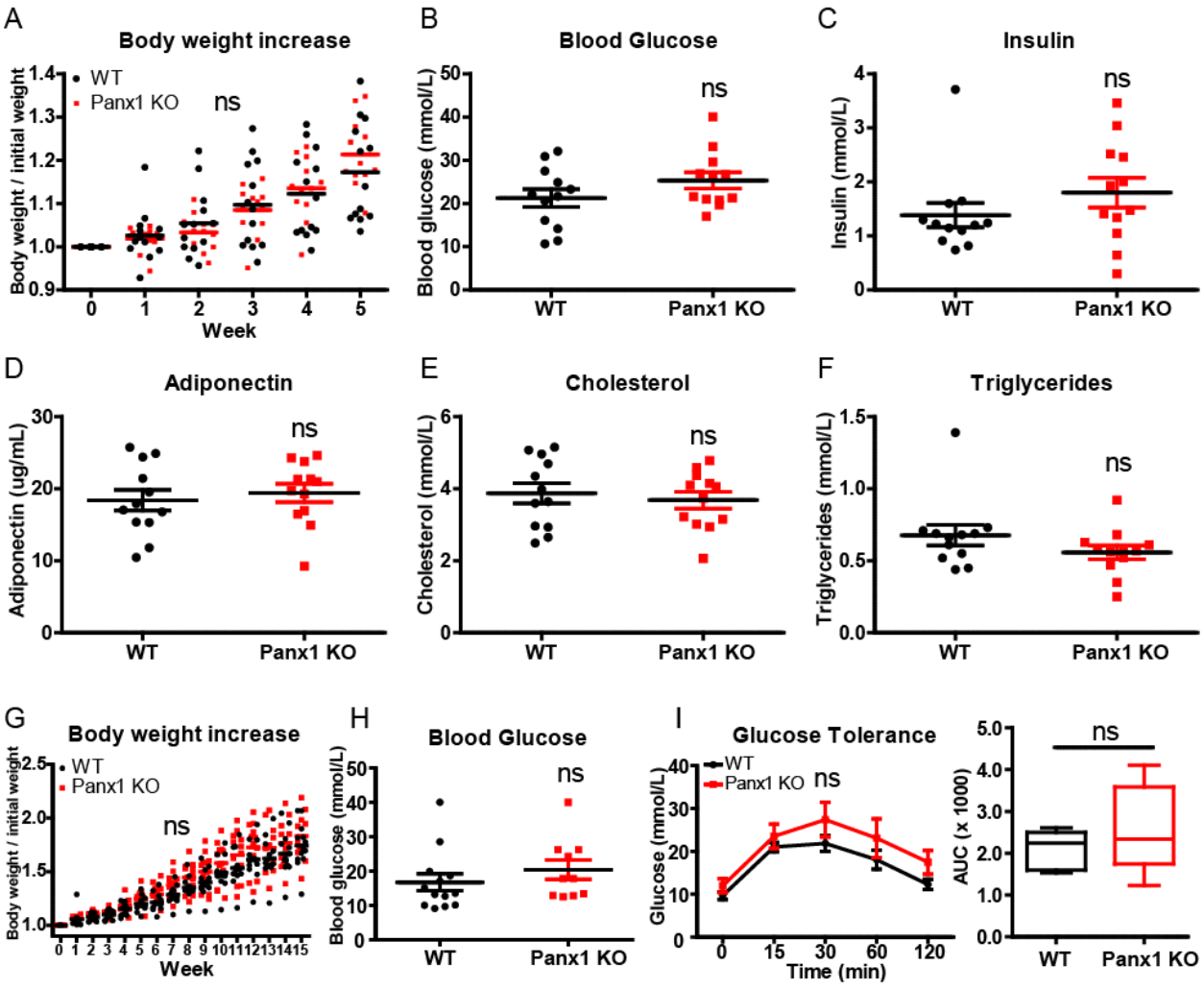
Figure 2.5 Panx1 expression is increased after adipogenic induction in murine ASCs and lack of Panx1 enhances adipogenic differentiation. ASCs from WT and Panx1 KO male mice fed on a HFD were cultured and induced to differentiate into adipocytes for 14 days. A. Western blot and respective quantification of Panx1 expression in WT ASCs showing that expression significantly increases after adipogenic induction. B. Oil red O staining of WT and Panx1 KO ASCs, control cells (left) and induced cells (right) scale bar= 100µm. C. Panx1 KO ASCs revealed increased oil red O content compared to WT ASCs. D. Panx1 KO ASCs showed significantly increased GPDH activity compared to WT ASCs. E-F. ELISAs for leptin and adiponectin performed on conditioned media of ASCs. Panx1 KO ASCs have significantly increased leptin and adiponectin content compared to WT ASCs. A-D. N=3, n=6, E-G. N=3, n=9,*$P<0.05$, different letters denote significant differences (b: $P<0.01$, c: $P<0.05$), means ± s.e.m.


A
WT ASCs
Control
Induced
50 kDa
37 kDa
Panx1
GAPDH
WT ASCs
Panx1/GAPDH
1.5
1.0
0.5
0.0
*
Control
Induced
B
Control
Induced
WT
Panx1 KO
C
Oil Red O Content
Oil Red O Content/ Control
5
4
3
2
1
a
b
Control
Induced
Control
Induced
WT
Panx1 KO
D
GPDH activity
GPDH activity (mU/mg)
60
40
20
0
a
b
c
Control
Induced
Control
Induced
WT
Panx1 KO
E
Leptin
Leptin/Total Protein (ng/mg)
4
3
2
1
0
a
b
c
Control
Induced
Control
Induced
WT
Panx1 KO
F
Adiponectin
Adiponectin/Total Protein (ng/mg)
40
30
20
10
0
a
b
c
Control
Induced
Control
Induced
WT
Panx1 KO

2.3.6 Panx1 KO mice on a HFD show no weight gain differences, but display increased activity

Based on our findings that Panx1 KO mice at baseline had significantly increased fat mass (Fig. 2), we set out to determine whether there would be overt phenotypic effects in the context of a high fat diet. We hypothesized that Panx1 KO mice on a high fat diet (HFD, 60% kcal from fat) would show increased weight gain. Initially, we placed congenic WT and Panx1 KO mice on either 5 or 15 week HFDs and observed no significant differences in body weight increase between WT and Kos (WT N=13, KO N=12) (Fig. 6A, G). We also looked at blood lipids and various metabolic markers associated with obesity and fat accumulation (blood glucose, insulin, adiponectin, cholesterol, and triglycerides) but found no significant differences between groups (Fig. 6B-F). Finally, we assessed glucose tolerance and found no significant differences between WT and Panx1 KO mice (Fig. 6H and I).

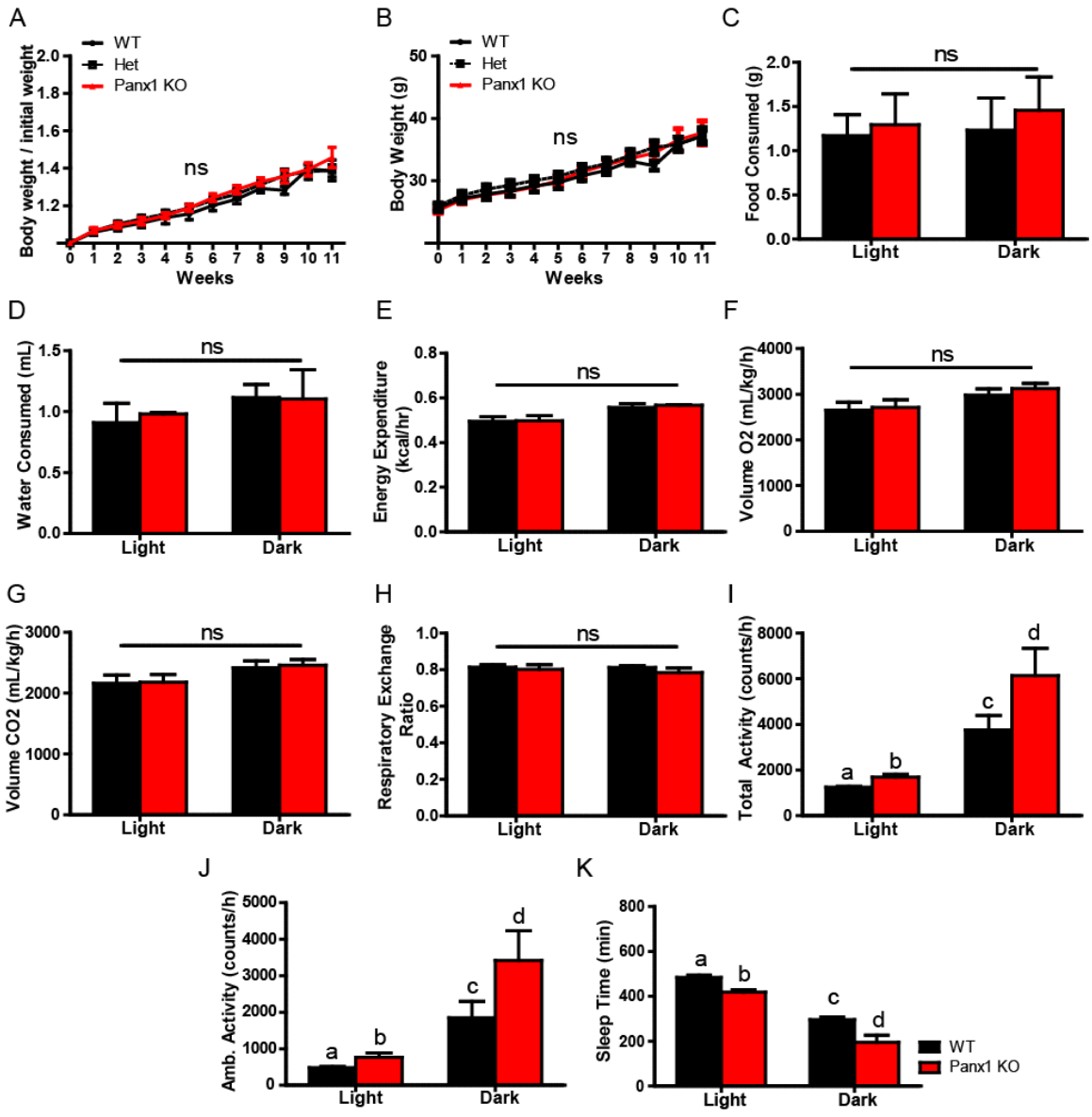
Figure 2.6 Panx1 KO mice on a HFD show no weight gain differences and no changes in blood markers. WT and Panx1 KO congenic male mice were fed on either a 5-week (A-F) or 15-week (G-I) high fat diet (60% kcal from fat) starting at 3 months of age. A, G. Panx1 KO mice show no difference in body weight increase in either 5- or 15-week HFD. B-F. Fasted blood glucose (measured by glucometer), insulin, adiponectin, cholesterol, and triglyceride hormone levels (serum ELISA of WT and Panx1 KO mice fed on a 5 week high fat diet). There were no significant differences between WT or Panx1 KO groups. H-I. Fasted blood glucose collected from WT and Panx1 KO mice fed on a 15-week high fat diet, in addition to a glucose tolerance test where mice were administered 1 g/kg of glucose by intraperitoneal injection and blood glucose was monitored over time. Fasted blood glucose and glucose tolerance test revealed no significant difference between WT or Panx1 KO mice with no difference in combined area under the curve (AUC) analysis. WT N= 13, Panx1 KO N=12, means ± s.e.m.


A
Body weight increase
WT
Panx1 KO
ns
Body weight / initial weight
Week
B
Blood Glucose
ns
Blood glucose (mmol/L)
WT
Panx1 KO
C
Insulin
ns
Insulin (mmol/L)
WT
Panx1 KO
D
Adiponectin
ns
Adiponectin (ug/mL)
WT
Panx1 KO
E
Cholesterol
ns
Cholesterol (mmol/L)
WT
Panx1 KO
F
Triglycerides
ns
Triglycerides (mmol/L)
WT
Panx1 KO
G
Body weight increase
WT
Panx1 KO
ns
Body weight / initial weight
Week
H
Blood Glucose
ns
Blood glucose (mmol/L)
WT
Panx1 KO
I
Glucose Tolerance
WT
Panx1 KO
ns
Glucose (mmol/L)
Time (min)
ns
AUC (x 1000)
WT
Panx1 KO

We also placed littermate (WT, heterozygotes, and Panx1 KO) male mice at 3 months of age on an 11 week HFD and assessed weight and metabolic parameters. We observed that littermates showed no significant differences in body weight increase (WT N=6, Het N=13, Panx1 KO N=13) (Fig. 7A) or raw body weight (Fig. 7B) during the HFD. We placed this cohort of mice individually in metabolic cages (WT N=4, Panx1 KO N=4) in order to assess multiple metabolic parameters during their active period (dark) and their sleep period (light) for 24 hours after acclimation. Food and water consumption was monitored but showed no significant differences between WT and Panx1 KO mice (Fig. 7C, D). Energy expenditure was also monitored, but there was no significant difference between groups of mice (Fig. 7E). Volume of O_2 and CO_2 showed no significant differences between mice (Fig. 7F, G). The respiratory exchange ratio (RER) (the rate at which CO2 is produced over the rate of O2 consumed) was monitored and we expected to see changes in RER because it is a measure of how efficiently the body oxidizes fuel sources (carbohydrates or fats). However, we did not observe any differences between our mice (Fig. 7H), suggesting that the diet affected the RER of both WT and Panx1 KO mice to an equal extent. We also measured total activity of the mice and interestingly, the Panx1 KO mice had significantly increased total activity both in the light (26% increase) and dark periods (38% increase) as compared to the WT mice ($P<0.01$) (Fig. 7I). Further, we saw that the Panx1 KO mice had significantly greater ambulatory activity both in light (38% increase) and in dark conditions (46% increase) ($P<0.01$) (Fig. 7J). Finally, we assessed sleep duration, and found that the Panx1 KO mice were sleeping significantly less than the WT mice during both the light (13% reduction) and dark (34% reduction) periods ($P<0.01$) (Fig. 7K). Taken together, the Panx1 KO mice showed no overt weight phenotypes compared to the WT

mice, however they showed significantly increased total activity, total ambulatory activity, and they slept significantly less.

Figure 2.7 Littermate WT, Het and Panx1 KO mice show no differences in weight increase on a high fat diet, but the Panx1 KO mice exhibit increased activity in metabolic cages. WT, Heterozygous (Het), and Panx1 KO, male littermate mice were fed on an 11-week high fat diet starting at 3 months of age. A-C. Panx1 KO mice show no difference in body weight increase, body weight, or food consumption. C-K. Mice were individually placed in metabolic cages to assess metabolism and activity during their sleep period (light) and during their active period (dark). Panx1 KO mice showed significantly increased total activity (I) and ambulatory activity (J), with significantly reduced sleep duration (K), while all other parameters remained unchanged. A-B: WT N=6, Het N=13, Panx1 KO N=13. C-K: WT N=4, Panx1 KO N=4. Different letters denote significant differences. $P<0.01$, means ± s.e.m.


A
WT
Het
Panx1 KO
Body weight / initial weight
ns
Weeks
B
WT
Het
Panx1 KO
Body Weight (g)
ns
Weeks
C
Food Consumed (g)
ns
Light
Dark
D
Water Consumed (mL)
ns
Light
Dark
E
Energy Expenditure (kcal/hr)
ns
Light
Dark
F
Volume O2 (mL/kg/h)
ns
Light
Dark
G
Volume CO2 (mL/kg/h)
ns
Light
Dark
H
Respiratory Exchange Ratio
ns
Light
Dark
I
Total Activity (counts/h)
a
b
c
d
Light
Dark
J
Amb. Activity (counts/h)
a
b
c
d
Light
Dark
K
Sleep Time (min)
a
b
c
d
Light
Dark
WT
Panx1 KO

2.4 Discussion

It has been well established that Panx1 plays important functions in proliferation and differentiation of many cell types, however there have been no reports on whether this is true for adipogenic cell populations. We have shown for the first time that Panx1 regulates the proliferation and differentiation of ASCs into mature adipocytes, and the germline deletion of Panx1 in ASCs leads to increased adipogenic differentiation and fat accumulation. We have also shown *in vivo* that the global Panx1 KO mouse model has significantly more fat mass than WT controls. However, the KO mice do not gain more weight under an intense high fat diet, which may be due to their increased activity and decreased sleep relative to their WT counterparts.

The first and only report on Panx1 being expressed in adipose tissue by Adamson et al., 2015 proposed an elegant mechanism in which insulin activates the release of ATP from the Panx1 channels, which in turn causes a signaling cascade indirectly allowing the transport of glucose into the cell[25]. They also established that blocking Panx1 channels using pharmacological inhibitors significantly reduced glucose uptake in 3T3-L1 pre-adipocyte cells. Additionally, they went on to use an adiponectin-inducible Cre recombinase to delete the *Panx1* gene from mature adipocytes, generating an adipocyte-specific Panx1 knockout mouse model[25]. With this model, they found slight diet-induced insulin resistance in the conditional KO, with no changes in body mass composition, metabolic parameters, or activity under a high fat diet[25].

This group also assessed body mass composition in Panx1 adipose-specific knockout mice on a 12-week high fat diet, and found no significant differences, but observed some trends towards increased circulating blood glucose and increased insulin resistance[25].

Our study differs from the previous study as we have chosen to utilize a global Panx1 KO mouse with a constitutive germline deletion of Panx1, whereas Adamson et al., 2015[25] assessed mature adipocyte-specific Panx1 knockout mice, driven from an adiponectin promoter.

Adiponectin is expressed only in terminally differentiated adipocytes, thus the knockout of Panx1 in this model occurs at the final stages of adipocyte development and would not affect early precursor cells[40], such as ASCs. Additionally, this adipocyte-specific promoter is induced by doxycycline, which is an additional variable for the assessment of metabolism and weight, potentially altering food consumption and possibly having off target effects[41]. Therefore, the global Panx1 KO mouse model provides a unique approach at assessing Panx1 during early adipocyte development and it highlights the striking differences in fat accumulation, proliferation and differentiation that were observed. On the other hand, global KO models also present limitations, since the germline deletion of the *Panx1* gene may be compensated throughout development by other members of the pannexin family[33,42], or other channel proteins with similar function, and the resulting phenotypes may be attenuated or masked by this effect.

We have demonstrated that the global Panx1 KO mice have significantly greater fat mass than WT mice fed on a regular chow diet. Similar to our results, it was demonstrated that Panx1 KO mice fed on a regular chow diet had a significantly increased area of hypodermal fat under a thinner dorsal skin[33]. We saw no differences in overall body weight, suggesting that the amount of fat that is increased in the Panx1 KO mice is subtle. However, we did observe that the overall lean mass in some cohorts was significantly decreased, which could compensate for the increases in fat mass. Panx1 is also expressed in bone[43], muscle[34] and other organs[24] that could be altered in this global KO, which may account for the lack of differences in body weight under normal chow.

Our studies confirm that Panx1 is present in adipose tissue visceral depots that are commonly associated with metabolic disorders in obesity. We have demonstrated that Panx1 is present not only in mouse white adipose tissue, but also in murine and human ASCs, and in the

pre-adipocyte 3T3-L1 cell line. Our preliminary data suggests similarity in the Panx1 expression patterns in both human and murine ASCs that reinforces the translational potential of our research.

In our study, the majority of Panx1 expression was found intracellularly, with the exception of adipose tissue, where it appeared to localize at the cell surface. However, the cytoplasm of adipose cells *in vivo* is difficult to visualize since they are filled with lipid droplets. Panx1 is typically at the cell surface when ectopically expressed, however it has been reported in the literature that endogenous Panx1 can localize to the intracellular compartments, in tissues such as skin[18], skeletal muscle[34], canine cardiac myocytes[29], or in the retina[44]. In primary cells and cell lines, it is common for endogenous Panx1 to localize intracellularly such as in: primary dermal fibroblasts[33], mouse melanoma cell lines[28] and when ectopically expressed in some reference cell lines[43]. Intracellularly, Panx1 has been proposed to act in the ER as a calcium leak channel[45]. Interestingly, intracellular calcium has been demonstrated to be key regulator of keratinocyte differentiation via many different signaling pathways by activation of kinases and phospholipases[46]. In addition, intracellular calcium release and uptake from the sarcolemma and sarcoplasmic reticulum signaling plays a role in embryonic stem cell-derived cardiomyocytes differentiation[47]. Finally, calcium from the endoplasmic reticulum has been shown to play a role in adipocyte differentiation, where pharmacological blockade of calcium release by thapsigargin results in a dose-dependent inhibition of adipocyte differentiation[48]. It is possible that Panx1 in ASCs may act through an intracellular function of the channels as a mediator of adipogenic differentiation.

Panx1 regulates the proliferation of primary cell types including dermal fibroblasts and keratinocytes[33], osteoblasts[24], and skeletal muscle myoblasts[34]. Our results show that lack of

Panx1 expression results in significantly reduced cell proliferation. Consistent with this data, in a previous study assessing Panx1 function in mammary gland development, lactating mice globally lacking Panx1 showed reduced alveolar development and reduced cell proliferation within the mammary glands[49]. Contrastingly, primary dermal fibroblasts isolated from the same Panx1 KO model proliferated more and were less responsive to TGFβ stimulation than WT controls, but presented already high levels of α-SMA, a marker of myofibroblasts differentiation, suggesting that they may have been primed towards differentiation[33] similar to our findings with the KO ASC differentiation pattern. In that same study, Panx1 expression was decreased in WT keratinocytes induced to differentiate *in vitro*[33], while we observe a slight increase in Panx1 levels of ASCs induced to differentiate. In skeletal muscle myoblasts, Panx1 is regulated throughout differentiation and overexpression results in cell differentiation *in vitro*[34]. Taken together, we propose that Panx1 regulates the processes of cellular proliferation and differentiation in a cell type-specific manner.

To date, little is known on the role of Panx1 in precursor or stem-like cells throughout differentiation. Most prominently, one group has reported that Panx1 is expressed in postnatal neural stem and progenitor cells, and by either inhibiting or overexpressing Panx1, they observed reductions or increases in cell proliferation, respectively[36]. Our results revealed that lack of Panx1 results in significantly decreased proliferation and increased differentiation in ASCs. We postulate that Panx1 KO ASCs may be more pre-committed to the adipogenic lineage, as evidenced by the more homogeneous differentiation response and mature phenotype observed over the 14 day culture period. Given that there is an inverse relationship typically observed between proliferation and adipogenesis, with the cells undergoing growth arrest at the onset of differentiation[9], the reduced proliferation rates observed in the Panx1 KO ASCs are consistent

with this interpretation. Additionally, it has been reported in the literature that mouse ASCs from either inguinal or perigonadal depots show differences in their ability to cope with HFD by either delineating between hypertrophy and hyperplasia. ASCs from inguinal depots have been shown to be more hyperplastic with greater amount of adipogenic progenitors (more amenable for isolation), compared to the perigonadal depots which are more prone to hypertrophy, suggesting depot dependent fat accumulation mechanisms[50]. Thus, lack of Panx1 may alter the response to HFD in inguinal ASCs, causing them to behave in a more hypertrophic (and less hyperplastic) manner as observed by the increased intracellular lipid content in the Panx1 KO cells. However, we also observed reduced cell numbers in epididymal ASCs from Panx1 KO perigonadal fat pads (Suppl. 1) indicating that the lack of Panx1 may affect other white adipose depots in a similar manner. Alternatively, the lack of Panx1 may also alter the composition of the ASC population causing greater uniformity and commitment towards the adipogenic lineage. There is evidence that multipotent mesenchymal stem cell (MSCs) clones are highly proliferative, whereas unipotent cells exhibit significantly slower growth rates[51], and lineage commitment leads to reduced proliferation[52]. This is consistent with our results where Panx1 KO ASCs have significantly reduced proliferation, which may indicate commitment towards the adipogenic lineage compared to WT ASCs.

Our data has shown that there were no differences in weight gain in the Panx1 KO mice. We expected that the excess fat mass in the KO mice under regular chow would contribute to increased weight gain when challenged with a significantly higher amount of fat in the diet. It is possible that the HFD that we chose at 60% kcal from fat (Western HFD chow is only 45% kcal) may have had a saturating effect on the weight phenotype[53]. We cannot rule out other effects of the global Panx1 deletion in other organs where the protein is expressed, or the effect of

compensation by other channel proteins that may prevent the detection of a more overt weight phenotype. However, the options for a tissue specific Cre-deleter are limited since the Adiponectin Cre is the most specific one, but as shown by Adamson et al., 2015[25] is only effective when adipocytes are fully mature and would miss the early stages of adipogenesis where Panx1 plays a key role. In our model, we did not detect differences in weight gain. However, overall activity and ambulatory activity were significantly increased in the KO mice, and they also slept significantly less. We propose that these increases in activity and reduction in sleep may counteract any differences in weight gain. In a different global Panx1 KO model[54], it has also been established that the mice exhibit greater anxiety[55] and greater motility[56], thus demonstrating a collective role for Panx1 in increased activity. It is well known in the literature that Panx1 is present in the brain[35], and thus a global Panx1 knockout could have a multitude of effects across the body, including at a behavioral level. It is possible that our global Panx1 knockout could be affecting neurological aspects of behavior in the mice, as it has been reported that the Panx1 KO mice possess mechanisms of neuroprotection against ischemia-induced neurodegeneration[57] and seizures[58]. This is an intriguing finding, as lack of Panx1 in the brain may result in changes in behavior, causing dysregulation in brain centers responsible for sleep and activity. Panx1 channels can be activated by extracellular potassium[59] and Panx1 has been reported to interact with the potassium channel subunit Kvβ3 mediating redox potentials[60]. In a knockout model of Kv3-type inhibitory fast-spiking potassium channels, mice exhibited increased seizure susceptibility[61] and significantly reduced sleep with associated hyperactivity[62]. As such, Panx1 may also interact with Kv3-type channels regulating activity and sleep. It should be noted that there are many different factors that regulate sleep and activity, which should be further pursued in the context of the Panx1 KO mouse.

In summary, we have shown for the first time that Panx1 regulates both the proliferation and differentiation of adipogenic cells resulting in increased fat accumulation. We have demonstrated that Panx1 KO mice have higher fat content but show no overt weight gain increase on a high fat diet, potentially due to their increased activity and sleep alterations. Taken together, we have identified Panx1 as a novel regulator of ASC proliferation and differentiation, and subsequently, a key component of the regulation of fat accumulation, representing a potential new target for obesity intervention.

2.5 Materials and Methods

3T3-L1 cell culture

3-day transfer, inoculum $3x10^5$ mouse embryonic fibroblast pre-adipocyte (3T3-L1) cells (ATCC) were grown in culture and induced to differentiate once confluent. Undifferentiated cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, 1% Pen-Strep, and 10% calf serum (Thermo Fisher Scientific) and cells beyond passage 10 were not included in studies. Adipogenic media was composed of: DMEM with 4.5 g/L glucose (Thermo Fisher Scientific), 10% fetal bovine serum (Thermo Fisher Scientific), 1% Pen-Strep, 100 µg/mL of IBMX, 390 ng/mL dexamethasone, and 5 µg/mL insulin (Sigma Aldrich), and was replaced or supplemented every other day as described previously[63] over 14 days.

Animals and ethics

Experiments performed on animals were approved by the Animal Care Committee of the University Council on Animal Care at the University of Western Ontario, London ON, Canada. Panx1 KO mice were originally obtained from Genentech (San Francisco, CA) and were previously described[37]. These mice were backcrossed with the WT mice: C57BL/6N strain mice from Charles River Canada (Saint-Constant, PQ) until a congenic line was obtained. Human samples were obtained from breast reduction surgeries from consented patients at the London Health Research Centre, according to an approved ethics protocol (REB# 105426).

High fat diet

Congenic and littermate WT and Panx1 KO male mice were placed on either a 5-, 15-, or 11-week high fat diet (HFD, 60% kcal from fat, TestDiet 58Y1) and fed *ad libitum*. Mice were 3 months old at the start of the experiments and initially fed regular chow diet (6.2% kcal from fat, Harlan 2018).

Body mass composition

Fat and lean mass composition of 12 month-old mice, in addition to 6 month-old littermate male mice were measured using quantitative magnetic resonance (echo-MRI) analysis with an echo magnetic resonance imaging mobile unit (Avian Facility of Advanced Research, University of Western Ontario, London, ON, Canada) as described by Guglielmo, et al., 2011[64] with the modification of placing live mice in the apparatus and measuring on the "bird" setting omitting water content. Measurements were taken in duplicate to ensure consistency of the results.

Immunofluorescence

White adipose tissue (WAT) was isolated from WT and Panx1 KO mice after a 15 week high fat diet, by incising the abdomen of the mouse and excising all visible epididymal fat. Adipose tissue was fixed in 10% neutral buffered formalin overnight, processed at the Robarts Research Pathology facility and embedded in paraffin. Paraffin sections were taken at 6 μm thickness, sections were deparaffinized and immune-labeled with Panx1-CT primary antibody in a (stock 1ug/ul) 1:500 dilution as described previously by Penuela et al., 2007[24]. For proliferation studies, cell were labelled with active cell cycle phase specific marker Ki-67 in a 1:1000 dilution (Abcam, Cambridge, UK), and for cell death assays cells were labelled with CellEvent (Thermo Fisher Scientific) Caspase-3 Green Detection Reagent following manufacturer's protocols, and counterstained with Hoechst nuclei stain in a 1:1000 dilution, as described previously after 5 days in culture[33]. Staurospoine was used to induce cell death in WT ASCs at day 5 in culture and was utilized as a positive control. Images of adipose tissue were collected using a Leica DM IRE2 inverted epifluoresence microscope equipped with a 20X objective, while images of cells were collected using an LSM 800 inverted confocal microscope equipped with a 40X water

objective (Carl Zeiss, Jena, Germany). Identical parameters were maintained to compare between samples.

Adipose-derived stromal cell isolation

ASCs were isolated as previously described by Yu et al., 2011[66] from WT and Panx1 KO littermate male mice fed on the 11 week HFD, with the modification of isolating cells from the inguinal adipose depot (or epididymal fat pads, supplemental figure 1) and cells were filtered through a 100μm filter to remove debris prior to cell seeding. Fat from up to three mice were pooled together for each separate isolation. Cells were seeded at high density (80 000 cells/cm^2) and rinsed 24 hours after isolation with sterile PBS, cells were passaged when confluent (approximately 7 days). ASCs were grown in DMEM: Ham's F-12 (Sigma Aldrich), supplemented with 10% fetal bovine serum and 1% Pen-Strep and growth medium was changed every 2 days. ASCs used for assays were grown up to Passage 2. Human ASCs were isolated from two female breast adipose tissue donors and isolation was conducted as described previously[65].

Western Blotting

Western blots of protein lysates (primary cells and cell lines) were conducted as described previously by Penuela et al., 2007[24]. Cell lysates were collected with a Triton-based extraction buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% Np-40, 100 mM NaF, 100 mM sodium orthovanadate, proteinase inhibitor mini-EDTA tablet (Roche-Applied Science, Laval, QC)] and subsequently run on a western blot, then probed using the Panx1-CT antibody at 1:5000 dilution or 0.2ug/ml, and anti-hPANX1 (412-426) antibody at 1:1000 dilution or 1.0ug/ml with the modification of using 50 μg of protein for all blots[24]. Human embryonic kidney (293T) cells ectopically expressing mouse Panx1 were used as a

positive control for all mouse blots as described by Penuela et al., 2008[43], while A375 human melanoma cells that express endogenous PANX1 were used as a positive control for human blots. Loading control used was GAPDH (Millipore, Billerica, MA). Detection of blots was established on an Odyssey infrared imaging system (LICOR).

Proliferation and differentiation assays

ASCs from WT and Panx1 KO mice were plated for differentiation in 12 well plates at a seeding density of 10 000 cells/cm^2. Cell counts were measured in triplicate every other day up until day 7 using trypan blue (1:1) and a Countess automated cell counter (Thermo fisher Scientific). For differentiation assays, ASCs were plated in 6 well plates at a seeding density of 30 000 cell/cm^2. Adipogenic media was made as previously described[66], with the modifications of substituting 1 µg/mL troglitazone and 0.25 mM isobutylmethylxanthine (IBMX) (instead of 0.5 mM) (Sigma Aldrich) for days 1-3. After day 3, a modified adipogenic media was made, lacking troglitazone and IBMX. Modified adipogenic media was changed every other day until differentiation was complete at day 14. Differentiation assays were performed on day 14. GPDH enzyme activity was measured using the GPDH Activity Measurement Kit (Kamiya Biomedical Corporation, Seattle, WA, USA) following the manufacturer's protocols and as described previously[67]. GPDH activity was normalized to total protein content measured using the Bio-Rad Protein Assay.

Oil Red O staining

For visual inspection of differentiating cells, plates were stained with oil red O (Sigma Aldrich) after 14 days of differentiation as previously described by Flynn et al., 2007[65], with the modification of an 8 minute oil red O incubation, followed by hematoxylin counterstaining for 2 minutes. Staining was visualized on a bright field microscope and images were recorded in 4

randomly selected areas from each well. In order to quantify oil red O content, the intracellular dye was extracted by incubating the stained cells in 100% isopropanol at room temperature for 15 minutes. The absorbance of the dye extract was measured at 492 nm using a CLARIOstar (BMG Labtech) plate reader and compared against groups.

ELISA assays

Conditioned media from induced ASCs and 3T3-L1 wells, as well as non-induced controls were collected at 14 days post-induction, where the media had been conditioned for 48 hours prior to analysis. Leptin and adiponectin content in the media was measured by ELISA following the manufacturer's protocols (Crystal Chem Inc., IL, USA). Total leptin and adiponectin content was normalized to total protein content measured using the Bio-Rad Protein Assay. Additionally, ELISAs (ALPCO, NH, USA) were performed following manufacturer's protocols for insulin and adiponectin, while cholesterol was assessed by CHOD-PAP kit (Roche Diagnostics, Indianapolis, IN) and triglyceride analysis was conducted by Triglycerol/Glycerol kit (Roche Diagnostics, Indianapolis, IN) following manufacturer's protocols. Analysis for *in vivo* studies were conducted using serum collected from congenic mice fed on a 5-week HFD.

Metabolic analysis

Blood glucose analysis was conducted at the time of termination, while glucose tolerance testing was conducted one week prior to termination. Male congenic mice that were placed on a 15-week HFD were fasted 4 hours prior to testing. Fasted blood glucose was measured via a glucometer (OneTouch Ultra) and glucose tolerance testing was conducted by administration of 1 g/kg of glucose by intraperitoneal injection and blood glucose was monitored at 15, 30, 60, and 120 minutes. Metabolic analysis was assessed using the Comprehensive Lab Animal Monitoring System (CLAMS) with the Oxymax software (Columbus Instruments, Columbus, OH, USA) at

the Robarts Research Institute. Mice were individually caged and acclimated for 24 hours prior to measurement of food consumption, water consumption, energy expenditure, volume of oxygen (VO2) and carbon dioxide (VCO2), respiratory exchange ratio (RER), total activity, total ambulatory activity, and sleep duration as described previously by[68].

Statistical analysis

Statistical analyses were performed using Graph Pad Prism (GraphPad, San Diego, CA). Student's t-test or ANOVA were performed with Tukey's *post hoc* comparisons. Data are presented as mean ± SEM. N values in *in vitro* ASC assays represent pooled cells from up to 3 mice, whereas, N values in *in vivo* experiments represent individual mice. Biological replicates are indicated by N. Technical replicates indicated by n.

Author contributions

VRL performed all cell culture, primary ASC, mouse experiments and data analysis. CB and KR assisted with the initial isolation of ASCs and adipogenic assays under the supervision of LF. KB did mouse breeding and animal husbandry. RG performed the metabolic caging. SP did the experimental design, supervised experiments, data analysis and edited the manuscript along with LF. All authors reviewed the manuscript.

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2.6 References

1. World Health Organization (WHO). *Global Status Report on Noncommunicable Diseases 2014*. Geneva, Switzerland; 2014.
2. Tao W, Lagergren J. Clinical management of obese patients with cancer. *Nat Rev Clin Oncol*. 2013;10(9):519-533.
3. Westermark O, Bernard S, Buchholz BA, et al. Dynamics of fat cell turnover in humans. *Nat Lett*. 2008;453:783-787.
4. Attie AD, Scherer PE. Adipocyte metabolism and obesity. *J Lipid Res*. 2009;50 Suppl:S395-S399.
5. Cinti S, Mitchell G, Barbatelli G, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res*. 2005;46(11):2347-2355.
6. Murano I, Barbatelli G, Parisani V, et al. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *J Lipid Res*. 2008;49(7):1562-1568.
7. Otto TC, Lane MD. Adipose Development : From Stem Cell to Adipocyte. *Crit Rev Biochem Mol Biol*. 2005;40(4):229-242.
8. Gregoire FM, Smas CM, Sul HS. Understanding Adipocyte Differentiation. *Physiol Rev*. 1998;78(3):783-809.
9. Pairault J, Green H. A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc Natl Acad Sci USA*. 1979;76(10):5138-5142.
10. Cawthorn WP, Scheller EL, MacDougald O a. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. *J Lipid Res*. 2012;53(2):227-246. doi:10.1194/jlr.R021089.
11. Hauner H, Skurk T, Wabitsch M. Cultures of human adipose precursor cells. In: *Methods in Molecular Biology*. Vol 155. ; 2001:239-247. doi:10.1385/1-59259-231-7:239.
12. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol*. 2006;7(12):885-896.
13. Altiok S, Xu M, Spiegelman BM. PPARγ induces cell cycle withdrawal: inhibition of E2F/ DP DNA-binding activity via down-regulation of PP2A. *Genes Dev*. 1998;11:1987-1998.
14. Tontonoz P, Hu E, Spiegelman BM. Stimulation of Adipogenesis in Fibroblasts by PPARy2, a Lipid-Activated Transcription. *Cell*. 1994;79:1147-1156.
15. Panchin Y, Kelmanson I, Matz M, Lukyanov K, Usman N, Lukyanov S. A ubiquitous family of putative gap junction molecules. *Curr Biol*. 2000;10(13):473-474.
16. Baranova A, Ivanov D, Petrash N, et al. The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. *Genomics*. 2004;83(4):706-716.
17. Le Vasseur M, Lelowski J, Bechberger JF, Sin W-C, Naus CC. Pannexin 2 protein expression is not restricted to the CNS. *Front Cell Neurosci*. 2014;8(392):1-13.
18. Celetti SJ, Cowan KN, Penuela S, Shao Q, Churko J, Laird DW. Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation. *J Cell Sci*. 2010;123(Pt 8):1363-1372.
19. Iwamoto T, Nakamura T, Doyle A, et al. Pannexin 3 regulates intracellular ATP/cAMP levels and promotes chondrocyte differentiation. *J Biol Chem*. 2010;285(24):18948-18958.
20. Ishikawa M, Iwamoto T, Fukumoto S, Yamada Y. Pannexin 3 Inhibits Proliferation of

Osteoprogenitor Cells by Regulating Wnt and p21 Signaling. *J Biol Chem*. 2014;289(5):2839-2851.
21. Boassa D, Ambrosi C, Qiu F, Dahl G, Gaietta G, Sosinsky G. Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane. *J Biol Chem*. 2007;282(43):31733-31743.
22. Bao L, Locovei S, Dahl G. Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett*. 2004;572(1-3):65-68.
23. Scemes E, Spray DC, Meda P. Connexins, pannexins, innexins: novel roles of "hemi-channels." *Pflugers Arch*. 2009;457(6):1207-1226.
24. Penuela S, Bhalla R, Gong X-Q, et al. Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. *J Cell Sci*. 2007;120(Pt 21):3772-3783.
25. Adamson SE, Meher AK, Chiu Y, et al. Pannexin 1 is required for full activation of insulin-stimulated glucose uptake in adipocytes. *Mol Metab*. 2015;4(9):610-618.
26. Diezmos EF, Sandow SL, Markus I, et al. Expression and localization of pannexin-1 hemichannels in human colon in health and disease. *Neurogastroenterol Motil*. 2013;25(6):e395-e405.
27. Penuela S, Harland L, Simek J, Laird DW. Pannexin channels and their links to human disease. *Biochem J*. 2014;461(3):371-381.
28. Penuela S, Gyenis L, Ablack A, et al. Loss of Pannexin 1 Attenuates Melanoma Progression by Reversion to a Melanocytic Phenotype. *J Biol Chem*. 2012;287(34):29184-29193.
29. Dolmatova E, Spagnol G, Boassa D, et al. Cardiomyocyte ATP release through pannexin 1 aids in early fibroblast activation. *Am J Physiol Heart Circ Physiol*. 2012;303:H1208-H1218.
30. Billaud M, Lohman AW, Straub AC, et al. Pannexin1 regulates α1-adrenergic receptor-mediated vasoconstriction. *Circ Res*. 2011;109(1):80-85.
31. Gulbransen BD, Bashashati M, Hirota SA, et al. Activation of neuronal P2X7 receptor – pannexin-1 mediates death of enteric neurons during colitis. *Nat Med*. 2012;18(4):600-604.
32. Orellana JA, Froger N, Ezan P, et al. ATP and glutamate released via astroglial connexin43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. *J Neurochem*. 2011;118(5):826-840.
33. Penuela S, Kelly JJ, Churko JM, Barr KJ, Berger AC, Laird DW. Panx1 regulates cellular properties of keratinocytes and dermal fibroblasts in skin development and wound healing. *J Invest Dermatol*. 2014;134(7):2026-2035.
34. Langlois S, Xiang X, Young K, Cowan BJ, Penuela S, Cowan KN. Pannexin 1 and Pannexin 3 Channels Regulate Skeletal Muscle Myoblast Proliferation and Differentiation. *J Biol Chem*. 2014;289(44):30717-30731.
35. Barbe MT, Monyer H, Bruzzone R. Cell-cell communication beyond connexins: the pannexin channels. *Physiology (Bethesda)*. 2006;21:103-114.
36. Wicki-stordeur LE, Dzugalo AD, Swansburg RM, Suits JM, Swayne LA. Pannexin 1 regulates postnatal neural stem and progenitor cell proliferation. *Neural Dev*. 2012;7(11):1-10.
37. Qu Y, Misaghi S, Newton K, et al. Pannexin-1 is required for ATP release during apoptosis but not for inflammasome activation. *J Immunol*. 2011;186:6553-6561.

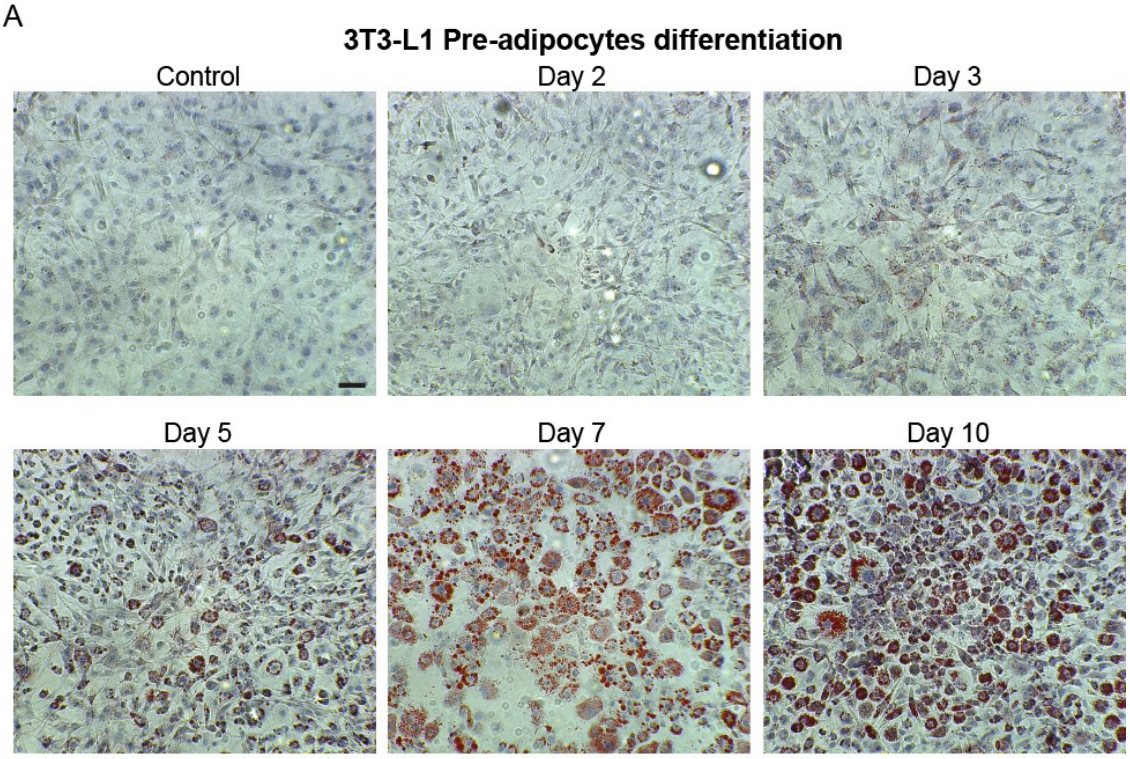
38. Forbes S, Bui S, Robinson BR, Hochgeschwender U, Brennan MB. Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. *Proc Natl Acad Sci U S A*. 2001;98(7):4233-4237.
39. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem*. 1995;270(45):26746-26749. http://www.ncbi.nlm.nih.gov/pubmed/7592907. Accessed July 13, 2016.
40. Wang Z V, Deng Y, Wang QA, Sun K, Scherer PE. Identification and Characterization of a Promoter Cassette Conferring Adipocyte-Specific Gene Expression. *Endocrinology*. 2010;151(June):2933-2939.
41. Brihoum M, Rollin F, Desmecht D, Detilleux J, Amory H. Clinical evaluation of cardiac effects of experimental doxycycline overdosing in healthy calves. *BMC Vet Res*. 2011;7(1):40.
42. Lohman AW, Isakson BE. Differentiating connexin hemichannels and pannexin channels in cellular ATP release. *FEBS Lett*. 2014;588(8):1379-1388.
43. Penuela S, Celetti SJ, Bhalla R, et al. Diverse Subcellular Distribution Profiles of Pannexin1 and Pannexin3 Diverse Subcellular Distribution Profiles of Pannexin1 and Pannexin3. *Cell Commun Adhes*. 2008;15(1-2):133-142.
44. Dvoriantchikova G, Ivanov D, Panchin Y, Shestopalov VI. Expression of pannexin family of proteins in the retina. *FEBS Lett*. 2006;580:2178-2182.
45. Vanden Abeele F, Bidaux G, Gordienko D, et al. Functional implications of calcium permeability of the channel formed by pannexin 1. *J Cell Biol*. 2006;174(4):535-546.
46. Bikle D, Xie Z, Tu C. Calcium regulation of keratinocyte differentiation. *Expert Rev Endocrinol Metab*. 2013;7(4):461-472. doi:10.1586/eem.12.34.Calcium.
47. Fu J, Yu H, Wang R, Liang J, Yang H. Developmental regulation of intracellular calcium transients during cardiomyocyte differentiation of mouse embryonic stem cells. *Acta Pharmacol Sin*. 2006;27(7):901-910. doi:10.1111/j.1745-7254.2006.00380.x.
48. Ntambi J, Takova T. Role of Ca2+ in the early stages of murine adipocyte differentiation as evidenced by calcium mobilizing agents. *Differentiation*. 1996;60(3):151-158.
49. Stewart MKG, Plante I, Penuela S, Laird DW. Loss of Panx1 Impairs Mammary Gland Development at Lactation : Implications for Breast Tumorigenesis. *PLoS One*. 2016;11(4):e0154162.
50. Joe AWB, Lin Y, Even Y, Vogl AW, Rossi FM V. Depot-specific differences in adipogenic progenitor abundance and proliferative response to high-fat diet. *Stem Cells*. 2009;27(10):2563-2570. doi:10.1002/stem.190.
51. Russell KC, Lacey MR, Gilliam JK, Tucker HA, Phinney DG, O'Connor KC. Clonal Analysis of the Proliferation Potential of Human Bone Marrow Mesenchymal Stem Cells as a Function of Potency. *Biotechnol Bioeng*. 2011;108(11):2716-2726. doi:10.1126/scisignal.2001449.Engineering.
52. Russell KC, Phinney DG, Lacey MR, Barrilleaux BL, Meyertholen KE, O'Connor KC. In vitro high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells*. 2010;28(4):788-798. doi:10.1002/stem.312.
53. Wilson CR, Tran MK, Salazar KL, Young ME, Taegtmeyer H. Western diet , but not high fat diet , causes derangements of fatty acid metabolism and contractile dysfunction in the heart of Wistar rats. *Biochem J*. 2007;406:457-467.
54. Dvoriantchikova G, Ivanov D, Barakat D, et al. Genetic Ablation of Pannexin1 Protects

Retinal Neurons from Ischemic Injury. *PLoS One*. 2012;7(2):e319991.
55. Dvoriantchikova G, Hanske J, Petrasch-Parwez E, et al. Pannexin1 Stabilizes Synaptic Plasticity and Is Needed for Learning. *PLoS One*. 2012;7(12):e51767.
56. Kurtenbach S, Whyte-Fagundes P, Gelis L, et al. Investigation of olfactory function in a Panx1 knock out mouse model. *Front Cell Neurosci*. 2014;8(266):1-8.
57. Bargiotas P, Krenz A, Hormuzdi SG, Ridder DA, Herb A, Barakat W. Pannexins in ischemia-induced neurodegeneration. *PNAS*. 2011;108(51):20772-20777.
58. Santiago MF, Veliskova J, Patel NK, et al. Targeting Pannexin1 Improves Seizure Outcome. *PLoS One*. 2011;6(9):e25178.
59. Silverman WR, de Rivero Vaccari JP, Locovei S, et al. The pannexin 1 channel activates the inflammasome in neurons and astrocytes. *J Biol Chem*. 2009;284(27):18143-18151.
60. Bunse S, Locovei S, Schmidt M, et al. The potassium channel subunit Kvβ 3 interacts with pannexin 1 and attenuates its sensitivity to changes in redox potentials. *FEBS J*. 2009;276(21):6258-6270.
61. Lau D, Miera EV De, Contreras D, et al. Impaired Fast-Spiking , Suppressed Cortical Inhibition, and Increased Susceptibility to Seizures in Mice Lacking Kv3.2+ K Channel Proteins. *J Neurosci*. 2000;20(24):9071-9085.
62. Espinosa F, Marks G, Heintz N. Increased motor drive and sleep loss in mice lacking Kv3-type potassium channels. *Genes, Brain Behav*. 2004;3:90-100.
63. Student AK, Hsu RY, Lane MD. Induction of Fatty Acid Synthetase Synthesis in Differentiating 3T3-Ll Preadipocytes. *J Biol Chem*. 1980;255(10):4745-4750.
64. Guglielmo CG, McGuire LP, Gerson AR, Seewagen CL. Simple, rapid, and non-invasive measurement of fat, lean, and total water masses of live birds using quantitative magnetic resonance. *J Ornithol*. 2011;152(1 SUPPL). doi:10.1007/s10336-011-0724-z.
65. Flynn L, Semple JL, Woodhouse KA. Decellularized placental matrices for adipose tissue engineering. *J Biomed Res Part A*. 2006;79(2):359-369.
66. Yu G, Wu X, Kilroy G, Halvorsen YC, Gimble JM, Floyd ZE. Isolation of Murine Adipose-Derived Stem Cells. Gimble JM, Bunnell BA, eds. *Methods Mol Biol*. 2011;702(5):29-36.
67. Flynn L, Prestwich GD, Semple JL, Woodhouse KA. Adipose tissue engineering with naturally derived scaffolds and adipose-derived stem cells. *Biomaterials*. 2007;28:3834-3842.
68. Guzman MS, De Jaeger X, Drangova M, Prado MAM, Gros R, Prado VF. Mice with selective elimination of striatal acetylcholine release are lean, show altered energy homeostasis and changed sleep/wake cycle. *J Neurochem*. 2013;124:658-669.

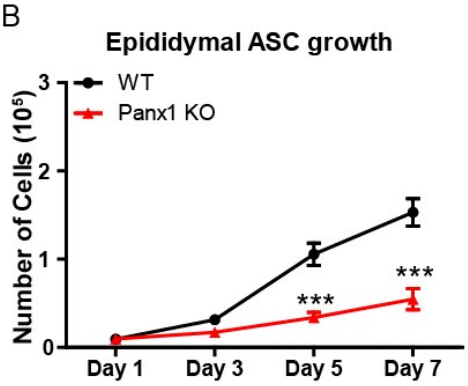
2.7 Supplemental Figure

Supplemental Figure 1. Representation of 3T3-L1 adipogenic differentiation over time and Panx1 KO ASCs from epididymal fat grow significantly slower than WT ASCs. A. 3T3-L1 mouse pre-adipocyte cells shown during a time course of adipogenic induction, 10 days in culture. Bright field images of cells stained with oil red O show gradual differentiation of cells into lipid-loaded (red) adipocytes, counterstained with hematoxylin (blue). Scale bar= 100µm. B. Panx1 KO ASCs from another fat depot (epididymal fat) also grow significantly slower than their WT counterparts in a growth curve assay over 7 days. N=3, n=3, ***P<0.001, means ± s.e.m.

A


3T3-L1 Pre-adipocytes differentiation
Control
Day 2
Day 3
Day 5
Day 7
Day 10

B


Epididymal ASC growth
WT
Panx1 KO
Number of Cells (10^5)
0
1
2
3
***
***
Day 1
Day 3
Day 5
Day 7

Chapter 3 – Supplemental Partial Manuscript

The role of Panx3 in adipocyte development and fat accumulation

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3.1 Introduction

The pannexin glycoprotein family is involved in the development of many different cell types and the wealth of knowledge surrounding them continues to grow. Since their discovery in 2000[1], it has been established that the members: Panx1, Panx2 and Panx3 are expressed in mammalian tissues, and emerging literature states that they exist in many different pathologies. The large pore pannexin channels participate in cellular communication through the extracellular environment via paracrine signaling[2]. There is evidence that Panx1 is expressed in adipocytes and involved in glucose metabolism and insulin sensitivity[3]. In humans, Panx1 and Panx3 share sequence homology (41%)[4] and are often co-expressed in the same cells and tissues. Panx3 has been documented to be expressed in cartilage[5], bone[6], muscle[7], the inner ear[8], and testes[9]. Panx3 plays a catabolic role in osteoarthritis[10], as was revealed by a study using the first ever mouse lacking Panx3 globally or conditionally in cartilage which showed resistance to the development of surgically-induced osteoarthritis[10].

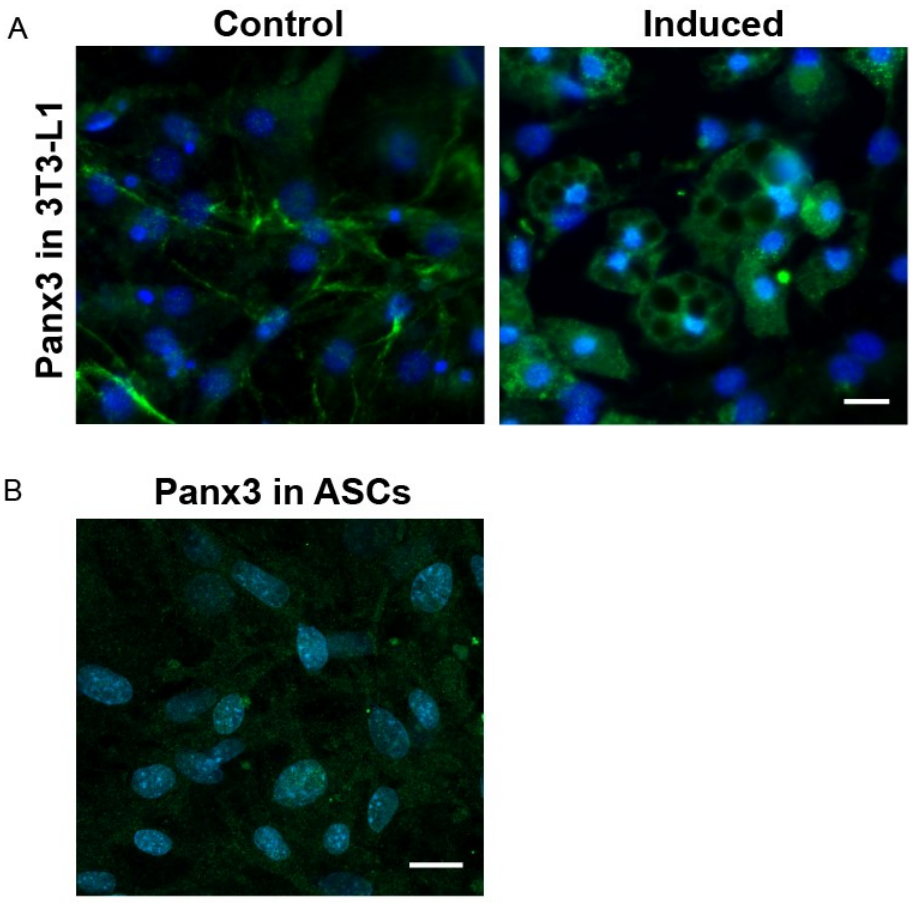
In mice, Panx3 has been reported to regulate functions of cell proliferation and differentiation in many cell types such as: osteoblasts[6], chondrocytes[5], keratinocytes[11], skeletal muscle myoblasts[12], and odontoblast[13]. In osteoblasts, Panx3 is a target for runt-related transcription factor (Runx2), essential for the formation of bone. Panx3 is also found in intramembranous bones in mouse embryos, and has been identified in chondrocytes[6]. It has been proposed that Panx3 is regulated throughout chondrocyte differentiation via the regulation ATP and cAMP content[5]. Most notably, Panx3 has been shown to play an essential role in skeletal long bone development in rodents, as lack of Panx3 results in significantly shorter long bones[14] and dysregulated chondrocyte and osteoblast development[15]. Similar to Panx1, Panx3 is involved in keratinocyte differentiation[11]and is also involved in the differentiation and proliferation of skeletal muscle myoblasts[12].

Based on the knowledge that Panx3 is highly involved in proliferation and differentiation of many cell types, and that it shares similar sequence homology to Panx1, we hypothesized that Panx3 could also regulate adipocyte proliferation and differentiation. In this study, we highlight for the first time the expression and function of Panx3 in adipose precursor cells and pre-adipocytes throughout adipogenic differentiation. Panx3 expression is regulated in the proliferation and differentiation of ASCs, underlining a potential function in fat accumulation and obesity.

3.2 Results

3.2.1 Panx3 is expressed in pre-adipocytes and mouse ASCs

Since it has been well established that Panx1 is involved in cell proliferation and differentiation of many different cell types in conjunction with Panx3[11,12,16], we hypothesized that Panx3 could also be expressed in adipocytes and play a role in adipocyte development. We labeled 3T3-L1 pre-adipocyte cells via immunohistochemistry and observed that Panx3 was clearly expressed both before and after adipocyte differentiation (Appendix 1a-b). Panx3 labelling in the control cells was observed mostly intracellularly. We isolated multipotent primary adipose derived-stromal cells (ASCs) from WT mice and subsequently labelled the ASCs for Panx3. We observed that Panx3 was also expressed intracellularly (Appendix 1c) Therefore, we show for the first time that Panx3 is expressed in the pre-adipocyte 3T3-L1 cell line before and after adipocyte differentiation and in mouse ASCs.

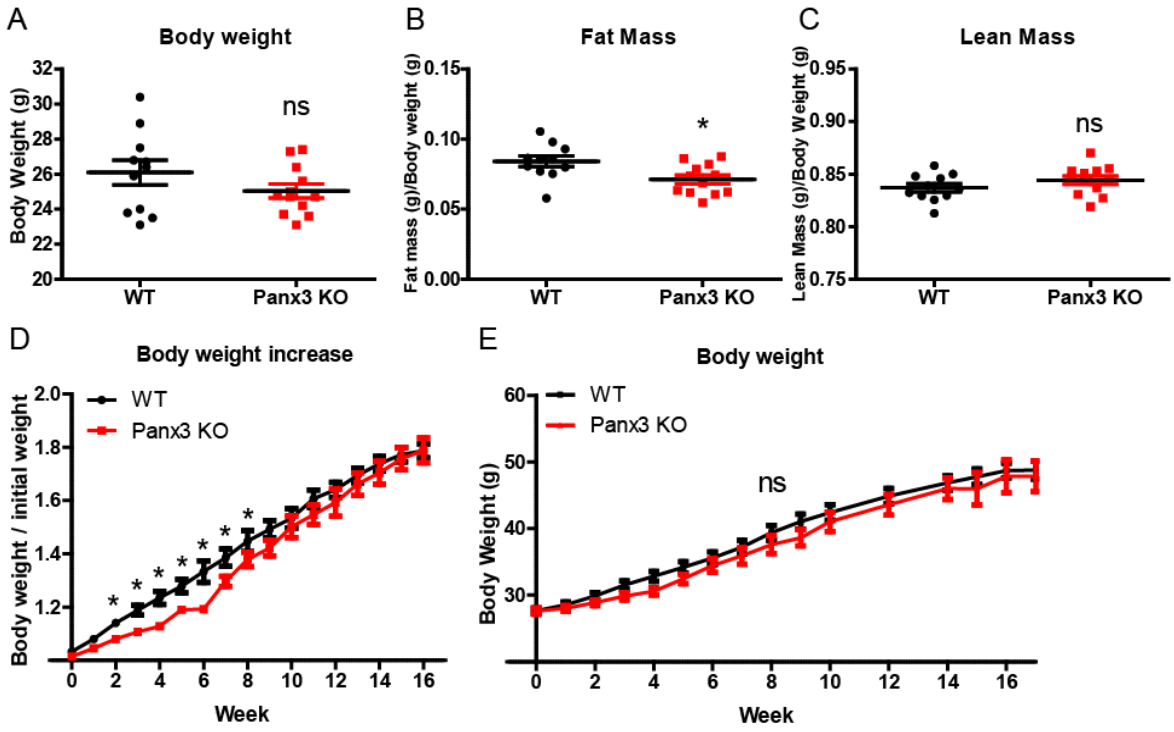


Appendix 3.1 Panx3 is expressed in pre-adipocytes and mouse ASCs. A. Fluorescent micrographs depicting Panx3 expression (green) in 3T3-L1 cells before (control) and after adipogenic induction (induced). B. Panx3 is expressed in WT ASCs (green) with Hoechst nuclei stain (blue). Scale bars= 20µm, 100µm. Identical microscope parameters were maintained to compare between samples.

3.2.2 Panx3 KO mice have less fat mass at baseline and gain slightly less weight on a HFD.

Since we observed that Panx3 was present during adipogenic differentiation of 3T3-L1 pre-adipocytes, we hypothesized that along with Panx1, it could also play a role in fat accumulation. With the use of the first global Panx3 KO mice, as described by Moon et al., 2015[10], we assessed overall body mass composition via echo-MRI. We first compared average body weight between WT and Panx3 KO mice and saw no significant differences (WT N=11, Panx3 KO N=12) (Appendix 2a). When assessing overall fat mass composition, we were surprised to see that the Panx3 KO mice had significantly less fat mass (15% reduction) compared to WT mice ($P<0.05$) (Appendix 2b), and no significant differences in overall lean mass (Appendix 2c). Therefore, the significant reduction in overall fat mass was due to the lack of Panx3 in the mice, indicating that Panx3 may play a role in fat accumulation that may be opposite to that of Panx1.

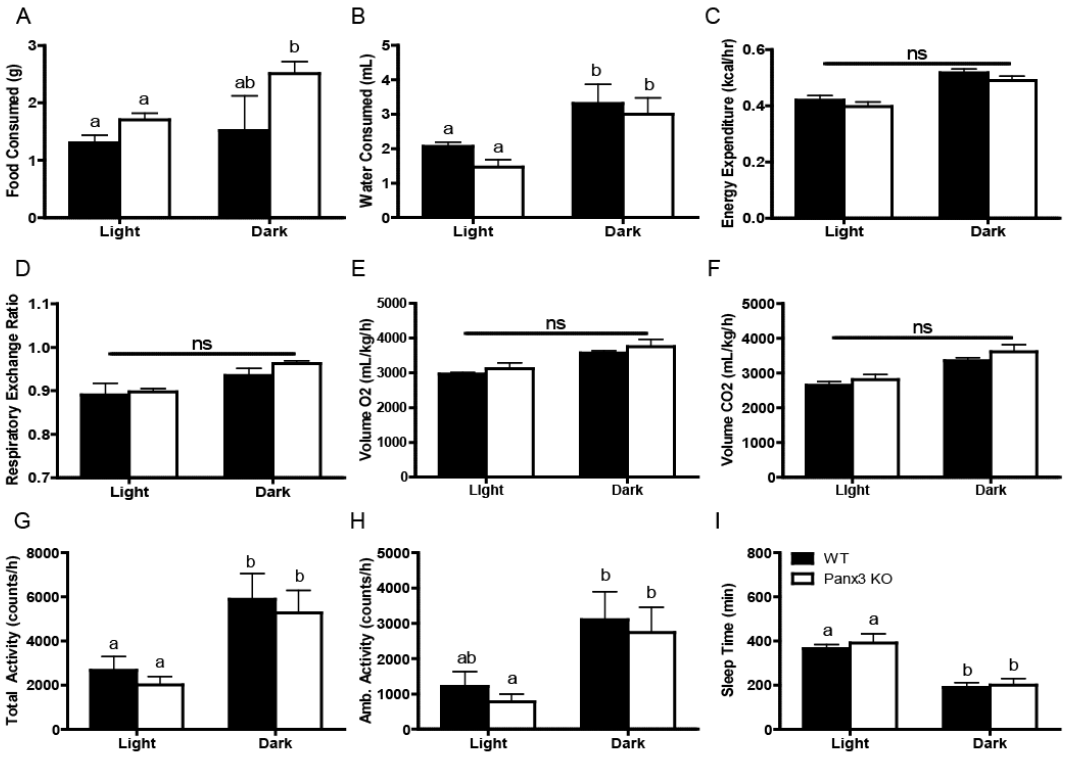
Since we saw differences in overall fat mass in Panx3 KO mice at baseline, we wanted to determine whether there would be any phenotypic effects in the context of a high fat diet. We hypothesized that by placing Panx3 KO mice on a high fat diet (HFD) they would show reduced weight gain. When we placed WT and Panx3 KO mice on a 16 week HFD we observed a slight reduction in weight gain for the Panx3 KO mice between weeks 2 and 8 of the HFD. Beyond that time frame there were no longer any significant differences (WT N=16, Panx3 KO N=13, $P<0.05$) (Appendix 2d). When we assessed raw body weight, we saw no significant differences between WT or Panx3 KO mice (Appendix 2e). Therefore, Panx3 seems to affect fat accumulation and may exert slight effects on weight gain.



Appendix 3.2 Panx3 KO mice have significantly less fat mass at baseline and gain slightly less weight on a HFD compared to WT mice. A-C. Panx3 KO mice fed ad libitum on normal chow diet (baseline) at 3 months of age, were placed in echo-MRI to determine body mass composition. There was no significant difference in average body weight or average lean mass between groups of mice, however Panx3 KO mice had significantly decreased fat mass compared to WT mice and relative to body weight. D-E. Pannexin 3 global knockout (Panx3 KO) mice fed on a high fat diet (60% kcal from fat) starting at 3 months of age for 17 weeks, and congenic WT controls. Panx3 KO mice had a slight reduction in weight increase (normalized to initial weight) between 2 and 8 weeks on HFD, but no change in raw body weight compared to WT mice. A-C. WT N=11, Panx3 KO N=12. D-E. WT N=16, Panx3 KO N=13, Asterisks denote significant differences, $P<0.05$, means ± s.e.m.

3.2.3 Panx3 KO mice at baseline exhibit normal metabolic behavior

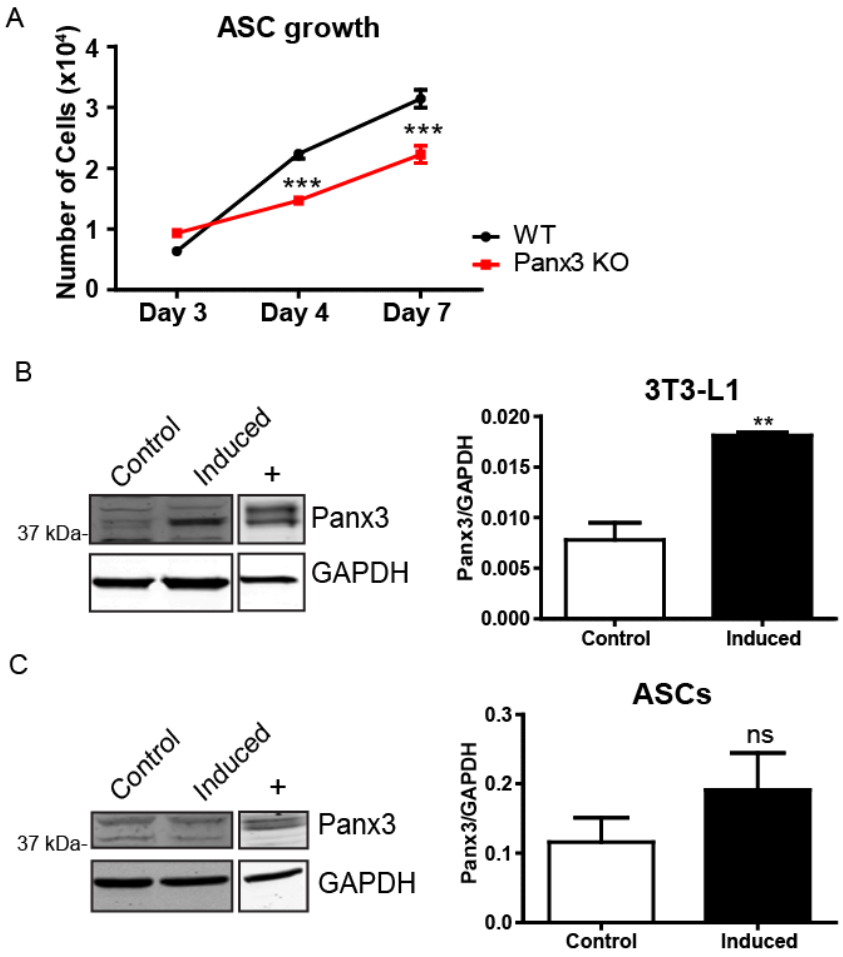
Based on the findings that Panx3 KO mice had significantly reduced fat mass, along with reduced weight gain on HFD, we determined that it would be beneficial to assess metabolic parameters at under normal chow diet conditions. When we placed WT and Panx3 KO mice individually in metabolic cages, we saw no significant differences in any of the measured parameters comparing WT and Panx3 KO mice during their active period (dark) or their sleep period (light) over 24 hours. Thus Panx3 does not seem to regulate any aspects of metabolism and activity such as mobility, or metabolism (WT N=4, Panx3 KO N=4) (Appendix 3a-i).



Appendix 3.3 Panx3 KO mice at baseline exhibit normal metabolic behavior. A-I. WT and Panx3 KO male mice fed ad libitum on a normal chow diet (baseline) were placed in metabolic cages to assess metabolism and activity during their sleeping period (light) and the active period (dark). There were no overt differences in any of the metabolic parameters between WT and Panx3 KO mice. WT N=4, Panx3 KO N=4. Different letters denote significant differences, $P<0.05$, means ± s.e.m.

3.2.4 Panx3 KO ASCs grow significantly slower than WT ASC, and Panx3 is expressed throughout adipogenic differentiation.

Since we observed that the reduction in fat accumulation and weight gain were not due to metabolic or activity parameters in the Panx3 KO mice, it led us to believe that Panx3 could be regulating fat accumulation processes at the level of the adipocytes. We isolated ASCs from epididymal fat pads of WT and Panx3 KO male mice on a HFD, grew them in culture and assessed their growth by systematic cell counts over 7 days. We observed that Panx3 KO ASCs grew significantly slower with approximately 30% reduction in cell number compared to WT ASCs at days 4 and 7 in culture ($N=3$, $P<0.001$) (Appendix 4a). We induced the 3T3-L1 cells and WT ASCs to differentiate using adipogenic differentiation media for 10 and 14 days respectively to assess Panx3 expression before and after differentiation. In the 3T3-L1 cells, we observed that Panx3 expression was significantly increased after differentiation by approximately 50% ($n=3$, $P<0.01$) (Appendix 4b). In ASCs, there was also a trend towards increased Panx3 levels upon differentiation but it was not statistically significant. (Appendix 4c).



Appendix 3.4 Panx3 KO ASCs grow significantly slower than WT ASCs, and Panx3 is expressed throughout adipogenic differentiation. ASCs from WT and Panx3 KO male congenic mice fed on a 16 week HFD (60% kcal from fat) were isolated from epididymal adipose tissue and grown in culture. A. Panx3 KO ASCS grow significantly slower than WT ASCs. B. Panx3 expression is significantly increased after terminal differentiation in 3T3-L1 pre-adipocytes. C. Panx3 expression is unchanged in WT ASCs induced to differentiate. N=3, n=3. **P<0.01, ***P<0.001, means ± s.e.m.

3.3 Discussion

We have shown for the first time that Panx3 is expressed in adipocytes, and also that it may play an opposing role compared to Panx1 on fat accumulation and weight gain, where lack of Panx1 was shown to result in significantly increased adipogenic differentiation, resulting in increased intracellular lipid content. We have also further established that Panx3 may be involved in adipocyte proliferation since Panx3 KO ASCs had reduced proliferation. Panx3 may also play a role in adipocyte differentiation since its expression is regulated before and after differentiation, however this remains to be further investigated. Finally, we have characterized body mass composition of the novel Panx3 KO mouse, in that they have significantly less fat mass at baseline and show slight resistance to weight gain on a HFD, with no differences in metabolism or activity.

Panx3 is present in pre-adipocytes and in adipocyte precursor cells (ASCs). We determined that the majority of Panx3 expression was found mostly intracellularly. In the literature, Panx3 expression has been shown both at the cell surface and also intracellularly in cell types such as the inner ear: dieter cells and pilar cells[17], mouse skin[11], calvarial cells[18] (precursor cells derived from the skull), which have cell surface expression, while cells in the testis express Panx3 intracellularly[9]. Thus it is not unusual for expression of Panx3 to be widespread and intracellular. Panx3 has been reported to act as an intracellular ER calcium channel during osteoblast differentiation that is activated by an interaction with PI3K/Akt signaling[19].

Our study has demonstrated for the first time that the global Panx3 KO mice have significantly less fat mass than WT mice fed on a regular chow diet. We saw no differences in overall body weight or lean mass (consistent with Moon et al., 2015[10]) suggesting that the amount of fat that is reduced in the Panx3 KO mice must be subtle. Although Moon et al., 2015 saw no overt phenotypes in skeletal formation of the KO mice[10], Caskenette et al., 2016

described that this Panx3 KO mice have significantly shorter diaphyseal shafts in both humerus and femoral bones, which were also more robust compared to WT mice, as shown by cross-sectional geometric properties analysis[14]. Panx3 KO mice also had larger areas of muscle attachment[14], suggesting that the mice were relatively stockier with potentially more muscle. Results from metabolic cages showed that there were no obvious differences in metabolic parameters or activity of mice, suggesting that Panx3 may not affect metabolism or behavior unlike the Panx1 KO mice, which showed greater activity and reduced sleep duration. Thus, Panx3 may affect different metabolic mechanisms not covered by the scope of this study. When we placed Panx3 KO mice on a HFD, they exhibited slight reduction in weight gain between 2 and 8 weeks. Therefore we have shown that Panx3 may play a role in reducing or inhibiting fat accumulation, but further studies are needed to reach more solid conclusions.

Since Panx3 plays key roles in the proliferation and differentiation of many progenitor cell types, we hypothesized that Panx3 could also be involved adipocyte development. Our results demonstrate that lack of Panx3 causes a significant reduction in cell growth. Additionally when we differentiated pre-adipocytes we observed that Panx3 expression was significantly upregulated, whereas there were no significant differences in Panx3 expression in the differentiated ASCs, but a similar trend was observed. Contrasting with these data, in a study assessing the function of Panx3 in osteoprogenitor cells, it was found that Panx3 overexpression inhibited cell proliferation[6] and promoted cell cycle arrest at G_0/G_1[6]. The proposed mechanism was that Panx3 releases ATP which induces signaling pathways involved in β-catenin degradation and Wnt signaling, promoting cell cycle exit and switching cells from proliferation to differentiative states[6]. Interestingly, in order for adipocytes to differentiate, cell cycle arrest occurs at the G_1 and G_0 cell cycle phases[20]. Additionally, the Wnt family has been shown to

influence cell fate and development in many cell types including adipocytes[21]. The function of Wnt is to inhibit adipocyte differentiation by blocking master transcription factor expression of: PPARγ and C/EBPα[22].Thus, lack of Panx3 may dysregulate cell proliferation and based on our studies, we have indications that fat accumulation is also potentially targeted.

In a study assessing keratinocyte differentiation, Panx3 expression increased when rat epidermal keratinocytes (REK) cells were grown in organotypic epidermis, suggesting a role for Panx3 in differentiation[11]. When assessing skeletal muscle myoblast differentiation and proliferation, it was observed that Panx3 expression was low in fetal tissue, but it was present in adult tissue. Overexpression of Panx3 caused inhibition of proliferation, but induced myoblast differentiation. However, when silenced by shRNAs, reduction of Panx3 caused inhibition of proliferation, similar to our data, but this seemed to also inhibit differentiation[12]. Panx3 has also been implicated in osteoblast differentiation, where Panx3 expression is consistently expressed during osteogenesis and upon overexpression, caused the upregulation of differentiation markers. Panx3 overexpression in newborn metatarsal bones caused increased growth in both length and width[18]. Similarly, Panx3 was found in chondrogenic cells both before and after differentiation, and upon overexpression, chondrogenic differentiation was induced. Finally, it has been shown that Panx3 is required in skeletal bone development and cartilage development in both mice and zebrafish. In knockout models of both species, embryos exhibited delays in chondrocyte and osteoblast differentiation manifesting in shortened long bones[15], similar to the model that Caskenette at al., 2016[14] had shown. Taken altogether, there have been no reports of Panx3 in adipocytes or adipocyte differentiation, however there is substantial evidence of its role in skin, bone, and cartilage development. Therefore, Panx3 could be regulated during both proliferation and adipogenic differentiation.

There is evidence that there is a relationship between fat accumulation and the development of bone in humans and rodents[23]. In humans, those with higher fat mass (overweight and obese) exhibit increased total body bone mineral content[24]. The mechanism and cross-talk between bone and fat is not well understood, however there are a few potential theories. Both *in vitro* and *in vivo*, it has been shown that osteoblasts contain insulin receptors[25], and administration of exogenous insulin causes osteoblast proliferation[26]. In male mice, addition of insulin to the calvariae (skullcap) resulted in increased bone formation[27]. Thus, throughout fat accumulation, it is proposed that increased insulin leads to increased bone density. Additionally, both osteoblasts and chrondrocytes possess leptin receptors[28, 29] another key hormone released from adipocytes. Similarly, exogenous addition of leptin causes proliferation and differentiation of osteoblasts and chondrocytes[29, 30], contributing to increased bone growth and density. Taken altogether with our data and the current body of knowledge of Panx3 in bone and cartilage development, it is possible that a cross-talk with fat may lead to dysregulated proliferation and differentiation of osteoblasts, chondrocytes, and adipocytes. Lack of Panx3 may ultimately lead to changes in fat accumulation and weight gain.

In summary, we have shown for the first time that Panx3 is expressed in pre-adipocytes and ASCs. Panx3 seem to play a role in the regulation of proliferation and differentiation of ASCs that may result dysregulated fat accumulation. We have further characterized the Panx3 KO mice showing that they have significantly less fat mass at baseline, and have a slight reduction in weight gain on a high fat diet. Based on this current data, it is clear that Panx3 is an interesting candidate for the study of fat accumulation, proliferation and differentiation of adipocyte. Therefore, further *in vitro* studies should be conducted assessing differentiation

capacity, cell proliferation, and the mechanisms affecting fat accumulation. Panx3 shows promise as a target for therapeutic interventions in obesity.

3.4 Materials and Methods

3T3-L1 cell culture

3-day transfer, inoculum $3x10^5$ mouse embryonic fibroblast pre-adipocyte (3T3-L1) cells (ATCC) were grown in culture and induced to differentiate once confluent. Undifferentiated cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, 1% Pen-Strep, and 10% calf serum (Thermo Fisher Scientific) and cells beyond passage 10 were not included in studies. Adipogenic media was composed of: DMEM with 4.5 g/L glucose (Thermo Fisher Scientific), 10% fetal bovine serum (Thermo Fisher Scientific), 1% Pen-Strep, 100 µg/mL of IBMX, 390 ng/mL dexamethasone, and 5 µg/mL insulin (Sigma Aldrich), and was replaced or supplemented every other day as described previously [31] over 14 days.

Animals and ethics

Experiments performed on animals were approved by the Animal Care Committee of the University Council on Animal Care at the University of Western Ontario, London ON, Canada. Panx3 KO mice were generated as described by Moon et al., 2015[10]. These mice were backcrossed until a congenic line was obtained against C57BL/6N strain mice from Charles River Canada (Saint-Constant, PQ).

High fat diet

Congenic WT and Panx3 KO male mice were placed on a 16-week high fat diet (HFD, 60% kcal from fat, TestDiet 58Y1) and fed *ad libitum*. Mice were 3 months old at the start of the experiments and initially fed regular chow diet (6.2% kcal from fat, Harlan 2018).

Body mass composition

Fat mass and lean mass composition was measured using quantitative magnetic resonance (echo MRI) analysis with an echo magnetic resonance imaging mobile unit (Avian Facility of

Advanced Research, University of Western Ontario, London, ON, Canada) as described by Guglielmo, et al., 2011[32] with the modification of placing live mice in the apparatus and measuring on the "bird" setting omitting water content. Measurements were taken in duplicate to ensure consistency of the results.

Adipose-derived stromal cells isolation

ASCs were isolated as previously described by Yu *et al*, 2011[34] from WT and Panx3 KO male mice fed on the 16 week HFD, and fat from up to three mice were pooled together for each separate isolation. Cells were filtered through a 100μm filter to remove debris prior to cell seeding. Cells were seeded at high density (80 000 cells/cm^2) and rinsed 24 hours after isolation with sterile PBS, cells were passaged when confluent (approximately 7 days). ASCs were grown in DMEM: Ham's F-12 (Sigma Aldrich), supplemented with 10% fetal bovine serum and 1% Pen-Strep and growth medium was changed every 2 days. ASCs used for assays were grown up to Passage 2.

Immunofluorescence

Coverslips containing ASCs were grown in culture for 3 days and 3T3-L1 cells were grown in culture for 14 days for analysis of before and after adipogenic differentiation. Cells were rinsed with PBS and fixed in 80% methanol and 20% acetone solution for 20 minutes at 4°C. Cells were then rinsed in PBS three times and immuno-labeled with Panx3-CT primary antibody (stock of 1ug/ml) diluted at 1:400 as described previously by Penuela *et al*., 2007[7]. Images were collected using an LSM 800 inverted confocal microscope equipped with a 40X water objective (Carl Zeiss, Jena, Germany). Identical parameters were maintained to compare between samples.

Western Blotting

Western blots of protein lysates (primary cells and cell lines) were conducted as described previously by Penuela et al., 2007[7]. Cell lysates were collected with a Triton-based extraction buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% Np-40, 100 mM NaF, 100 mM sodium orthovanadate, proteinase inhibitor mini-EDTA tablet (Roche-Applied Science, Laval, QC)] and subsequently run on a western blot, then probed using the Panx3-CT antibody at 1:5000 dilution or 0.2ug/ml with the modification of using 50 μg of protein for all blots[7]. Human embryonic kidney (293T) cells ectopically expressing mouse Panx3 were used as a positive control for all mouse blots as described by Penuela et al., 2008[33]. Loading control used was GAPDH (Millipore, Billerica, MA). Detection of blots was established on an Odyssey infrared imaging system (LICOR).

Proliferation and differentiation assays

ASCs from WT and Panx1 KO mice were plated for differentiation in 12 well plates at a seeding density of 10 000 cells/cm^2. Cell counts were measured in triplicate every other day up until day 7 using trypan blue (1:1) and a Countess automated cell counter (Thermo fisher Scientific). For differentiation assays, ASCs were plated in 6 well plates at a seeding density of 30 000 cell/cm^2. Adipogenic media was made as previously described[34], with the modifications of substituting 1 μg/mL troglitazone and 0.25 mM isobutylmethylxanthine (IBMX) (instead of 0.5 mM) (Sigma Aldrich) for days 1-3. After day 3, a modified adipogenic media was made, lacking troglitazone and IBMX. Modified adipogenic media was changed every other day until differentiation was complete at day 14. Differentiation assays were performed on day 14.

Metabolic analysis

Metabolic analysis was assessed with the usage of the Comprehensive Lab Animal Monitoring System (CLAMS) with the Oxymax software (Columbus Instruments, Columbus, OH, USA). Mice were individually caged and acclimated for 24 hours prior to measurement of food consumption, water consumption, energy expenditure, volume of oxygen (VO2) and carbon dioxide (VCO2), respiratory exchange ratio (RER), total activity, total ambulatory activity, and sleep duration as described previously by Guzman *et al,* 2007[35].

Statistical analysis

Statistical analyses were performed using Graph Pad Prism (GraphPad, San Diego, CA). Student's t-test or ANOVA were performed with Tukey's *post hoc* comparisons. Data are presented as mean ± SEM. N values in *in vitro* ASC assays represent pooled cells from up to 3 mice, whereas, N values in *in vivo* experiments represent individual mice. Biological replicates are indicated by N and technical replicates indicated by n.

3.5 References

1. Panchin Y, Kelmanson I, Matz M, Lukyanov K, Usman N, Lukyanov S. A ubiquitous family of putative gap junction molecules. *Curr Biol*. 2000;10(13):473-474.
2. Scemes E, Spray DC, Meda P. Connexins, pannexins, innexins: novel roles of "hemi-channels." *Pflugers Arch*. 2009;457(6):1207-1226.
3. Adamson SE, Meher AK, Chiu Y, et al. Pannexin 1 is required for full activation of insulin-stimulated glucose uptake in adipocytes. *Mol Metab*. 2015;4(9):610-618.
4. Baranova A, Ivanov D, Petrash N, et al. The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. *Genomics*. 2004;83(4):706-716.
5. Iwamoto T, Nakamura T, Doyle A, et al. Pannexin 3 regulates intracellular ATP/cAMP levels and promotes chondrocyte differentiation. *J Biol Chem*. 2010;285(24):18948-18958.
6. Ishikawa M, Iwamoto T, Fukumoto S, Yamada Y. Pannexin 3 Inhibits Proliferation of Osteoprogenitor Cells by Regulating Wnt and p21 Signaling. *J Biol Chem*. 2014;289(5):2839-2851.
7. Penuela S, Bhalla R, Gong X-Q, et al. Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. *J Cell Sci*. 2007;120(Pt 21):3772-3783.
8. Wang X-H, Streeter M, Liu Y-P, Zhoa H-B. Identification and characterization of Pannexin expression in the mammalian cochlea. *J Comp Neurol*. 2010;512(3):336-346.
9. Turmel P, Dufresne J, Hermo L, Smith CE, Penuela S, Laird DW. Characterization of Pannexin1 and Pannexin3 and Their Regulation by Androgens in the Male Reproductive Tract of the Adult Rat. 2011;138:124-138.
10. Moon PM, Penuela S, Barr K, et al. Deletion of Panx3 Prevents the Development of Surgically Induced Osteoarthritis. *J Mol Med*. 2016;93(8):845-856.
11. Celetti SJ, Cowan KN, Penuela S, Shao Q, Churko J, Laird DW. Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation. *J Cell Sci*. 2010;123(Pt 8):1363-1372.
12. Langlois S, Xiang X, Young K, Cowan BJ, Penuela S, Cowan KN. Pannexin 1 and Pannexin 3 Channels Regulate Skeletal Muscle Myoblast Proliferation and Differentiation. *J Biol Chem*. 2014;289(44):30717-30731.
13. Fu D, Song F, Sun H, Pei D, Wang Y, Lei J. Archives of Oral Biology Expression of Pannexin3 in human odontoblast-like cells and its hemichannel function in mediating ATP release. *Arch Oral Biol*. 2015;60(10):1510-1516.
14. Caskenette D, Penuela S, Lee V, et al. Global deletion of Panx3 produces multiple phenotypic effects in mouse humeri and femora. *J Anat*. 2016;288(5):746-756.
15. Oh S, Shin J, Baek J, et al. Pannexin 3 is required for normal progression of skeletal development in vertebrates. *FASEB J*. 2015;29:4473-4484.
16. Bond SR, Lau A, Penuela S, et al. Pannexin 3 is a novel target for Runx2, expressed by osteoblasts and mature growth plate chondrocytes. *J Bone Miner Res*. 2011;26(12):2911-2922.
17. Zhao H. Expression and function of pannexins in the inner ear and hearing. *BMC Cell Biol*. 2016;17(Suppl 1).
18. Yamada Y. Pannexin 3 functions as an ER Ca 2+ channel, hemichannel, and gap junction to promote osteoblast differentiation. 193(7):1257-1274.
19. Ishikawa M, Iwamoto T, Nakamura T, Doyle A, Fukumoto S, Yamada Y. Pannexin 3 functions as an ER Ca(2+) channel, hemichannel, and gap junction to promote osteoblast

differentiation. *J Cell Biol*. 2011;193(7):1257-1274.
20. Pairault J, Green H. A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc Natl Acad Sci USA*. 1979;76(10):5138-5142.
21. Catriona Y. Logan, Roel Nusse. The Wnt Signaling Pathway in Development and Disease. *Annu Rev Cell Dev Biol*. 2004;20:781-810.
22. Ross SE, Erickson RL, Gerin I, et al. Microarray analyses during adipogenesis: understanding the effects of Wnt signaling on adipogenesis and the roles of liver X receptor alpha in adipocyte metabolism. *Mol Cell Biol*. 2002;22(16):5989-5999.
23. Reid IR. Relationships between fat and bone. *Osteoporos Int*. 2008;19(5):595-606.
24. Goulding A, Taylor RW, Jones IE, McAuley KA, Manning PJ, Williams SM. Overweight and obese children have low bone mass and area for their weight. *Int J Obes Relat Metab Disord*. 2000;24(5):627-632.
25. Pun KK, Lau P, Ho PW. The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line. *J Bone Miner Res*. 1989;4(6):853-862.
26. Hickman J, McElduff A. Insulin promotes growth of the cultured rat osteosarcoma cell line UMR-106-01: an osteoblast-like cell. *Endocrinology*. 1989;124(2):701-706.
27. Cornish J, Callon KE, Reid IR. Insulin increases histomorphometric indices of bone formation In vivo. *Calcif Tissue Int*. 1996;59(6):492-495.
28. Steppan CM, Crawford DT, Chidsey-Frink KL, Ke H, Swick AG. Leptin is a potent stimulator of bone growth in ob/ob mice. *Regul Pept*. 2000;92(1-3):73-78.
29. Cornish J, Callon KE, Bava U, et al. Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. *J Endocrinol*. 2002;175(2):405-415.
30. Gordeladze JO, Drevon CA, Syversen U, Reseland JE. Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: Impact on differentiation markers, apoptosis, and osteoclastic signaling. *J Cell Biochem*. 2002;85(4):825-836.
31. Student AK, Hsu RY, Lane MD. Induction of Fatty Acid Synthetase Synthesis in Differentiating 3T3-Ll Preadipocytes. *J Biol Chem*. 1980;255(10):4745-4750.
32. Guglielmo CG, McGuire LP, Gerson AR, Seewagen CL. Simple, rapid, and non-invasive measurement of fat, lean, and total water masses of live birds using quantitative magnetic resonance. *J Ornithol*. 2011;152(1 SUPPL). doi:10.1007/s10336-011-0724-z.
33. Penuela S, Celetti SJ, Bhalla R, et al. Diverse Subcellular Distribution Profiles of Pannexin1 and Pannexin3 Diverse Subcellular Distribution Profiles of Pannexin1 and Pannexin3. *Cell Commun Adhes*. 2008;15(1-2):133-142.
34. Yu G, Wu X, Kilroy G, Halvorsen YC, Gimble JM, Floyd ZE. Isolation of Murine Adipose-Derived Stem Cells. Gimble JM, Bunnell BA, eds. *Methods Mol Biol*. 2011;702(5):29-36.
35. Guzman MS, De Jaeger X, Drangova M, Prado MAM, Gros R, Prado VF. Mice with selective elimination of striatal acetylcholine release are lean, show altered energy homeostasis and changed sleep/wake cycle. *J Neurochem*. 2013;124:658-669.

Chapter 4 – Discussion and Conclusions

4.1 Overall study conclusions

Panx1 and Panx3 are important players in cellular proliferation and differentiation, regulating tissue development and having implications in disease. Here, we have shown for the first time the role of Panx1 and Panx3 in adipocyte development and fat accumulation. Panx1 and Panx3 are present in adipocytes, and they function in mediating cellular proliferation of adipocyte progenitors. When assessing differentiation capacity and fat accumulation, Panx1 KO ASCs show enhanced differentiation along with increased fat accumulation or lipid content. This remains to be explored in the context of Panx3. We have also shown that Panx1 and Panx3 are regulated throughout differentiation, with Panx1 protein expression being slightly increased in ASC differentiation, whereas Panx3 expression is significantly increased in pre-adipocyte differentiation. Finally our *in vivo* studies have revealed a function for Panx1 and Panx3 in fat accumulation under normal conditions, where Panx1 KO mice have significantly greater fat mass, however Panx3 KO mice have significantly less fat mass compared to WT mice. When placed on a high fat diet, Panx1 KO mice do not exhibit changes in weight gain, which could be due in part to their increased mobility and reduced sleep. Contrastingly, Panx3 KO mice show slightly reduced weight gain, however no changes at baseline in metabolism or activity. Metabolism and activity of the Panx3 KO mice on a high fat diet is currently unknown and will be explored in the future. Our overall data suggests that Panx1 and Panx3 are both regulated in adipocyte development, and may play opposing or contrasting roles. Panx1 seems to control the extent of fat accumulation and adipocyte differentiation tightly regulating early adipogenic events, while Panx3 may be important in the onset of fat accumulation promoting growth or differentiation. Consistent with our findings, it has been established that Panx1 and Panx3 play

opposing roles in keratinocyte differentiation where Panx1 is highly expressed in embryonic mice but decreases in aged skin, however Panx3 expression is unchanged throughout maturity. Ectopic expression of both Panx1 and Panx3 in REKs reduce cell proliferation, but overexpression of Panx1 dysregulates organotypic epidermis[1]. Both Panx1 and Panx3 are highly upregulated during keratinocyte differentiation, suggesting their collective role in mediating differentiation in addition to proliferation[2]. In skeletal muscle myoblasts, Panx1 and Panx3 are co-expressed however, prior to differentiation low levels of Panx1 are detected, but at the onset of differentiation Panx1 is significantly increased. Contrastingly, Panx3 expression is high in undifferentiated cells, and is reduced during differentiation. Upon Panx1 overexpression, skeletal muscle myoblasts exhibit enhanced differentiation, and Panx3 overexpression shows a similar effect with reduced proliferation and increased differentiation. However, blocking Panx1 causes inhibition of differentiation whereas knockdown of Panx3 causes inhibition of proliferation but does not affect differentiation[3]. Therefore, similar to our results, Panx1 and Panx3 regulate proliferation and differentiation of skeletal muscle myoblasts in a contrasting fashion, where it appears that Panx1 regulates differentiation to a greater degree.

4.1 Limitations and Future directions

This study has shown for the first time both Panx1 and Panx3 as novel regulators of adipocyte proliferation and differentiation, which also leads to the regulation of fat accumulation. There are a few limitations that should be addressed in the studies outlined in this thesis.

Panx1 KO and Panx3 KO mice fed on a regular chow diet were not fully characterized, and most of the data comes from mice on a high fat diet which represents a limiting factor to this study. The data collected on ASCs outlines the function of Panx1 and Panx3 in ASCs isolated from mice on high fat diets. The reason being is that a greater number of cells can be harvested

from larger amounts of fat. Therefore it would be necessary in the future to assess proliferation and differentiation of cells from regular chow fed mice in addition to assess metabolic activity (metabolic cages), to rule out the diet as a confounding factor. Ideally more studies on epididymal ASCs should be conducted but a limitation of these cells is that they do not grow well after a couple of passages and therefore are limited in their usefulness for our studies.

We were fortunate to be able to assess PANX1 expression in human ASCs derived from patients undergoing reduction surgeries. However, it is difficult to translate our Panx1 KO mouse model to humans. Future experiments could focus on human and mouse studies for a better understanding of cellular mechanisms. Human ASCs and pre-adipocytes (3T3-L1 cells) could be isolated and *Panx1* could be subjected to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) deletion[4]. Alternatively, known pharmacological blockers of PANX1/Panx1 could be used to assess potential changes in proliferation or differentiation, further reinforcing the role of Panx1 in adipocyte development. Cell cycle markers and alternative markers of apoptosis such as TUNEL could be utilized to further understand the mechanism of reduced growth in Panx1 KO and Panx3 KO ASCs. An extension of this study would be an *in vivo* model using WT and Panx1 KO mice on a high fat diet, with a subgroup of mice being fed the pharmacological blockers. I would expected that WT mice fed blockers would show possible weight increases or changes in activity. Using a global knockout model also has its limitations regardless of the study due to its broad spectrum of effects that could affect a multitude of organs, tissues, and systems. Thus, an additional next step would be utilizing a conditional or adipogenic specific Panx1 knockout mouse (other than the Adiponectin Cre[5]) and isolate adipogenic progenitors to verify whether there are outside mechanisms or compensation affecting proliferation or differentiation.

It is possible that Panx1 and Panx3 may compensate for each other. Panx2 has been shown to potentially be compensated in Panx1 KO mice and vice versa leading to ischemic damage in a stroke model. However, when both Panx1 and Panx2 are ablated, mice are neuroprotected[6]. Thus, a limitation of our study is the effect of compensation in our mouse models and cell lines. We have shown that both Panx1 and Panx3 are present in adipocytes and ASCs, however in either knockout model, it is unknown whether the opposing Panx family members are upregulated in response. Our group has published that Panx3 is upregulated in young Panx1 KO dorsal skin[7]. Thus, it would be beneficial to assess the role of compensation in adipocyte development and eventually a double Panx1/3 knockout could be used as a useful tool to account for Panx compensation and additionally assess metabolism, obesity and overall activity.

Currently, there is only one known human germline mutation of PANX1, where a patient presents with severe cognitive, reproductive, development delays, and notably has fatty liver disease of unknown etiology. This report is interesting, specifically noting the presence of fatty liver disease, potentially hinting at dysregulated fatty acid metabolism or overproduction of fat in the liver. Additionally, this study has reported defective PANX1 channel ATP release and dye uptake, in addition to reduced channel function[8]. It would be beneficial to screen for PANX1 and PANX3 mutations, specifically in those who are overweight or obese which could potentially act as an indicator for obesity.

It would be important to determine if Panx1 and Panx3 are also localized to the cell surface of ASCs or just intracellularly, and if that is the case, then it would be imperative to perform functional ATP release or dye uptake studies to evaluate channel function alterations in the KO ASCs. In the event that all the pannexin is intracellular, it would be important to determine their

function inside the cells (e.g. ER calcium channel) and the potential interactions with other intracellular proteins that may be behind the phenotypes observed.

Finally, as obesity is an inflammatory disease and it is well established that Panx1[9] and Panx3[10] are involved in inflammation and release of nucleotides, it would be valuable to assess immune cell infiltration and changes in anti or pro-inflammatory cytokines within Panx1 KO or Panx3 KO mice. This could be assessed at the level of adipocytes, where localized inflammation is commonly reported, or in whole body analysis with a focus on metabolically related tissues such as muscles and the liver.

4.2 Summary

In summary, we have shown for the first time the role of Panx1 and Panx3 in early adipocyte development and its effects on fat accumulation. We have shown that not only are Panx1 and Panx3 expressed in adipocytes, but expression is also regulated during adipogenic differentiation. Panx1 KO mice have significantly greater fat mass, however Panx3 KO mice have significantly less fat mass compared to WT mice on a regular diet. Lack of Panx1 and Panx3 causes a reduction in ASC proliferation. Panx1 regulates differentiation capacity of ASCs, and lack of Panx1 significantly enhances adipogenic differentiation capacity leading to increased fat accumulation. Finally, Panx1 KO mice on a high fat diet show no differences in weight gain, but they exhibit enhanced total activity, ambulatory activity, and sleep less than WT mice. Comparatively, Panx3 KO mice on a regular chow diet show no differences in metabolism or activity, however when fed on a high fat diet they have slightly reduced weight gain. Therefore both Panx1 and Panx3 are novel regulators of adipocyte precursor cell proliferation and differentiation, while regulating fat accumulation *in vivo*. Thus, Panx1 and Panx3 represent novel and unique targets for obesity intervention.

4.3 References

1. Celetti SJ, Cowan KN, Penuela S, Shao Q, Churko J, Laird DW. Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation. *J Cell Sci*. 2010;123(Pt 8):1363-1372.
2. Cowan KN, Langlois S, Penuela S, Cowan BJ, Laird DW. Pannexin1 and Pannexin3 Exhibit Distinct Localization Patterns in Human Skin Appendages and are Regulated during Keratinocyte Differentiation and Carcinogenesis. *Cell Commun Adhes*. 2012;19(3-4):45-53.
3. Langlois S, Xiang X, Young K, Cowan BJ, Penuela S, Cowan KN. Pannexin 1 and Pannexin 3 Channels Regulate Skeletal Muscle Myoblast Proliferation and Differentiation. *J Biol Chem*. 2014;289(44):30717-30731.
4. Cong L, Ran FA, Cox D, et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science (80-)*. 2013;339(6121):819-823.
5. Wang Z V, Deng Y, Wang QA, Sun K, Scherer PE. Identification and Characterization of a Promoter Cassette Conferring Adipocyte-Specific Gene Expression. *Endocrinology*. 2010;151(June):2933-2939.
6. Bargiotas P, Krenz A, Hormuzdi SG, Ridder DA, Herb A, Barakat W. Pannexins in ischemia-induced neurodegeneration. *PNAS*. 2011;108(51):20772-20777.
7. Penuela S, Kelly JJ, Churko JM, Barr KJ, Berger AC, Laird DW. Panx1 regulates cellular properties of keratinocytes and dermal fibroblasts in skin development and wound healing. *J Invest Dermatol*. 2014;134(7):2026-2035.
8. Shao Q, Lindstrom K, Shi R, et al. A germline variant in PANX1 has reduced channel function and is associated with multisystem dysfunction. *J Biol Chem*. 2016;291(24):12432-12443.
9. Chekeni FB, Elliott MR, Sandilos JK, et al. Pannexin 1 channels mediate “find–me” signal release and membrane permeability during apoptosis. *Nature*. 2010;467(7317):863-867.
10. Pillon NJ, Li YE, Fink LN, et al. Nucleotides released from palmitate-challenged muscle cells through pannexin-3 attract monocytes. *Diabetes*. 2014;63(11):3815-3826.

Curriculum Vitae

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1. *The effects of gill denervation on ionocyte function in the goldfish (Carassius auratus).* 23rd Annual Comparative Physiology and Biochemistry Workshop. Keene, Ontario. February 1, 2014.
2. *The role of pannexins in fat accumulation and metabolism.* 9th Annual Nexin Research Forum. University of Western Ontario. London, ON. March 5, 2015.
3. *The role of pannexins in fat accumulation and metabolism.* Anatomy and Cell Biology Research Day. University of Western Ontario. London, ON. October 23, 2015.
4. *The role of pannexins in fat accumulation and metabolism.* American Society of Cell Biology Meeting Subgroup. San Diego, California, USA. December 12, 2015.
5. *The role of pannexins in fat accumulation and metabolism.* 10th Annual Nexin Research Forum. University of Western Ontario. London, ON. February 18, 2016.

Poster Presentations:
1. **Lee VR,** Barr K, Laird DW, and Penuela S. The Role of Pannexins in Fat Accumulation and Metabolism. American Society of Cell Biology Meeting. San Diego, California, USA. December 13, 2015.
2. **Lee VR,** Barr K, Laird DW, and Penuela S. The Role of Pannexins in Fat Accumulation and Metabolism. London Health Research Day. London, ON. April 1, 2015. *Poster Award Winner.
3. Lee VR, Barr K, Laird DW, and **Penuela S**. The Role of Pannexins in Fat Accumulation and Metabolism. International Gap Junction Meeting. Valparaiso, Chile. March 30, 2015.