Investigating metabolic disease in human induced pluripotent stem cells : apidocyte size, insulin signaling and hepatic lipids

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Chapter 1: Introduction

Inappropriate cellular response to insulin is an affliction called insulin resistance (IR). Insulin signaling maintains metabolic homeostasis through mechanisms such as blood glucose clearance, regulating lipid homeostasis, and effects on appetite. IR leads to hyperglycemia due to decreased glucose uptake in peripheral tissues, most notably the muscle, liver and fat. Hyperglycemia, along with other mechanisms, causes IR to pose an increased risk for Type II Diabetes (T2D) and cardiovascular disease (CVD) (Rask-Madsen and Kahn, 2012). Obesity often accompanies IR, and its incidence has doubled over the last 30 years as shown in Figure 1. The disease group of IR, T2D, CVD and obesity together has often been called metabolic disease or metabolic syndrome (Alberti et al., 2005).

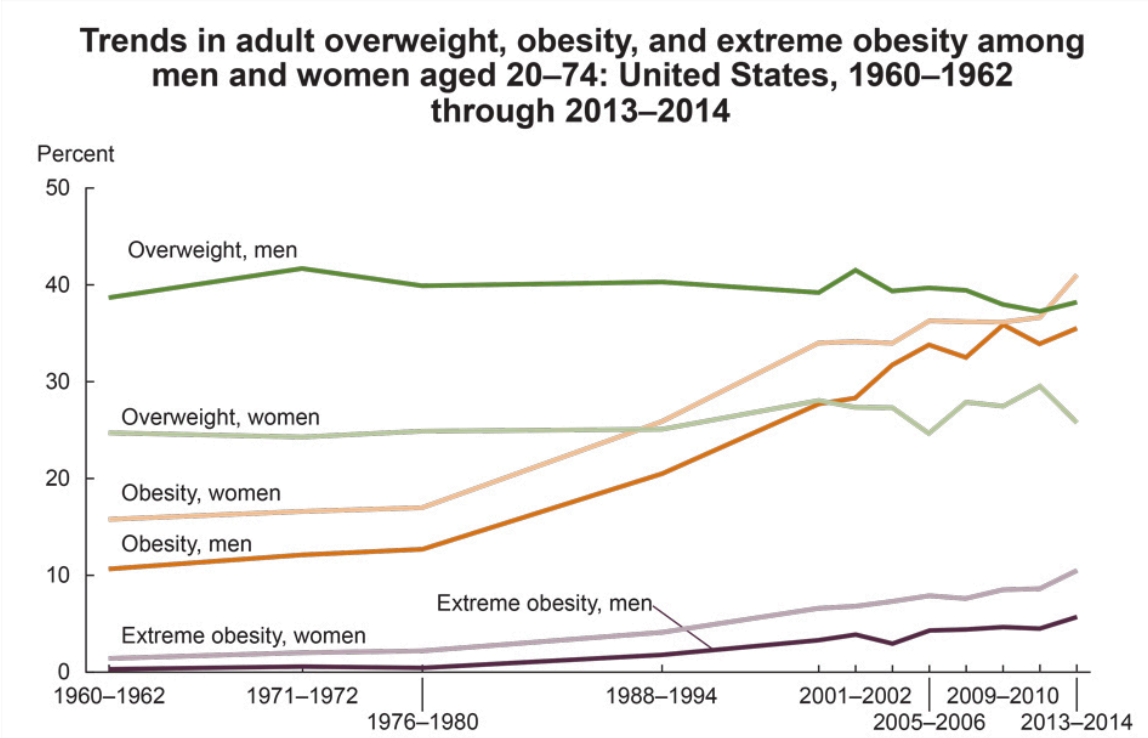

Figure 1. Age-adjusted obesity rates for people in the United States. Age groups are 20-39, 40-59, 60-74. Overweight is BMI >25kg/m^2, obesity is BMI >30kg/m^2, extreme obesity is BMI >40kg/m^2. Source: NIH NIDDK.

The etiology of IR is incredibly complex, due to the interplay of genetics and environment. Most genetic contribution to IR is polygenic, with each variant contributing only a minor amount to the overall pathology. On top of this multigenic landscape there is a strong contribution of environmental factors, including diet, exercise and lifestyle. Here we focus on the genetics and molecular mechanisms of insulin resistance and related metabolic diseases.

Metabolic homeostasis

Metabolism comprises the chemical reactions by which the body converts nutrients into energy to maintain life. It encompasses a vast amount of processes meant to break down substances to yield energy, catabolism, or to synthesize the building blocks necessary for life, anabolism. One of the premier players in metabolism is glucose, the first-choice energy-generating molecule for most cells in the body, and the energy-carrying unit into which most dietary macronutrients are converted. The main organs involved in postprandial glucose uptake are skeletal muscle, the liver and adipose tissue (Petersen et al., 2017). Adipose tissue and the liver also subsequently store glucose by utilizing it to further synthesize lipids (Ameer et al., 2014). Through this process long-term energy storage can be achieved by accumulating lipid reserves. This regulates and impacts whole-body metabolic homeostasis and energy levels. Though both organs generate triglycerides out of glucose, adipocytes have the specialized function of storing large fat reserves for extended periods of time. This process is closely regulated by a number of hormones, most notably glucagon and insulin, which affect both glucose and lipid metabolism (Cherrington and Vranic, 1971). To regulate the body's intake of nutrients, these hormones affect a hypothalamic circuit. Through the same brain axis ghrelin and leptin affect hunger, satiety and appetite (Massadi et al., 2017). In a healthy body under hypoglycemic conditions, liver glycogen and fat triglycerides are broken down into glucose and free fatty acids and released into the bloodstream for energy use by other organs (Nguyen et al., 2008). However, improper glucose control can give rise to many conditions. For example, hypoglycemia can lead to heart palpitations, shakiness, confusion, seizures and loss of consciousness, which can be terminal if untreated (Cryer, 1999). Hyperglycemia on the other hand, is one of the hallmarks of diabetes, and extended exposure to high blood glucose levels leads to cardiovascular complications, diabetic nephropathy, retinopathy and cataracts (Ruderman et al., 1992). It has also been shown that hyperglycemia leads to microvascular complications, which most likely precedes and contributes to ensuing cardiovascular disease. This is hypothesized to be because of superoxide accumulation (Brownlee, 2001). The increase of reactive oxygen species occurs due to cells attempting to combat the hyperglycemia by overstressing their mitochondria, which induces mitochondrial dysfunction and endoplasmic reticulum stress. This promotes cellular damage and in turn potentiating development as well as progression of diabetic complications.

Lipodystrophy

Lipodystrophy is defined as a medical condition in which the body is unable to maintain appropriate adipose tissue (Hegele et al., 2007). Lipodystrophy is a disease group that can be divided into congenital and acquired lipodystrophies, with multiple types of each (Decaudain et al., 2009; Leiter and Semple, 2017). Here we focus on one type of congenital lipodystrophy, namely Familial Partial Lipodystrophy Type 2 (FPLD2), also called the Dunnigan Variety (Hegele et al., 2007). It is a rare genetic metabolic condition characterized by a loss and abnormal distribution of subcutaneous adipose tissue. In this type specifically, there is an

atrophy of trunk and limb subcutaneous tissue, while there is excess supraclavicular fat. This lack of adipose causes insulin resistance, driving progression to T2D as well as fatty liver and hyperlipidemia followed by atherosclerosis. It also leads to reduced serum concentrations of adiponectin and leptin, further exacerbating these deleterious metabolic effects.
FPLD2 is a monogenic disease caused by mutations in *LMNA*, with the most common genetic variation being the missense mutation R482W (Capanni, 2005; Decaudain et al., 2009). Mutations in *LMNA* can lead to a wide range of syndromes called laminopathies, which includes premature aging syndromes, myopathies and neuropathies (Worman and Bonne, 2007). The R482W mutation displays no association with laminopathies outside of lipodystrophy. The mutation is known to be in the C-terminal tail of the protein where it does not alter three-dimensional structure, but its mechanism of causing lipodystrophy is unknown (Krimm et al., 2002). It is possible this mutation causes structural defects in the nuclear envelope and disrupts protein-protein interactions that are important for adipogenesis.

Insulin signaling

Insulin is the most critical hormone for energy homeostasis, as it regulates both glucose and lipid metabolism. Upon high blood glucose levels, such as after a meal, pancreatic beta cells secrete insulin into the bloodstream (Goren, 2005). When it reaches its target tissues, it promotes glucose and fatty acid uptake, as well as glycogen and triglyceride synthesis (Saltiel and Kahn, 2001; Wong and Sul, 2010). Oppositely it suppresses adipose lipolysis and hepatocyte gluconeogenesis. Altogether the actions of insulin favor energy storage. Once insulin reaches the cell, it binds the insulin receptor, a tyrosine kinase receptor, which starts a phosphorylation chain (Haeusler et al., 2018; Krüger et al., 2008; Menting et al., 2013). The receptor auto-phosphorylates, activating a family of adaptor proteins including the insulin receptor substrate (IRS) family (Boucher et al., 2014). These further activate downstream signaling, one of the constituents of which is the protein kinase B (AKT) family (Taniguchi et al., 2006). This family in turn affects a multitude of downstream signaling pathways, both functionally and transcriptionally. For example, it leads to increased insulin-stimulated glucose uptake through the trafficking and translocation of glucose transporter type 4 (GLUT4) vesicles to the cell surface, causing an influx of glucose into the cell and lowering blood glucose levels (Klip et al., 2014). Similarly, it stimulates amino acid uptake and protein synthesis while inhibiting protein degradation, both processes mostly regulated by AKT-activation of glycogen synthase kinase 3 (GSK3) (James et al., 2017). The transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) mediates the lipid response, causing fatty acid uptake and lipogenesis, while simultaneously inhibiting lipolysis (Wong and Sul, 2010). Insulin also affects adipocyte differentiation through AKT-mediated forkhead box O1 (FoxO1) nuclear exclusion, releasing FoxO1 inhibition of adipogenic transcription factors (Nakae et al., 2003; Rosen and MacDougald, 2006). However, in the liver FoxO1 regulates gluconeogenesis, shutting down expression of necessary enzymes after nuclear

exclusion (Langlet et al., 2017). This is an immaculate display of how the same factor is used for different purposes in separate insulin-sensitive tissues.

One of the main mechanisms to shut down intracellular insulin signaling is the degradation of PIP3, and the attenuation of its production through the 3' phosphatase phosphatase and tensin homolog (PTEN) and the SH-2 containing inositol 5' polyphosphatase (SHIP) family of proteins, including 5' phosphatases SHIP1 and SHIP2 (Haeusler et al., 2018; Pal et al., 2012). To terminate signaling, the receptor internalizes inside a vesicle, and can be degraded or recycled back to the cell surface, while the bound insulin is being broken down. Most of the players in this pathway, with the exception of endocytosis regulation, are summarized in the pathway in Figure 2.

Many studies have assessed insulin signaling in a range of tissues. Most of these experiments have been performed in mouse models, by tissue-specific knockout of various players in the insulin signaling pathway, by assessing natural genetic heterogeneity, or by high-throughput screening for diabetic pathology of large collections of mouse mutants (Kubota et al., 2017; Parks et al., 2015). The most significant disruption of insulin signaling is the deletion of the insulin receptor, which has been studied in various tissues (Kubota et al., 2017). As expected these models present with deleterious effects on the animal's metabolism and overall quality of life. However, one controversial set of reports found that knocking out the adipose insulin receptor had a positive effect (Blüher et al., 2003; 2002). The fat mass in these animals was reduced, and they were protected from obesity and associated glucose intolerance. Presumably based on this protection, the mice also displayed increased median and maximum lifespan. Oppositely it has also been shown that increasing insulin sensitivity is beneficial for mice (Morley et al., 2015). Sadly, the mechanism for this protective effect has not yet been elucidated, spurring further research into this topic.

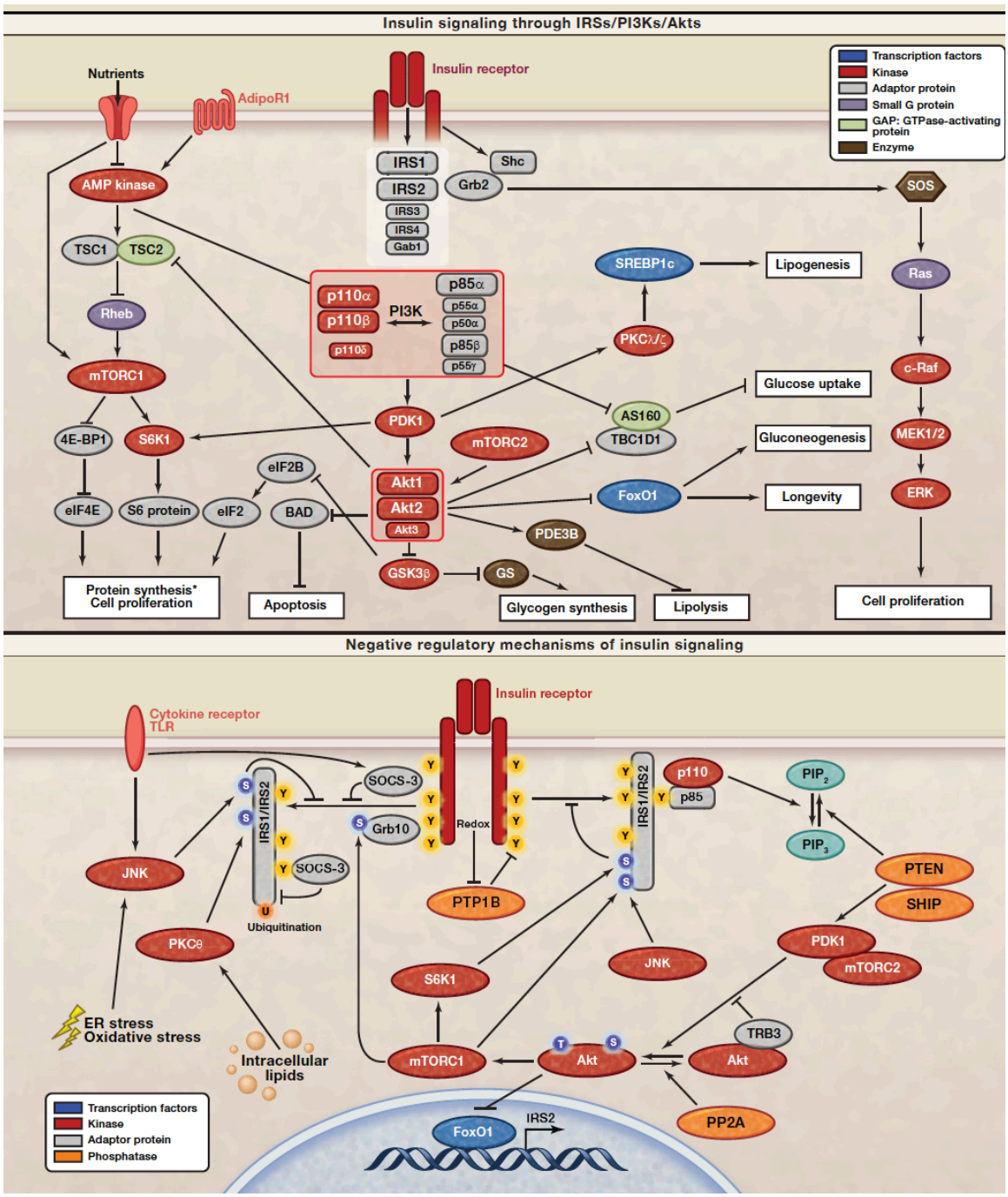

Figure 2. An overview of the insulin signaling pathway and its negative regulatory mechanisms. The top half shows the activation of main nodes (IRS family, AKT family) and the downstream metabolic effects. The bottom half shows the players acting upon the pathway to downregulate insulin signaling. (Kadowaki et al., 2012)

Inflammation

Many studies have shown that obesity is often accompanied by the development of inflammatory diseases (Tilg and Moschen, 2008). The disruption of metabolic homeostasis in nutritionally overloaded cells leads to inflammation through many paths (Chitraju et al., 2017; Hotamisligil, 2017). Due to their size, obese adipocytes experience hypoxia, undergo mechanic pressure by extracellular matrix constriction, and become insulin resistant. All of these processes activate a range of transcriptional pathways, including cytokines, chemokines and inflammatory mediators (Deng et al., 2013; Eguchi et al., 2011; Mansuy-Aubert et al., 2013; Moraes-Vieira et al., 2014; Murahovschi et al., 2014; Perry et al., 2015). The downstream effects of this transcriptional regulation consist of activation of adipose tissue-resident macrophages and infiltration of immune cells (Kälin et al., 2017; Schipper et al., 2012). Upregulated inflammatory kinases such as JNK and IKK further inhibit insulin signaling and glucose uptake, exacerbating the metabolic dysregulation (Glass and Olefsky, 2012; Osborn and Olefsky, 2012). This process also increases pro-inflammatory cytokine expression, worsening the metabolic condition of the adipocytes (Rajbhandari et al., 2018; Zhao et al., 2018). These cytokines lead to a necrosis-like state for a portion of obese adipocytes, after which macrophages will attempt to engulf the adipocyte (Lee et al., 2018). Obesity is known to be a disease associated with inflammation and necrosis, leading to a chronic illness in humans. In turn this becomes a vicious cycle with obesity exacerbating the metabolic dysfunction (Reilly and Saltiel, 2017). The only way to break this cycle is to aggressively treat either condition
(Morley et al., 2015; Oral et al., 2017; Quarta et al., 2017; Wu et al., 2017).

Stem cells & genome editing

Human pluripotent stem cells (hPSCs) exist in various forms. The two major groups used in human research are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are derived directly from embryos, while iPSCs can be made directly from any somatic cell through reprogramming by introducing genes to revert the cell back into a pluripotent cell (Zhu and Huangfu, 2013). The use of iPSCs has been regarded as the ethical way of conducting stem cell research for human studies, due to the ease they can be derived with from readily available adult cells. ESCs on the other hand have thrown up ethical roadblocks because they have to be derived from human embryos. iPSC technology has been discovered in 2006 (Takahashi and Yamanaka, 2006). hPSCs are pluripotent because they can differentiate into all three of the major germ layers; endoderm, mesoderm and ectoderm (Zhang et al., 2012). They also have the capability to replicate indefinitely, meaning they are an infinite source of cellular material. Since the discovery of hPSCs, significant research has been done on the generation of different tissues (Turksen and Troy, 2006). To fully leverage the power of hPSCs for metabolic disease modeling it has to be possible to make mature tissues to study human diseases.

Recent advances have fully unlocked the full power of hPSCs, by enabling genome editing directly *in vitro* (Peters et al., 2013). With the advent of TALENs, and

especially the discovery of CRISPR/Cas9, the possibilities for disease modeling are now endless (Cermak et al., 2011; Cong et al., 2013; Mali et al., 2013). It is now possible to generate hPSC lines with any mutation or deletion, causing the over-activation or knockout of genes. Through endogenous cellular DNA repair mechanisms, it is possible to introduce indels, either short insertions or deletions by leveraging the error-prone non-homologous end joining repair mechanism. On the other hand, specific mutations in the genome can be made by taking advantage of homology-directed repair by presenting exogenous DNA as a repair template. These tools open up the possibility of generating any disease-causing mutation, and with the capability to differentiate genome-edited cells into any tissue, we can study human cells *in vitro* to understand the pathological mechanisms behind the disease. This means we've entered a new era, one where we are able to develop therapeutics based on mechanistic understanding of the faulty gene driving the affliction.

Adipocytes

The main energy-storing cells in the body are adipocytes, which make up the majority of the volume of fat tissue. These cells contain triglycerides as a metabolic reserve and respond to nutritional status by storing or releasing fatty acids (Rosen and Spiegelman, 2014). Adipocytes are exquisitely insulin sensitive as one of their important functions is clearing glucose from the blood upon insulin stimulation to maintain whole-body metabolic homeostasis (James et al., 1988). Subsequently they form triglycerides out of the glucose through lipogenesis and store these fatty acids in large lipid droplets (Collins et al., 2011). Not only does insulin stimulate the uptake of glucose, it also stimulates the fatty acid synthesis on both a protein and transcriptional level (Saltiel and Kahn, 2001).

Obesity is due to expansion of adipocytes, thereby increasing fat and body mass (González-Muniesa et al., 2017). The two mechanisms by which this takes place are hyperplasia and more importantly hypertrophy, which are increased proliferation and adipocyte differentiation, or cell size increase of adipocytes respectively (Rosen and MacDougald, 2006). Obesity and insulin resistance are closely related, as one will cause the other (Garg, 2006). Oversized adipocytes in obese people cause a dysregulation of hormones and lipid handling, eventually leading to adipocyte insulin resistance. On the other hand, insulin resistance causes adipocytes to continually uptake glucose and convert it into stored fat, thereby ballooning in size and causing obesity. Eventually both will lead to chronic inflammation as well (Reilly and Saltiel, 2017).

Adipocytes also have major endocrine functions, which have only recently been appreciated. Hormones released by fat are called adipokines, with most released factors related to metabolic homeostasis (Wozniak et al., 2009). The most well-known of these is leptin, which was also the first adipokine to be discovered (Zhang et al., 1995). It is critical in regulation of appetite and acts as a satiety factor, mainly by signaling through the hypothalamus in the brain (Zhang and Chua, 2017). It also regulates other metabolic processes, including the glucose-fatty acid homeostasis (Perry et al., 2018).Similarly important is adiponectin, an adipokine that also heavily modulates metabolic processes (Christou and Kiortsis, 2013; Nakashima et al.,

2008). It decreases gluconeogenesis while increasing glucose uptake, plus it promotes lipid catabolism by stimulating beta-oxidation and triglyceride clearance (Yamauchi et al., 2001). Through affecting insulin sensitivity and upregulation of uncoupling proteins, it can also modulate body weight. It confers these functions by circulating through the bloodstream and primarily acting upon skeletal muscle, the adipose tissue itself, and the liver.

Adipocytes have traditionally been arduous to study, as the excessive lipid storage complicates most biochemical assays. Additionally, it is hard to access human adipose tissue, unless the subject is obese. Therefore, most human research has only focused on unhealthy adipose tissue. Due to these restrictions, most adipocyte biology has been studied in mice, considering the tractability of the system and the ability to study multi-organ interactions. However, especially in metabolic research findings are often not consistent between mice and men, owing to differing metabolic biology. It has always been a focus of the field to study human adipocytes in both healthy and metabolically dysregulated states. With the advent of iPSC technology, it has become possible to study human adipocytes in a dish. The creation of a differentiation protocol from hPSCs to adipocytes has allowed us unlimited access to biological material for human fat (Ahfeldt et al., 2012). These adipocytes have proven to be transcriptionally extraordinary similar to human adipocytes, display appropriate mitochondrial metabolism, and can be used to study glucose and lipid metabolism (Ahfeldt et al., 2012; Ding et al., 2013). With genome editing we can also model metabolic genetic diseases found in patients, and investigate the mechanisms underlying these pathological states. These adipocytes can also be implanted into mice to assess their function in an organismal setting and interrogate various genetics and functionalities of the adipocytes.

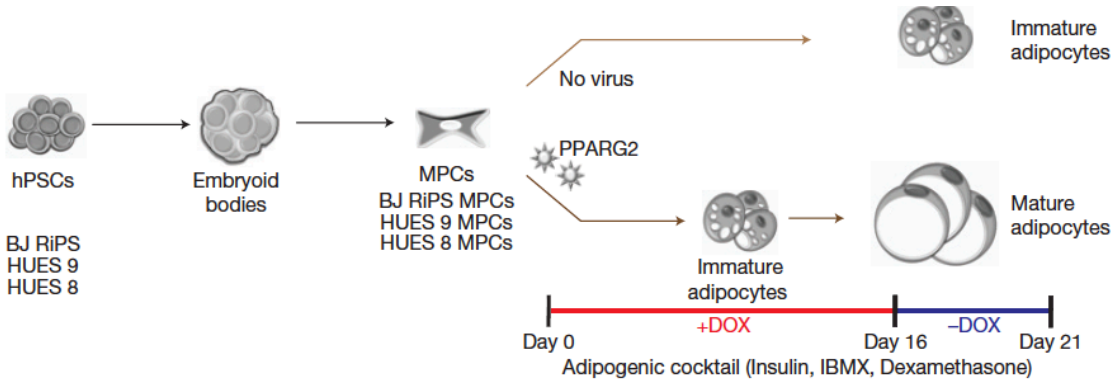

Figure 3. Experimental scheme for the differentiation of hPSCs into adipocytes. hPSCs are differentiated into embryoid bodies, re-plated and passaged to generate mesenchymal progenitor cells. These cells are then transduced with *PPARG2* lentivirus. Adipocytes are differentiated over a 3 week period in adipogenic medium before analysis. (Ahfeldt et al., 2012)

Hepatocytes

Hepatocytes, comprising the majority of the liver's mass, are the cell type responsible for metabolic functions of the organ (Rui, 2014). The hepatocyte is the main source of serum albumin, fibrinogen, and clotting factors. This excessive protein production strains the cells, leading to polyploidy and multinucleate hepatocytes being common (Gentric and Desdouets, 2014). Hepatocytes also regulate lipid metabolism, for example by synthesis and recycling of lipoproteins (Nguyen et al., 2008). They metabolize chylomicrons, synthesize cholesterol, release low-density lipoprotein and take up high-density lipoprotein particles. The liver is also the sole producer of bile salts (Chiang, 2017). Lastly, hepatocytes play an instrumental role in detoxifying the body by metabolizing and inactivating compounds such as drugs, but also endogenous steroids (Guillouzo et al., 1993; Waxman et al., 1988).

One contributor to lipid metabolism in hepatocytes specifically is the 1p13 rs12740374 common noncoding polymorphism. In a GWAS this genetic variant was found to be associated with both plasma low-density lipoprotein cholesterol (LDL-C) and myocardial infarction risk (Teslovich et al., 2010). Through creation of a CCAAT/enhancer binding protein transcription factor binding site it alters the expression of the *SORT1* gene. It has been shown that Sort1 alters plasma LDL-C and very low-density lipoprotein (VLDL) particle levels by regulating hepatic VLDL secretion (Musunuru et al., 2010).

Compared to adipocytes, more research has been done on differentiating hepatocytes from hPSCs. Many protocols have been developed which generate liver cells (Ding et al., 2013; Hannan et al., 2013; Loh et al., 2014). Most do not fully recapitulate the *in vivo* hepatocytes found in the human body, but the resultant cells are sufficiently functional to model glucose and lipid metabolism (Ding et al., 2013). Various approaches have been taken to generate a more mature hepatocyte population, including small molecule additions to the differentiation protocol, specialized culture conditions or hepatocyte purification steps (Mallanna et al., 2016; March et al., 2015; Schepers et al., 2016; Shan et al., 2013).

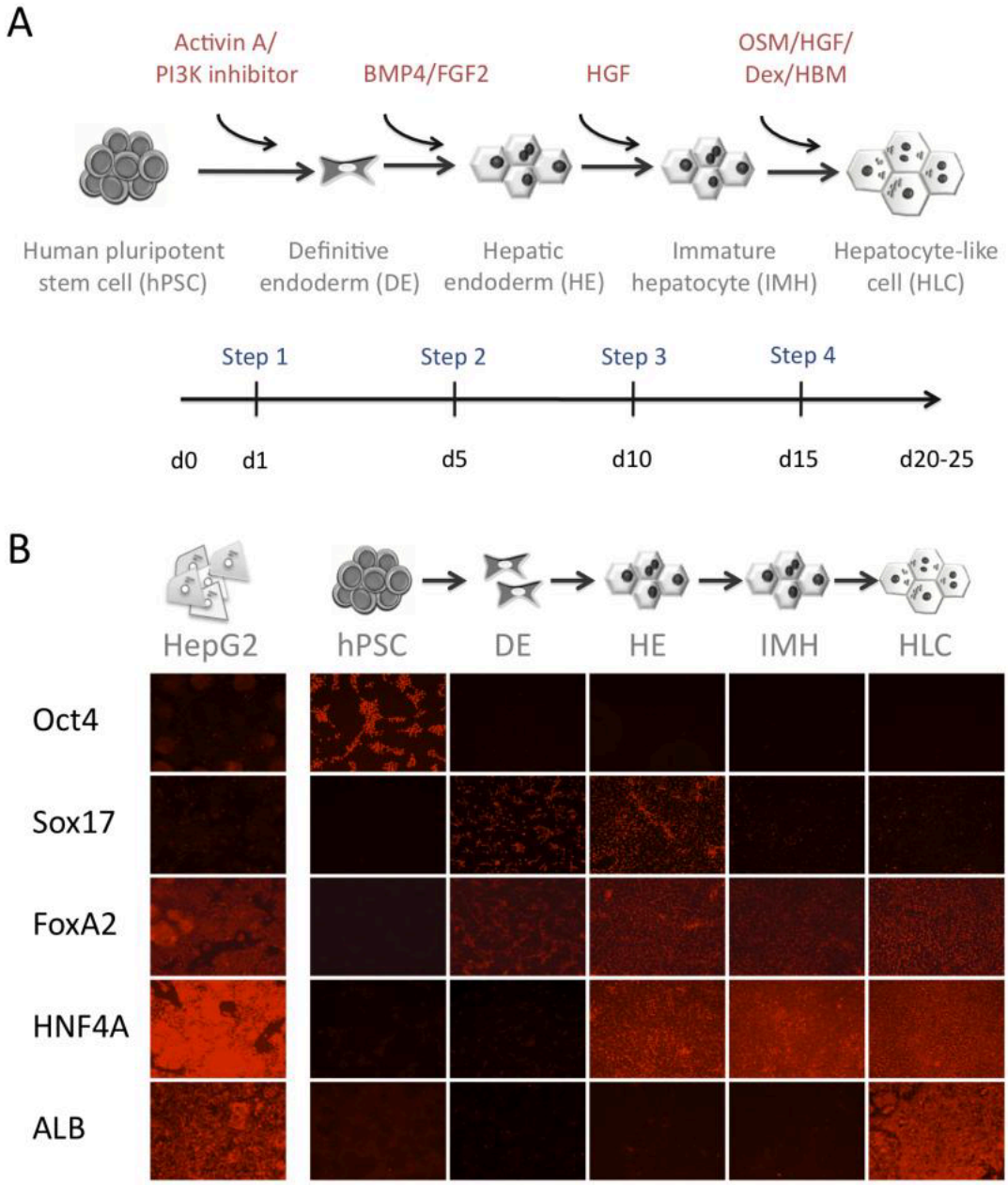

Figure 4. Differentiation scheme and experimental validation of hepatocytes. A) Differentiation protocol of hPSCs into hepatocytes in a four-stage progression. B) Representative hepatocyte differentiation with marker staining at various stages of progress. Cultured human HepG2 cells are used as a comparison. (Ding et al., 2013)

Aim and outline of the thesis

In this thesis we aim to explore the mechanisms of metabolic disease. Given the incredibly broad range of pathologies in this class, there are two main areas we focus on. The first is adipocyte metabolism, which we explore in chapter 2 through 4. In chapter 2 we investigate lipodystrophy by generating iPSCs from patients with a *LMNA* mutation. These cells have a deficiency in adipocyte differentiation, lipolysis, mitochondrial metabolism and dysregulated insulin signaling that promotes adipose wasting. This in turn copies the whole-body phenotype of the patients. In chapter 3 we delve into the mechanism of insulin signaling, by examining the implications of knocking out the insulin receptor specifically in adipocytes. In a murine model lacking the adipose *INSR* we observe severe effects on body weight, driven by a lack of adipocytes, which leads to glucose metabolism dysfunction. While these animals are protected from high fat diet-induced obesity, the severity of metabolic complications is such that it causes a dramatically reduced lifespan. Chapter 4 digs into the relationship between obesity and inflammation, as we attempt to identify a transcription factor that is upregulated during obesity. We find *IRF1* to be highly expressed in obese adipocytes, and *in vitro* differentiated adipocytes overexpressing this factor recapitulate obesity phenotypes. The *IRF1* adipocytes have more unilocular lipid droplets and have attenuated insulin signaling. When implanted into mice they also attract more inflammatory immune cells. This establishes a gene expression link between inflammation and obesity.
The second half of this thesis switches focus to explore liver metabolism. We have optimized a differentiation protocol for hepatocytes in chapter 5. Not only have we standardized the protocol, we also make the case for purification based on surface marker ASGPR1 cell sorting. These purified hepatocytes mimic primary human hepatocytes much closer compared to unpurified bulk hepatocytes. This new protocol sets up the tools required for chapter 6. For this project we needed a supremely robust differentiation protocol to ensure the purity of 68 hepatocyte differentiations of 34 separate iPSC lines. The concept of this study was to recapitulate *in vitro* the findings of a genome-wide association study in which a single nucleotide polymorphism affected *SORT1* expression specifically in hepatocytes. We differentiated hepatocytes with our established protocol, and pairwise differentiated adipocytes as a negative control. The iPSC lines used for this study were generated from 17 donors with a major genotype for the genetic variation affecting *SORT1*, and 17 donors with a minor genotype. We were able to confirm the GWAS findings and demonstrate that it is in fact possible to model SNPs *in vitro* using iPSCs. As a follow-up experiment we identified hepatic metabolites that were significantly regulated by the genotype of these cells.
Finally, at the end of this thesis we present a discussion of the results, the implications for metabolic disease, and the outlook on future research with an aim towards therapeutic interventions for obesity, T2D, CVD and insulin resistance.

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