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

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Discussion

In this thesis we explored the underlying mechanisms of metabolic disease, with a particular focus on adipocyte insulin signaling and liver metabolism. We evaluated the effect of lipodystrophy and obesity on metabolic signaling, as well as the role of the adipocyte insulin receptor in maintaining metabolic homeostasis. In the introduction we laid out the background of the field and rationale for our studies.
The global burden of metabolic disease is overwhelming healthcare systems around the world as the number one cause of death among non-communicative diseases. Medications exist to curb the progression of atherosclerosis, and to alleviate the symptoms of diabetes. However, no treatments exist to fully cure patients of metabolic disease, or to revert the pathogenesis to a fully metabolically healthy condition. The obstacle to developing therapeutics for this set of diseases is that we do not fully understand the mechanisms, or the early beginnings of disease progression. Insulin resistance's advance into obesity or diabetes is not yet understood on a molecular level, and often diagnoses are only made once the disease has reached an irreversible state. Similarly, cardiovascular disease patients are often only encountered when atherosclerosis has progressed too far for prevention. At this point, only the symptoms can be aggressively treated, or heart failure alleviated through surgical intervention. Even though these afflictions are not immediate killers, they lead to health deterioration over time. Especially if present in younger people this leads to a major quality of life decrease, eventually leading to an early death.
In this work we investigated early onset mechanisms of metabolic disease, and the underlying molecular pathology. We examine the contributors to the progression into eventual full-blown diabetes and/or cardiovascular disease. We elaborate on how the insulin signaling pathway contributes to pathological insulin resistance, and how this is affected by lipodystrophy or obesity. As shown by their involvement in obesity, inflammatory pathways play a big role in this process as well. Secondly, we investigate a genetic variant protective against cardiovascular disease, by constructing an *in vitro* system tractable for genome-wide association studies in a dish. Utilizing a large cohort of iPSC lines, we model the effect of a SNP on phenotypes of cardiovascular interest. To this end we developed a protocol amenable to high-throughput differentiation and purification of hepatocytes.

Insulin resistance

The first part of this thesis dealt with the assessment of mechanisms preceding or underlying insulin resistance. We took three approaches to this problem, all centered on adipocytes. We focused on lipodystrophy, obesity, and adipose-specific knockout of the insulin receptor.
In chapter 2 we investigated lipodystrophy by generating iPSCs from patients with a *LMNA* R482W mutation. These donors presented with abnormal adipose tissue distribution, leading to metabolic deficiencies such as diabetes and hypertriglyceridemia. We recapitulated some of these phenotypes *in vitro* with iPSC-derived adipocytes and shed light on the molecular effects underlying these phenotypes. We showed a decrease in adipogenesis and insulin signaling, while mitochondrial and lipid metabolism were heightened. Consistent with symptoms of

lipodystrophic patients these adipocytes also seemed to accumulate markers of autophagy. Altogether this is suggestive of a mechanism that causes the lack of adipose tissue in FPLD2 patients.

In chapter 3 we focus specifically on insulin signaling in adipocytes. It was reported that knocking out insulin receptor function in murine adipocytes improved lifespan and glucose tolerance (Blüher et al., 2003; 2002). Though the mechanism had not been elucidated. In this study we attempted to verify this data and follow up on the beneficial mechanisms driving the improved lifespan. We generated the adipose-specific insulin receptor knockout (AIRKO) mouse using an *Adipoq*-Cre driver, which is more specific than the previously used *FABP4*-Cre (Jeffery et al., 2014). In contrast to this earlier work, we found that AIRKO mice from a young age displayed insulin resistance and reduced glucose tolerance. Interestingly, the insulin sensitivity of these mice was unaffected by age or dietary regimen, which both greatly increase insulin resistance in WT mice. AIRKO mice were inherently equally insulin resistant as obese or geriatric WT mice. The AIRKO mice also had a dramatically reduced lifespan due to their metabolic problems. When we reexamined the phenotype of the previously published *FABP4*-driven fat insulin receptor knockout (FIRKO) mouse we found it phenocopied our AIRKO results. The mechanism by which the AIRKO mouse was protected from diet-induced obesity was a 90% reduction of adipose depots. This adipose mass reduction was accompanied by hepatomegaly and increased hepatic steatosis. This data proves that adipocyte insulin receptor signaling has a profound effect on whole body metabolic homeostasis, with repercussions for other organs. In addition it is required for normal adiposity and energy storage, regulates hepatic lipid homeostasis and ensures a longer lifespan.

Chapter 4 dealt with obesity, the opposite of chapter 2's lipodystrophy. The aim of this study was to elucidate mechanisms contributing to human obesity *in vivo*. By transcriptionally comparing primary adipocytes from obese donors, donor-matched stromovascular cells, and *in vitro* stromovascular cell-differentiated adipocytes, we identified *IRF1* as one of the factors most strongly regulated by obesity. This suggests a possible role for *IRF1* as a connection between obesity and the oft-associated chronic inflammation. After we verified this finding in an independent cohort of people, we sought to discern the molecular effects of this particular gene. Overexpressing *IRF1* in differentiated adipocytes made them more transcriptionally similar to primary human adipocytes. As expected this overexpression increased the expression of inflammatory markers. Surprisingly *IRF1* expression altered lipid droplet morphology and adipocyte function. The adipocytes became less responsive to insulin and were less adept at lipolysis. Their total lipid content did not change, but the lipid droplets were more unilocular and showed higher long-chain fatty acid saturation. When implanted into mice, *IRF1*-adipocytes expressed higher levels of inflammatory markers and displayed increased macrophage infiltration. Altogether this shows a single transcription factor driving the link between an inflammatory state and an obese phenotype, which displays through the lipid droplet morphology.

In this first part of the thesis we investigated insulin signaling in the adipocyte. Not only did we directly interrogate the function in adipocytes, we also observed its workings in both obesity and lipodystrophy, two major adipocyte-caused diseases. We showed that in both of these conditions insulin signaling is disturbed, confirming that the insulin pathway is of paramount importance to maintain metabolic homeostasis. Both too little or too much activity in the

insulin pathway causes metabolic dysfunction, and only a narrow range of stimulation leads to a healthful state.

Liver metabolism

In the second part of this thesis we developed a platform for high-throughput phenotypic assessment of iPSC-derived tissues. There have been several issues with performing large-scale iPSC studies. If the genetic variant to be studied only has a minor contribution to the disease phenotype, the sheer number of iPSC lines required leads to large clonal variation which might obscure the desired readout. Standardization of differentiation and purification strategies has long been thought of as an approach to normalize results between different cell lines. Especially in the field of hepatocyte differentiation, many studies have sought to optimize the differentiation of these cells, with the aim of generating more mature hepatocytes and standardizing the output of a differentiation.

In chapter 5 we presented a robust protocol to differentiate hepatocytes, and a way to purify a mature population leading to reduced variability between cell lines. To this end we utilized a four-stage differentiation protocol, with a final FACS-purification step. Having identified a hepatocyte-specific cell-surface marker, *ASGR1*, we compared ASGR1+ cells versus non-purified hepatocytes. All purified hepatocytes expressed canonical liver markers, as opposed to only a fraction of non-purified cells. While the percentage of ASGR1+ cells in the population differed by more than a log-factor between different iPSC lines, the purified output was remarkably similar in gene expression. Purified hepatocytes were much more similar to primary human hepatocytes. We also showed that the sorted cells can be re-plated, and afterwards displayed dramatically higher functionality by albumin and urea secretion, as well as CYP3A4 activity. With this robust differentiation and purification protocol it is now possible to accurately compare the transcriptome and functionality of hepatocytes generated from many different iPSC lines. No longer does clonal variation threaten to obscure the phenotype investigated.

We leveraged this protocol in chapter 6, where we showed a proof-of-concept approach to functionally validate GWAS-identified disease variants through differentiation of iPSCs. In this case we studied cardiometabolic disease by investigating the effect of GWAS-identified variant 1p13 rs12740374 in differentiated hepatocytes and adipocytes. This SNP has been shown to increase gene expression of *SORT1*, *PSRC1*, and *CELSR2* in hepatocytes through a CAAT-enhancer-binding protein recognition site (Musunuru et al., 2010). Increased hepatic *SORT1* expression leads to a decrease in LDL-C secretion, which proves to be beneficial for myocardial infarction risk. We received 34 iPSC donors each for the major and minor genotype at this variant locus from the Framingham Heart Study. From each of these donors two separate iPSC lines were made, which were subsequently differentiated into adipocytes and hepatocytes in duplicate. Using our previously established purification protocol, we saw a vast difference in ASGR1+ cell percentage of each differentiation, but a negligible hepatocyte marker gene expression difference after purification. The *in vivo* described effect of the SNP on *SORT1* gene expression held true *in vitro*. The minor haplotype exhibited increased *SORT1* expression, and decreased APOB secretion. As a discovery effort we also identified a number of trans-eQTLs in hepatocytes that were related to the SNP genotype. Secondly, we interrogated the metabolome in these hepatocytes. We found that many lipid metabolites were significantly regulated between the major and minor genotypes, with some lipid species enriched up to 4-

fold. The genotype-driven lipid dysregulation occurred over a wide spectrum of lipid species, hinting at a generalized genotype-related lipid homeostasis mechanism instead of a pinpointed single metabolite target. With this study we proved it is now possible to functionally study GWAS variants in a dish, and the option now exists to undertake genome-wide studies on iPSC cohorts *in vitro*. These studies are also more tractable to discover molecular mechanisms, as GWAS studies do not illuminate underlying causes of the SNP's contribution to disease. Here we showed it is possible to correlate common genetic variants with cellular function, and we predict that future studies with larger cohorts will elucidate the function of many as of yet unexplained loci.

Future perspectives

Metabolic disease is an incredibly complicated affliction, and we are not even close to fully explaining the fundamental molecular mechanisms despite significant progress in the field. This thesis aimed to contribute to the elucidation of mechanisms involved in insulin resistance and lipid homeostasis. We have shown disrupted insulin signaling in both lipodystrophy and obesity and investigated the effects of insulin receptor deletion in adipocytes, now-known to be deleterious. However, we have not yet shown the causal driver of either pathology and are still missing vital information regarding the transcriptional effects of insulin resistance. While the lack of adipose tissue in lipodystrophy and insulin receptor deletion clearly disturbs metabolic homeostasis, the communicative factor by which other tissues sense the lack of fat tissue has not yet been found. It could be something as trivial as hyperglycemia or hyperlipidemia, but we believe there is a whole slew of undiscovered players warranting further research. The characterization of the signals emanating from the adipose that regulate metabolic homeostasis is of utmost importance to combat the global epidemic of obesity and diabetes. Further studies that assess the transcriptomic and proteomic changes brought on by abrogated adipose insulin signaling will be incredibly informative. We, as well as other groups, have begun to characterize the phenotype of these metabolic diseases.

Here we have showed several improvements on existing tools to study metabolic disease. However, this does not mean no additional progress can be made on the tool-building front. We have presented an optimized, robust differentiation protocol for both hepatocytes and adipocytes, with an extra purification step for hepatocytes. However, both of these cell types do not yet fully recapitulate their primary human counterparts. Adipocytes suffer from the fact that we cannot generate them without transcription factor overexpression, leading to an artificial adipogenesis process. Future work will have to devise a differentiation protocol independent of exogenous factor overexpression. Secondly, the two major adipose depots, visceral and subcutaneous, show dramatic differences *in vivo*, while the *in vitro* fat as of yet is unable to accurately model this distinction. On the hepatocyte side, the cells are able to display phenotypes related to glucose metabolism and lipid homeostasis. However, many groups are working on maturing these hepatocytes, especially to increase their capacity for drug metabolism and toxicology studies. The ASGR1+ hepatocytes we presented here are a revolutionary step forward compared to any previous protocols and do display a more mature phenotype. However, the final differentiation stage is still missing key functions, including features such as hepatocyte zonation.

For our purposes the hepatocytes generated after purification were more than sufficient to prove the phenotype desired. The gene expression effects of the assessed SNP fully phenocopies *in vivo* results, and we were able to investigate the downstream regulation of lipid species metabolism. As a proof-of-concept study the results were everything we had hoped for. This was the first demonstration of a GWAS in a dish, where we are able to interrogate the molecular mechanism, an opportunity impossible in human GWAS. We have applied this large-scale iPSC cohort methodology to one particular SNP for which a mechanism was previously elucidated. However, this opens the door to similar studies on other SNPs where a mechanism has not yet been established. This methodology is still not tractable for most of the scientific community, with the need for excessive resources, manpower and large cohorts of iPSC lines harboring the appropriate genotype. Technological developments will alleviate this problem in the future. Follow-up studies are being conducted to scale down the cellular material needed, and with the advent of single-cell RNA-sequencing and single-cell proteomics this possibility draws ever nearer. Additionally, growing repositories of commercially available iPSC lines of various genotypes, and the increasing tractability of genome editing will soon make any genomic variation accessible for study. With these tools for studying genetic variation, deep investigation of the insulin signaling pathway, and assessment of lipid metabolism homeostasis, we are hopeful that in the near future it will be possible to therapeutically intervene in the progression of insulin resistance. The tide of obesity, diabetes and associated cardiovascular disease will soon be turned.