The Culture and Differentiation of Human Pluripotent Cells into Brown and White Adipocytes

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1. Introduction:

1.1 Developmental Biology:

One of the major transitions during the course of evolution was the development of multicellularity. The possibility of cellular specialization permitted a whole new level of complexity and initiated a process that culminated in the amazing variety of life we observe today. At one end of the spectrum of complexity, on the border of single- and multi-cellular organisms, resides the slime mold *dictyostelium discoideum*, which can switch back and forth between both life styles when necessity dictates it[1, 2]. At the other end of this complexity scale are organisms like *homo sapiens*, consisting of trillions of cells organized into an estimated 30,000 highly specialized cell types.

At the heart of developmental biology lies the question of how organisms grow and develop, how a single cell can become an organism in all its complexity. As with solving most challenges in basic science, there is a practical dimension to this question. With the understanding of development comes the possibility to utilize this knowledge for modern medicine and the treatment of human disease.

Over the last century, work on model organisms like *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, *Xenopus laevis*, *Gallus gallus* and the previously mentioned *Dictyostelium discoideum*, has provided invaluable insights into the intricate interplay of endogenous and exogenous factors that coordinate cell differentiation, morphogenesis and growth. This research further helped illuminate the underlying masterpiece of temporal and spatial control of gene expression at the heart of development. Intriguingly, most of the mechanisms identified were highly conserved and were readily confirmed in the prominent mammalian model organism *Mus musculus*. Yet, differences emerged. Development in mammals is slow and involves substantial growth due to maternal

involvement. Therefore, the mouse model became central to the study of mammalian embryogenesis[3].

Be it as it may, model organisms can only give insight up to a certain point. The closer we get to the prospect of useful applications for human benefit, the clearer it becomes that next to the work on mice, flies, chicken and frogs, work on human cells can make important contribution to the scientific process. To validate and further expand the knowledge of mammalian and specifically human developmental biology, the recapitulation of development using human *in vitro* cell-culture systems has become an important cornerstone.

Out of the various *in vitro* culture models, the culture of human pluripotent stem cells has emerged as one of the most useful research tools. Human pluripotent stem cells possess two important characteristics: the capacity to generate cells of all three primary germ layers and the ability to proliferate for extended periods of time in culture while maintaining an undifferentiated state. Because of these attributes, human pluripotent stem cells may not only prove to be valuable for developmental biology research but may also have the potential to revolutionize medicine.

This potential and the possible roadblocks ahead will be the central topic of this thesis, with a special focus on the field of adipocyte biology.

1.1.1 Mammalian Development

In mammals, development begins with the fertilization of an egg, progresses through growth and specialization and is finalized with the adult organism. Specialized cells are formed by a one-way process in which the developmental potential of any cell decreases as it becomes more committed to its fate[4]. In this process cell types progressively lose their developmental potencies. Starting with totipotent cells which are able to form the

whole organisms, and ending with terminally differentiated cells which are more or less fixed in their acquired cell fate.

This loss of plasticity is why in mammals only the zygote is considered totipotent, defined by the potential to give rise to all of the specialized cells, including the extra-embryonic tissue[5, 6]. The zygote, following several rounds of division, develops into the blastocyst consisting of two parts, the outer layer termed trophoblast and the cells of the inner cell mass (ICM). ICM cells are considered pluripotent since they can form all embryonic tissues, namely ectoderm, endoderm and mesoderm, but have lost the ability to give rise to extra-embryonic tissues such as the placenta[7].

Multipotent cells are further restricted in their potential but can still give rise to multiple different cell type within their lineage. In adult organisms these cells are termed adult stem cells and act as a maintenance and repair system for the body by replacing lost and damaged cells. Examples are skin stem cells, neural stem cell, intestinal stem cells and hematopoietic stem cells[8, 9]. Unipotent cells only have one developmental option and are thus only capable in sustaining one cell type, as for instance hepatocytes[10, 11].

1.1.2 Pluripotent Stem Cells

Out of this succession of cells of varying potencies, pluripotent stem cells emerged as one of the most studied cell type and a promising research field. This is partly due to their potential to form every tissue of the organism proper and due to their ability to self-renew, to maintain this potential over an indefinite amount of cell divisions.

Hereafter the term pluripotent stem cell (PSC) will be used as an umbrella term for embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) respectively. If the cell lines have their origin in human cells, the term human pluripotent stem cell (hPSC) will be used as reference.

1.1.2.1 Embryonic Carcinoma Cells

One of the early breakthroughs in the study of pluripotent stem cells was the establishment of immortal pluripotent stem cells lines from teratomas, termed embryonic carcinoma cells (ECC). Teratomas are malignant germ cell tumors comprised of an undifferentiated and a differentiated compartment which can include all three germ layers. Although described centuries ago, teratomas were difficult to study simply due to their extreme rarity. This changed in the 1950s, when the observation that the males of a certain mice strain have a high incidence of testicular teratomas made those cells amenable to experimental analysis[12-14]. Because of their origin as cancerous cells, most of the ECC lines were genetically abnormal (aneuploid) and only inefficiently contributed to the adult somatic tissues and rarely to the germline[15-17]. Nonetheless, this early work on ECC significantly improved our understanding of pluripotent stem cells and helped identify the appropriate conditions to culture these cells in research.

1.1.2.2 Embryonic Stem Cells

The observation that teratomas can be experimentally induced by the transplantation of implantation-stage mouse embryos into histocompatible host mice, inspired the successful isolation of ESCs from the ICM of a mouse blastocyst[18-20]. Other than the ECC lines, the ESC lines were karyotypical normal and contributed efficiently to all germlayers and the germline. Furthermore ESCs have the potential to form an entire ESC-derived animal via tetraploid embryo complementation, thus passing the most stringent developmental assay of pluripotency in mice. The tetraploid blastocyst has the ability to develop an extraembryonic tissue but fails to develop into an embryo proper. Therefore, to develop into an animal the introduced ESCs have to give rise to the complete embryo[21, 22].

Since the first derivation of ESC, a range of other pluripotent stem cells lines have been derived from origins other than the ICM of the blastocyst. Of notice here are the epiblast-

derived stem cells (EpiSC) isolated from the post-implantation epiblast stage. While exhibiting a range of molecular commonalities, EpiSCs are not able to generate the whole embryo in a tetraploid complementation assay. This is thought to be caused by an imbalance in parental imprints[23-25].

1.1.2.3 Human Embryonic Stem Cells:

Similar to the mouse equivalent, human embryonic stem cells have their origin in the ICM of the blastocyst and are generally derived from human embryos generated in surplus by the *in vitro* fertilization process. The derivation of these cells was first successfully performed around two decades ago and since then several independent groups were able to establish a range of hESC lines[26, 27]. This allowed hESCs to become an important tool to study human development at the cellular and molecular level.

hESC are characterized *in vitro,* based on their molecular properties, their pluripotency and their ability to self-renewal[28]. Work both on mouse ESC (mESC) and hESC lead to the identification of the core network of transcription factors, consisting of octamer-binding factor 4 (OCT4), sex-determining region Y-box2 (SOX2), and NANOG, which are necessary for maintaining ESC pluripotency[29]. A transcription factor (TF) is a protein which binds to the DNA sequence and thereby promotes or blocks the recruitment of RNA polymerases. Each cell type and differentiation state is thought to possess a characteristic TF expression signature, and the presence of a TF network responsible for maintaining pluripotency, is considered an important ESC characteristic[29, 30]. These pluripotency markers are generally accesses by expression analysis, immuno-staining and by examining the epigenetic state of the promoter region.

The most stringent evaluation of any PSC is to test their ability to form an organism proper through tetraploid embryo complementation experiments. However, ethical and technical reasons prevent this kind of evaluation with hESCs. Therefore the most rigorous assay for hESCs is an *in vivo* assay in which hESC are injected under the kidney

capsule of immunocompromised mice, leading to a subsequent formation of a teratoma. Additionally, to further demonstrate their pluripotency hESCs are often differentiated *in vitro* into a variety of cell types of all three germlayers.

1.1.2.4 Comparison of Epiblast-Derived and Embryonic Stem Cells

Human and mouse ESCs are derived from similar developmental stages. Nonetheless, they show differences in morphology, cell proliferation rate, growth factor requirements and cell surface markers[31]. In all these characteristics, hESCs share features with mouse EpiSCs rather than with mESC. Recently, several groups were able to demonstrate the derivation of hPSCs lines with mouse ESC-like character and convert hPSCs from one state to the next, thus identifying two discrete and interconvertible states of pluripotency in culture[32-35].

One of these states, represented by hPSCs and mouse EpiSCs, seems to make their maintenance in culture challenging. hPSCs are less robust, resistant to single-cell dissociation, are prone to spontaneous differentiation and possess reduced karyotypical stability when compared to mESC[23, 24, 36-38]. Therefore converting this "classical" state into a "mouse-like" state of hPSCs, could potentially significantly facilitate the culture and maintenance of hPSCs. However, the conversion is dependent on exogenous expression of at least one reprogramming factor (KLF4), a caveat which has to be resolved before this technique can become a useful alternative to "classical" hPSC culture[31, 39].

1.1.3 The Road to Induced Pluripotent Stem Cells

The derivation of induced pluripotent stem cells is considered the major breakthrough in the last decade in regenerative medicine[40]. By introducing the technology to generate pluripotent stem cell lines from terminally differentiated cells, Yamanaka and colleagues

at once overcame a range of major hurdles in the field of stem cells research. The fundamental insights on the road to this seminal discovery, were derived from the study of nuclear reprogramming and involved important contributions of three distinct experimental approaches: nuclear transfer, cell fusion and lineage reprogramming[6].

1.1.3.1 Nuclear Transfer

The development of a technique called somatic-cell nuclear transfer (SCNT) (often referred to as nuclear cloning), enabled to test the developmental potential of a nucleus isolated from a late stage embryo by transplanting it into an enucleated oocyte[41]. Before this discovery, the loss of developmental potency in differentiation was thought to be accompanied by a reduction of genetic information or by a permanent inactivation of genes. Based on the results of these SCNT experiments, this view was quickly overturned. By generating the complete animal from cells which were differentiated, it became clear that these cells retain the full genetic information necessary to form an adult organism[4, 42-44]. These experiments were performed mainly in amphibian cells and it took decades before they were confirmed for the mammalian lineage with the cloning of Dolly the sheep[45]. Since then, cloning of animals has been repeated in a range of other mammals, but thus far yielded little success in the primate lineage[46-49].

In summary, these experiments demonstrated that specialization and the accompanying decrease of developmental potential, involves changes in gene expression and not gene content, and under circumstances, a developmental state can be fully reversed[6, 50]. In addition, the observation that by exposing nuclei of differentiated cell to the cytoplasma of enucleated oocytes, their developmental fate can be changed, implicated that the oocyte contains factors responsible for this change. These observations were in accordance with insights gained by another method of nuclear reprogramming, cell fusion.

1.1.3.2 Cell Fusion

Cell fusion involves the merging of two or more cells to one entity. It can occur naturally as for instance in fertilization or the maturation of myocytes. However, the fusion of two cells with differing cell identities is generally induced by experimental conditions. This technique was initially used for the first successful identification of tumor suppressor genes[51, 52]. In addition the process of cell fusion was utilized to examine the effect of one differentiation state on another through merging cell with differing developmental potential. Intriguingly, the fusion of a pluripotent cell with a differentiated somatic cell, led to a reversion of the differentiated cell into an undifferentiated state, a finding which could be reproduced with hESC[53-55]. Therefore this approach helped confirm the observation gained by SCNT, that mammalian somatic cell states can be altered.

1.1.3.3 Lineage Reprogramming

Other clues to a certain plasticity of cellular fate, emerged from a range of elegant transplantation studies performed in *Drosophila melanogaster*. By explanting cells from one area of the embryo and exposing them to another microenvironment of the same embryo, the transplanted cells underwent "transdetermination". Cells which were destined to become one specific cell-type change their fate. These results highlighted, that in some instances, the developmental fate of a cell is dictated by their new cellular neighbors, not by their original position in the embryo[56-58].

In summary, experiments utilizing SNCT, cell fusion and transdetermination techniques all provided evidence that cell fate is reversible and that cytoplasmic factors and cellular microenvironment are able to alter the differentiation state of the cell. The next step in illuminating this phenomenon was the identification of the factors which are responsible for the maintenance or reversion of the differentiation state. Through the fusion of myocytes with fibroblasts, myogenic differentiation 1 (MyoD) was the first of these regulatory factor which was identified[59, 60]. Subsequent experiments demonstrated that

ectopic expression of the transcription factor MyoD was sufficient to induce the formation of myofibers in fibroblast cell lines[61]. Up to this day, the realization that selected transcription factors can change the fate of a cell when ectopically expressed, leads to the identification of key regulatory transcription factors. Examples include the successful conversion of primary B- and T- cells into macrophages by CCAAT/enhancer-binding protein alpha (CEBPA), the *in vivo* derivation of insulin producing β-cells from pancreatic acinar cells by ectopic expression of MafA, Pdx1 and Ngn3, and the generation of neurons from fibroblasts via the expression of Ascl1, Brn2 and Myt1l[62-66]. In summary, these experiments showed that the differentiation state is subject to a dynamic control by transcriptional regulators. Therefore, by perturbing the stoichiometry of these factors, a differentiation state could potentially be altered[6].

1.1.3.4 The Derivation of Induced Pluripotent Stem Cells

The generation of induced pluripotent stem cells from terminally differentiated cells is one of the major breakthroughs in pluripotent stem cell research. This discovery was made possible by the advancements of techniques to derive, culture and study pluripotent stem cells. Furthermore, the realization that terminally differentiated cells maintain their genetic information and the identification of a key transcriptional network for pluripotency represented key insights on the way to the first generation of hiPSCs[20].

In the spirit of the lineage reprogramming experiments, Yamanaka and Takahashi devised a screen which identified four factors Oct3/4, Sox2, Klf4 and c-Myc, which were able to reprogram fibroblasts into pluripotent stem cells. While these cells could be differentiated into all three germlayers and possessed many important characteristics of pluripotent cells, the initial iPSCs had some shortcomings including the inability to generate postnatal chimeras or contribute to the germline[40]. However, these issues were soon overcome by the field at large, including by Yamanaka's group itself, which was able to reproduce and expand on the results of the first study[67-69].

This insight sparked an immense interest in pluripotent stem cells and lead to a string of high profile publications improving on the technical challenges of iPSC technology. The initial low efficiency, only about 0.1% of transduced fibroblast successfully reprogrammed into iPSC, has been consistently improved[70-73]. The introduction of the reprogramming factors via retro-virus has been substituted by methods which avoid mutagenesis through integration into the genome[74, 75]. Parts of the underlying reprogramming mechanism have been illuminated and iPSC have been thoroughly analyzed and compared to ESC[76]. Various iPSC lines have been derived from scores of somatic cell populations and were successfully differentiated into a range of adult tissues[77-82]. Furthermore the technique has been used to create iPSC from a range of organisms such as rats and rhesus monkeys[83, 84]. Finally, hIPSCs have been derived simultaneously by a range of laboratories[85-87].

1.1.4 The Promise of Human Pluripotent Stem Cell Biology

1.1.4.1 *In vitro* Model of Human Development

Human pluripotent stem cells can be important for developmental biology and for regenerative biology in a variety of ways. Developmental science may profit through the ability to analyze early developmental processes and through comparison of knowledge gained by work on model organisms to a human system. In addition, the ability to genetically manipulate hPSCs enables the design of knock-out and knock-in experiments to study normal and abnormal development.

Additionally, hPSCs allow the investigation of early development *in vitro* and have the potential to provide cell types which are so far inaccessible for propagation in culture. The proliferation capacity of hPSCs makes large scale experiments feasible, including transplantation of hPSC-derived tissue into immunocompromised mice.

1.1.4.2 Cell Replacement with Human Pluripotent Stem Cells

The ability of pluripotent stem cells to self-renew indefinitely and to differentiate into every cell type of the organism proper enables them to serve as a valuable source for cells, tissues and organs in regenerative medicine. The notion to replace damaged cells, tissue and even whole organs with healthy ones, has led to incredible improvements in transplantation medicine in the last decades, with thousands of patient lives extended every year. hPSCs could benefit transplantation medicine by providing an *in vitro* source to overcome the chronic organ donor shortage. In addition, the use of patient specific hiPSC lines can prevent rejection of transplanted tissue by the host immune-system and eliminates the need for lifelong treatment with immunosuppressant drugs. Furthermore, fatal complications such as host vs. graft disease can be avoided.

There are a variety of tissue types which are very challenging to harvest from organ donors, an issue prescient in neuro-degenerative diseases. For these disorders, cells are difficult to obtain in sufficient numbers and transplantation often achieves little to no success. Parkinson's disease, Alzheimer's disease or amyoltrophic lateral sklerosis (ALS) are the most common diseases of a range of severe and fatal neurodegenerative disorders. These diseases could potentially be cured by differentiating hPSCs into the damaged neurons or into earlier progenitor cell population which still possess the capability to integrate into the host nervous system. Additionally, hPSC could be beneficial for diseases in which cells are lost to injury or disease, such as diabetes mellitus type 1, cardiomyopathies or spinal cord injury.

The combination of PSC technology and gene therapy allows the repair of genetic defects in stem cells *in vitro* via genome editing, and a subsequent transplantation of the restored cells could potentially cure a range of genetic diseases. That this is indeed a possible strategy, has been successfully shown in a murine proof-of-principle experiment[88, 89]. The first attempt to utilize of hESC as a source for cell replacement therapy in the clinic was performed in 2009 by the company Geron. The use of hESCs for cellular therapy of

spinal-cord injury moved into a phase I safety study, but was soon stopped after microscopic cysts were found around the injury site in animal studies. This problem highlights a variety of conditions which need to be established before hPSCs can serve as a routine source for cell replacement therapy: controlled cell proliferation, efficient cell differentiation and maintenance of genetic integrity[90] (→1.1.5). On the upside, a recent trial utilizing hESC-derived retinal pigment epithelium in the treatment for macular degeneration showed very promising results without any observed complications[91, 92].

1.1.4.3 Disease Modeling with Human Pluripotent Stem Cells

Diseases are due to complex interplay of environmental and genetic factors. To disentangle this vast complexity underlying human diseases, science has had great success by reducing this complexity into controllable experimental settings. This reduction has been commonly achieved by selecting simple model organisms or by utilizing *in vitro* culture systems. These approaches have provided important insights which otherwise would have been close to impossible to achieve.

As fellow mammals, mice have become the prominent model organism in modeling human disease. They are comparatively close in evolutionary terms, can be easily accommodated, and allow investigating gene function by reverse genetic approaches. Yet the differences between human and mouse biochemistry, physiology and anatomy are profound and are visible in the failure to recapitulate many human diseases in the mouse model[93, 94]. In the cases certain aspect of the human disease can be experimentally induced in mice, it is often subject to controversy whether these phenotypes can be considered equivalent to the disease in human[95].

The ability to establish hPSC lines from patients affected by genetic disorders provides the opportunity to study the disease in an *in vitro* setting while avoiding the pitfalls of animal models. Additionally, the unlimited self-renewal of hPSC, allows the generation

of large quantities of the affected cells, and their pluripotency enables the generation of a wide variety of cells types, including those usually only accessible post mortem.

Patient specific hPSC lines were initially established through the generation of hESC lines of embryos carrying various severe genetic disorders, such as cystic fibrosis or Huntington's disease. These embryos were identified through preimplantation genetic diagnostics (PGD) in an *in vitro* fertilization setting[26, 96-98]. Alternatively mutations in normal hESC lines were successfully introduced via homologous recombination[99, 100]. Both approaches utilizing hESC lines had major limitations, namely the scarcity of embryos carrying disorders, ethical concerns regarding the derivation of hESC lines from these embryos and the inefficiency in gene targeting in hESCs[93]. Furthermore, diseases in which the genetic cause is unknown were inaccessible.

The first derivation of hiPSC was able to overcome these obstacles, by providing a method to generate patient specific hPSC lines from somatic cell types. It became possible to generate large quantities of hiPSC lines of a disease with minimal-invasive techniques and with relative ease and speed. This in turn, provided alternative *in vitro* systems to study disease phenotype, progression and underlying causation. Importantly, hPSC allow the study of afflictions in which a phenotype only presents itself after the disease already progressed to the final stages. For instance, patients suffering from Parkinson's disease generally show little to no symptoms before a large part of the dopaminergic neurons of the substantia nigra is already degenerated[101]. Disease modeling with hPSCs has therefore the potential to create a window to observe the progression as well as the final stages of a disease.

In vitro disease modeling utilizing hPSCs may not be feasible for all diseases, since the disease must have a manifestation at the cellular level and a phenotype assessable in cell-culture conditions. Moreover, diseases which depend on a complex interplay of various tissues, are slow in developing symptoms, have a low penetrance or a strong unknown environmental component, will be very difficult to model. It is therefore no surprise that

most of the established hPSC-based *in vitro* disease models are monogenic diseases with robust cellular-phenotypes, such as spinal muscular atrophy (SMA), Duchenne muscular dystrophy, fragile X syndrome, Rett-syndrome, cystic fibrosis or Huntington disease[87, 102-112]. Nonetheless, the impressive establishment of a Schizophrenia *in vitro* model showed that even multi-factorial, multi-genic diseases are suitable for disease modeling with hPSCs. The additional identification of a previously unknown cellular disease phenotype highlights the opportunity to study the underlying mechanism by disclosing new relevant biological signatures of the disease[112, 113].

One of the keys to the successful use of hPSCs in studying diseases is the identification of a cellular phenotype which relates to the known aspects of the disease pathology. This phenotype is often only observable in mature cell types. Therefore, the lack of efficient and robust *in vitro* differentiation protocols for many functionally mature cell types may indeed be the greatest challenge for the utilization of hPSCs in disease modeling at present (→1.1.5.6).

1.1.4.4 Drug Discovery with Human Pluripotent Stem Cells

The screening of large compound libraries against single enzymes or receptors is currently the most common approach for target driven drug discovery. Typically, a promising compound is then tested in relevant *in vitro* and/or animal models. However, only few drugs make it through all the clinical trial phases and eventually become an available drug. This high attrition rate is caused in large part by the lack of suitable *in vitro* and *in vivo* disease models which prevents a detailed study of the drug effects. Candidate drugs therefore are often insufficiently characterized and often fail to exhibit strong beneficial effects or cause unanticipated detrimental side-effects in a clinical setting[114-116].

hiPSC could in principle support drug discovery in several important ways. The ability to generate patient specific cell lines should allow the establishment of powerful new *in*

vitro disease models which closely resemble the actual disease phenotype. In addition, hPSC-based cell system could help identify detrimental drug side effects early in the development of a new drug. For example cardiomyocytes and hepatocytes, the two most common cell types affected from drug-toxicity, are difficult to obtain in sufficient quantities as primary tissue and have only a finite lifespan in culture. Both cell types can and are generated in large quantities from hPSCs which might soon enable to test compound for toxicity during preclinical drug development. hPSC-based cell models can be useful at every level of the preclinical studies by generating drug efficiency and toxicity information, and can help to avoid unnecessary trials by more reliably predicting clinical outcomes[106, 117-119].

Another possible role of hPSC in drug development concerns the observation of a surprisingly high variability in the patient response to drugs in the clinic. While a drug works perfectly fine for some patients, it has sometimes little to no effects on the next and even exhibiting severe toxic side effects on others. Generating hiPSC lines of these patients may help to identify the possible molecular causes, as well as offer the possibility to study the heterogeneity of patient populations due to gender or ethnicity[106]. The feasibility of this approach has been demonstrated by two recent publications. The first was able to predict the sensitivity to a drug in schizophrenic patients, while the second successfully screened for cardiotoxic effects of substances based on genetic background[105, 112]. Similar approaches may one day minimize the often difficult and harmful process of identifying the right drug and dose for a patient. In the future, the use of iPSC-based approaches may enable a form of personalized drug development, in which substances are screened based on their effect on specific genetic backgrounds in the human population. The treatment for each patient could then be refined based on the best possible genetic match.

Drug discovery faces a variety of challenges, which prevented hPSC-based models to live up to their full potential. For example the configuration of an assay needs an easily

accessible cellular phenotype, and robust and efficient differentiation protocols. In addition, the setup needs to scalable and amendable to automation[106]. The differentiation of iPSCs into specialized target cell types is, with few exceptions, a complex and timely process. This long preparation time for each assay, increases the variability and inconsistency of results and exacerbates the problem of variance and heterogeneity observed in differentiation protocols. Thus far successful drug screens have only been achieved using mESCs and mouse iPSCs[120].

1.1.5 Roadblocks and Challenges

hPSCs may one day be able to revolutionize drug discovery and cell replacement therapy[121]. Nonetheless, several roadblocks and challenges need to be overcome. Next to the specific challenges of each experimental design, such as the identification of a cellular disease phenotype in disease modeling, there are a few general technical issues which have to be overcome before hPSCs can be routinely used as a research tool.

1.1.5.1 Culture and Maintenance of Human Pluripotent Stem Cells

hPSCs are difficult to culture. Their growth and maintenance have remained challenging since their first derivation and when compared to mPSC, hPSCs are less robust, more prone to spontaneous differentiation, difficult to propagate as single cells, and less amenable to genetic manipulation. These observations have led some scientist to propose that switching from the "classical" state to a "mouse-like" pluripotency state, could facilitate the culture and maintenance of hPSCs (→1.1.2.4).

Another issue in the culture of hPSCs has been the difficulty to reproduce data and/or the transfer of protocols. This is thought to be caused by variations in the culture condition i.e. different batches of animal sera, mouse embryonic fibroblast (MEF) lines and the demanding handling of the cells. Therefore, the introduction of defined media conditions

as well as feeder free systems using various protein matrices, led to significant advances in the general ability to culture hPSCs[122-125]. Additionally, improvements facilitating survival as single cells have been achieved recently[125-127]. While these improvements helped to stabilized hPSC culture, an overall efficient and robust culture methodology is still amiss.

1.1.5.2 Genomic Integrity

Due to the arduous effort, time and resource investment necessary for hPSC experiments, facilitating and improving culture techniques would greatly benefit the hPSC research. Additionally, an enhanced culture platform could also reduce the time each cell remains in culture. This is especially important since hPSC seem to be prone to accumulate karyotypical aberration over time, especially when expanded clonally[128-130]. These abnormal cells may influence experimental results by introducing an artificial heterogeneity into the cell population. Especially regarding the use of hPSCs in clinical therapy, the observation that the culture conditions may select for specific genetic abnormalities over time is concerning[131]. Moreover, the same abnormalities reoccurred in various cell lines and were also in testicular germ cell tumor samples. The potential selection for aberrations associated to cancerous growth, further highlights the need to reduce the time cell remain in culture as well as reducing the selective pressure by optimizing culture conditions[27, 36, 90, 128, 129, 132, 133].

1.1.5.3 Genetic Manipulation

The lack of an easy and efficient way to genetically manipulate hPSCs is another obstacle. The ability to alter hPSC is a prerequisite for a range of experiments such as the generation of reporter cell lines or for gain- and loss-of function approaches[134]. Viral transduction has been achieved in hESCs using ultra concentrated virus, however, the efficiency remained generally low[135, 136]. Gene targeting via homologous recombination (HR), a standard procedure in mouse embryonic stem cells, was also achieved in

2003.However, only few successful studies have been published since[136-141]. A number of recent publications try to bypass this challenge, by utilizing zinc finger nucleases or transcription activator-like effector nucleases (TALENs) to increase recombination frequency[142-145]. An alternative approach is the induction of hPSCs to attain a "mouse-like" pluripotency, state which could allow an efficient delivery of the targeting construct[34, 35, 146, 147] (→1.1.2.4). While these methods have shown to facilitate targeting hPSCs to some degree, the setups remain laborious or entail other disadvantages, preventing the genetic modification of hPSCs to become a routine practice[134].

1.1.5.4 Control of Cell Proliferation

One characteristic of PSC, an abbreviated G1 phase of the cell circle, results in a sustained proliferation[90]. While this proliferation capacity is one of the reasons why PSCs are so valuable for science, when hPSC are used for cell replacement therapies, this ability has to be con1sidered a double-edged sword[148]. The ability hPSCs to induce teratomas *in vivo* underline the potential of hPSC to cause unwanted and deleterious side-effects. The issue of unwanted *in situ* cell proliferation and uncontrolled *in situ* differentiation is a problem not only associated with the direct use of hPSC but also concerning every hPSC-derived tissue[90,148]. Typical strategies to avoid this problem involve cell sorting and positive or negative selection strategies. If these are rigorously applied, teratoma growth can usually be avoided[149]. Nonetheless, the danger of a more differentiated tumor-like growth persists and the emergence of unwanted cell-types is possible[150, 151]. These issues will only be solved with stringent screens for the desired cell type and by differentiating cells with high efficiency into mature, terminally differentiated cells.

1.1.5.5 Reproducibility of Results

The current differentiation protocols vary widely between laboratories and routinely rely on a variety of undefined culture components. Even protocols considered robust, often

only produce heterogeneous cell populations enriched in the desired cell type. It is therefore no surprise that most protocols in hPSC culture are difficult to reproduce and dependent on specific lots of FBS or similar variables. Moreover, inconsistency are introduced by genetic heterogeneity (→1.1.5.2) and a high variability between different hPSC lines[152, 153]. Since the reproduction of results is central to scientific process in general, this has hindered the progress in the field significantly. Additionally, the comparisons between diseased and normal cell lines is impaired if an observed phenotype cannot be reliably associated to the disease and may represent an artifact caused by this variation. While these issues can be overcome to some extent by increasing the sample size, there is still an urgent need for a robust hPSC cell culture platforms, robust and efficient differentiation protocols and an improved quality control in PSC research[154].

1.1.5.6 Cell Differentiation

One of the single greatest barriers for the widespread utilization of hPSCs in cell-replacement therapy, disease modeling and drug discovery, is the difficulty to robustly and efficiently direct the differentiation into relevant cell types. While in principle hPSCs can give rise to every cell type in the adult human body, in practice *in vitro* differentiation protocols have only been developed for a small subset of cell-types, such as some subtypes of neurons, hematopoietic progenitors, cardiomyocytes or keratinocytes[102, 155-160]. If protocols exist, the differentiation process is often inefficient and produces populations of cells heterogeneous concerning their cell types and maturity. Understanding the mechanisms that control the induction, expansion and maturity of specific cell types will be a prerequisite for the implementation of many hPSCs-based technologies. It is therefore considered one of the major challenges for the utilization of hPSCs to date[134, 154, 155].

Strategies for Human Pluripotent Stem Cell Differentiation

The differentiation of PSCs is commonly based on the recapitulation of *in vivo* development via activation/inhibition of specific signaling pathways. In the early studies of the *in vitro* potential of ESCs, fetal bovine serum (FBS) has been a central component for the induction of and direction through the differentiation. However, adding a mix of unknown factors prevented the identification of the involved mechanisms and protocols based on FBS were only feasible for the differentiation of a few cell types in a subset of cell lines. Furthermore, the variability between batches of serum caused inconsistent results and prevented the independent reproduction of the differentiation setups[134]. In the last decade, these problems led to replacement of FBS with specific agonists and antagonists of signaling pathways known to function throughout development. The realization that a step by step recapitulation of *in vivo* development in the dish could be a successful strategy to achieve lineage specific differentiation was the next important breakthrough. For example, the derivation β-pancreatic cells by D'Amourr mimicked with 5 separate differentiation steps the *in vivo* development from an embryo to adult pancreatic islet cell, through addition of unique combinations of signaling molecules[161]. These cells could be transplanted into mice rendered diabetic by treatment with the β-pancreatic cell selective cytotoxin streptozotocin, and were able to reduce the blood glucose level in these animals[162-164]. This proof-of-principle experiments first demonstrated that hESC-derived cells have the capacity to be functionally integrated into a living organism.

However, this success has been rather an exception. For most cell types the necessary developmental steps are not known and the important signaling pathways remain to be identified. This limited knowledge is one of the reasons for the dearth of robust and efficient differentiation protocols[165]. Furthermore, the inability to mimic minor concentration variations or to influence complex cell-cell interactions renders the generation of homogenous populations of specific subtypes simply by recapitulating *in vivo* development, very difficult.

One possibility to overcome this problem may be by combining the recapitulation of *in vivo* development with a transient ectopic expression of lineage specific transcription factors. The differentiation state is subject to dynamic control by transcriptional regulators and the perturbation of the stoichiometry of these factors, may alter this differentiation state (→1.1.3.3). That this is a viable approach to change the cellular fate, has been demonstrated by the derivation of iPSC though the expression of key transcription factors, and by converting fibroblast to myocytes via MyoD expression (→1.1.3.4). In addition, a range of recent publications illustrated the potential to change the cell fate of terminally differentiated cells by direct lineage conversion[65, 66, 165-168].

These results indicated that the differentiation into specific cell types can be guided by the ectopic expression of key transcription factors. This could potentially increase efficiency, homogeneity and maturity of the differentiated cells, and further allow the generation of cell types which are inaccessible with the currently existing methodology. That the directed differentiation through over-expression of lineage specific transcription factors is possible way has recently been demonstrated in neuronal subtypes[169-171].

1.2. Adipocytes Biology:

The ability to store energy in times of nutritional excess and to provide this energy in times of need is a major evolutionary advantage. Therefore, it is not surprising that virtually all animal species store energy in the form of triglycerides for future needs[172, 173]. In complex organisms consisting of three germ layers the fat storage generally takes place in the mesodermal germ layer. In mammals, this organ is termed white adipose tissue (WAT). Mammals further possess another type of adipose tissue, termed brown adipose tissue (BAT). BAT emerged parallel to the development of homeothermy and the capacity for non-shivering thermogenesis[174]. In contrast to the function of white adipocytes, the function of brown adipocyte is to regulate the body temperature.

The involvement of adipose tissue in energy storage, thermal insulation, body temperature regulation, immune response, inflammation, reproduction and metabolism in general makes adipose biology an important field of research, especially in regards to the obesity epidemic the world faces at present. This importance is especially highlighted by patients suffering from extreme forms of adipose disorders: those afflicted by severe obesity and those suffering from lipodystrophy. While over the last few decades major improvements in understanding metabolic disease have been achieved, there is still a lack of simple methods for preventing or treating adipocyte associated disorders.

1.2.1 White Adipose Tissue

WAT is an endocrine organ that is central to a number of physiological processes including energy homeostasis and insulin signaling. It is the only tissue in the body that can markedly change in size after adult age is reached. The adipocyte mass as a percentage of total body mass can range from 2% in athletic to more than 70% in severely obese individuals. The normal distribution ranges from 9-18% in males, and 14-

28% in females[175]. In obese individuals the fat mass typically exceeds 22% of body mass in males and 32% in females respectively[176]. While the generation of adipose tissue is an important factor in the growth during childhood, the total adipocyte number does not seem to change during adulthood[177]. The increased adipocyte mass in patients with adult-onset obesity is mainly caused by an increase in adipocyte size (hypertrophy). In contrast, the increased adipose mass in patients with early-onset obesity is caused by both hypertrophy as well as an increase in adipocyte number (hyperplasia)[178, 179].

The basic organization of a white adipose depot consists of mature adipocytes, stromal-vascular cells (SVC), blood vessels and neuronal innervations. White adipocytes are the major component of this tissue, and lipids, predominantly triglycerides (>90%), comprised 85% of their mass. WAT is located principally in three anatomical areas: subcutaneous, dermal, and intra-abdominal (also often referred to as visceral fat). In recent years it has become clear that not only the total amount of adipose tissue is important but also the relative abundance of these anatomical locations is of importance. For instance, increased intra-abdominal fat is associated with a high risk of metabolic disorder, whereas increased subcutaneous fat seems to entail little to no risk[180, 181]. In addition to variably affecting metabolic disorder, the different WAT depots show distinct responses to hormones. For example, breast and thighs are very responsive to sex hormones, while other depots, like the neck and upper back, are more receptive to glucocorticoids[182]. This may explain why fat distribution, even with constant body mass index (BMI), changes with age, since aging is accompanied by several changes in hormone levels. With this emerging understanding of the importance of adipocyte depot localization in metabolic syndrome and obesity, a range of previously neglected questions arose. In particular, what are the differences in developmental origin and what controls the relative amounts and functional heterogeneity among the different depots[183]? To answer these questions accurately, it may not be sufficient to examine adult adipocyte tissue alone. Rather, it may be necessary to look into the exact pathways and intermediate cell types involved in the differentiation from embryonic tissue into the mature adipocyte.

1.2.1.1 Developmental Origin and Transcriptional Regulation of White Adipocytes

White adipose tissue is generally regarded to possess a mesodermal origin, but only a few precise lineage tracing studies for the various adipocyte types and depots have been performed[174]. Based on the relatively rapid turnover of adipocytes the existence of adipoblast preadipocytes has long been suspected[184]. However, a distinct stem cell population has only recently been identified. Whether these cell are able give rise to all the distinct adipocyte depots as well as "brite" adipocyte remains to be shown (→1.2.2.7)[174, 185-187]. Furthermore, while mesenchymal stem cells can be readily differentiated into adipocytes, recent reports of neural crest cells giving rise to adipocytes in culture indicates that the picture might not be as simple as first believed[188].

Work on the murine lines 3T3-L1 and 3T3-F442A revealed that the transcription factor peroxisome proliferator-activated receptor gamma 2 (PPARG2) is central for the maintenance and terminal differentiation of adipocyte fate at a transcriptional level[189-192]. It was shown to be both necessary and sufficient for adipocyte differentiation, and an inducible knock-out of PPARG2 leads to the death of both brown and white adipocytes[184, 193]. Other important factors in adipocyte differentiation include members of the CEBP family. CEBPB and CEBPG have been shown to induce expression of PPARG2 and CEBPA early in adipocyte differentiation. PPARG2 and CEBPA then act in a self-regulatory feedback loop and activate a range of genes involved in terminal adipocyte differentiation. They seem to be required for both adipogenesis and maintenance of the adipocyte cell fate[190].

1.2.1.2 Obesity:

The ability to accumulate and retain energy is a major evolutionary advantage. However, in most contemporary societies the permanent availability of calorie-dense food has driven an unprecedented weight gain. The resulting obesity is now an emerging

worldwide epidemic with dramatic effects on health, longevity and quality of life[194, 195]. The major underlying cause of obesity is thought to be a chronic imbalance of food intake and expenditure, leading to the storage of excess energy in adipocytes in the form of triglycerides[179]. These triglycerides accumulate to excessive amounts in obese individuals and interfere with the normal function of adipocytes. This in turn can interfere with the function of WAT as an active endocrine and paracrine organ and cause an alteration in the release of a large number of cytokines and bioactive mediators. The dysregulation of factors such as leptin, adiponectin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) has detrimental effects on body weight homeostasis, insulin resistance, lipid levels and blood pressure and may lead to inflammation and atherosclerosis[196-198]. Not surprisingly, obesity is a major risk factor for a range of conditions, such as cardiovascular disease, stroke, congestive heart failure and myocardial infarction as well as fatty liver disease and type 2 diabetes[199-203]. Furthermore, the obese phenotype is strongly associated with Alzheimer's disease, gallstones and cancer.

Obesity is a complex, multi-factorial disease involving environmental, genetic, and behavioral components[204]. Twin and population studies have found that both BMI and waist to hip ratio (WHR) are heritable traits, which suggests that genetics strongly contribute to the amount and distribution of fat throughout the body[205]. This notion is further supported by the striking phenotype of WAT distribution in patients suffering from heritable forms of lipodystrophy (→1.2.1.3)[206].

As the prevalence of obesity continues to increase worldwide, understanding the central role of adipocytes in the development of metabolic disease becomes more and more critical[207-209].

1.2.1.3 Lipodystrophy

Lipodystrophies are a heterogeneous group of diseases, commonly associated by localized or general loss of white adipose tissue. In the localized form of the disease, the loss of one depot is often associated with hypertrophy in other depots. For instance, patients suffering from familiar partial lipodystrophies (FPLD) caused by mutations in Lamin A (FPLD2) or PPARG2 (FPLD3) show an absence of subcutaneous fat, especially in the limbs and trunk, but exhibit increased storage in the neck and face[210]. These abnormal fat distributions are generally associated by a range of metabolic alterations such as insulin resistance, glucose tolerance, lowered plasma high-density lipoprotein (HDL) and an accumulation of plasma triglycerides often, resulting in premature cardiovascular complications[211]. Notably, the observed detrimental effects are consistent with the influence of differential fat distribution in the distinct WAT depots (→1.2.1). Additionally, the incapability of large parts of the WAT depots to store triglycerides leads to the storage of those molecules in muscles and liver, which can cause an increased incidence of diabetes mellitus type-2, liver failure and other complications.

The diseases may be divided in two forms: congenital and acquired lipodystrophies. Aquired lipodystrophies can range from harmless small dents in the skin due to repeated injections at the same spot to severe lipid redistributions as a common side effect of antiretroviral drugs. For congenital lipodystrophies, a range of causative mutations has been identified and the resulting phenotype can vary substantially in distribution and metabolic effects[212].

It is an interesting fact that both the paucity and overabundance of fat lead to severe metabolic complications and cause similar afflictions. Studying lipodystrophies and deciphering normal and abnormal adipose tissue development in particular, is therefore likely to assist in the investigation of all adipose disorders and adipose biology in general. Regrettably, attempts to study the disease in the mouse model have had little success

because no phenotype comparable to the human disorders could be observed. The additional difficulty in maintaining and culturing human primary adipocytes has prevented the establishment of any *in vitro* system to study adipocyte dysfunction in lipodystrophy thus far (→1.2.1.6). A possible alternative approach may be the development of hiPSC lines carrying lipodystrophic mutations, given the fact that an efficient and robust differentiation protocol can be developed and a cellular disease phenotype can be identified.

1.2.1.4 Treatment of Obesity

The common belief that obese individuals should just eat less and exercise more to lose weight and they only lack discipline seems to be at odds with scientific evidence. Twin and population studies strongly suggest that, although environmental factors contribute to the incidence of obesity over time, the individual differences in weight can be largely attributed to genetic factors[201, 213, 214]. In fact, obesity has a heritability similar to height, and its heritability exceeds that of many other conditions including schizophrenia, breast cancer and heart disease[215-219].

Food Intake

Body weight is regulated by a robust physiological system that maintains weight within a narrow range. Weight loss is met with compensatory responses that decrease metabolism and increase hunger. The body's nutritional state is constantly communicated by the WAT through the secretion of the adipokine leptin and assessed by the hypothalamus, the brain area responsible for the regulation of satiety and metabolism[219]. The force of this feedback mechanism becomes obvious when it is interrupted. Patients that suffer from congenital leptin deficiency experience an insatiable appetite and, if left untreated, quickly become morbidly obese[214, 220-222].

In an intact leptin feedback system, weight loss generally results in a fall in blood leptin levels, which in turn reduces energy expenditure and increases hunger. Most patients that

lose weight by dieting alone tend to regain their lost weight over time as a result of that mechanism[223-226].

An alternative approach to reduce energy uptake is through pharmacological interventions that interfere with nutrition uptake. For instance, the most commonly prescribed anti-obesity drug Orlistat prevents fat absorption by inhibiting digestion. However, pharmacological treatment has a relatively low long term compliancy and high relapse rates, which both can probably be attributed to the leptin feedback mechanism. A promising alternative may be drugs which interfere with this feedback mechanism, such as catecholamine's or cannabinoid receptor antagonists which directly suppress appetite. However, the existing remedies cause a variety of unwanted side effects[227-229]. In severe cases of obesity, a common alternative to dieting is intervention through bariatric surgery, a procedure in which the gastrointestinal anatomy is altered to reduce caloric intake. Although patients on average lose a significant amount of weight, they usually remain clinically obese, implicating a change in metabolic state independent of their individual weight[230].

Energy Expenditure

The other side of the energy balance equation, energy expenditure, is another target to promote weight loss. Energy expenditure is highly variable among individuals, and individuals with high rates of energy expenditure are less likely to become obese than individuals with low metabolic rates[231-233]. In response to weight loss, the metabolic rate falls significantly, which is another important reasons for the high relapse rate in dieting[224]. Therefore increasing the metabolic rate by therapeutic intervention may represent a promising alternative for inducing weight loss[219]. To that end, an assessment of drugs has been tested, including dinitrophenol, thyroid hormones, rosiglitazone and β-adrenergic agonists[234-238]. While all of these substances were successful in reducing weight to various extents, they also exhibited severe toxic side effects rendering them unsuitable for clinical application.

With the discovery that brown adipose tissue is prevalent in adult humans and may have an important contribution to the energy balance, it became a promising alternative target for pharmaceutical intervention (→1.2.2). Yet the lack of any human *in vitro* model for brown adipocytes as well as a suitable *in vitro* models for white adipocytes, have prevented the thorough investigation of BAT as a potential anti-obesity drug target so far (→1.2.1.6).

At present, only very few drugs that treat obesity are available, and these drugs generally show low efficiency in treatment. Also, due to their side effects, these drugs are only recommended if the benefits outweigh the risks. Given the challenges the growing obesity epidemic will have on society as a whole and on patient health in particular, there is a scarcity of treatment options.

1.2.1.5 Exercise and Obesity

One way to increase energy expenditure and thereby influence the energy imbalance underlying the development of obesity is through exercise. It can prevent obesity from developing and act as an effective treatment against metabolic syndrome and obesity [239]. Intriguingly, the energy expenditure in exercise exceeds the calories used in the actual work performed, indicating the involvement of organs other than skeletal muscles[240]. Recently, by simulating exercise at a molecular level through forced expression of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α) in skeletal muscles, the PGC1α-dependent myokine irisin has been identified[241]. Secreted by the muscle, this hormone is able to induce a brown adipocyte program in white adipocytes leading to increased thermogenesis and subsequent energy expenditure. While this phenotype has only been thoroughly established in the mouse model, the study as a whole suggests that it may be possible to induce certain benefits of exercise through pharmacological intervention in humans, thus providing another potential tool in the treatment of obesity.

1.2.1.6 Existing Adipocyte *In vitro* Models

The mouse model is a valuable tool in many experimental settings but in modeling metabolic disease it has proven to be suboptimal. First, mice do not show the same phenotype-genotype connection as humans. Also, when compared to humans, mice exhibit significant differences in lipid metabolism and metabolism in general[94, 242, 243]. Mutations leading to lipodystrophies in humans fail to induce comparable afflictions in mice. Furthermore, even though a mouse model for obesity does exist, it often fails to adequately recapitulate human disease processes[244]. However, the establishment of the first murine *in vitro* cell line 3T3-L1, capable of differentiating into adipocytes, has led to important insights concerning cell differentiation and transcriptional regulation. Still, the interspecies differences hold true as much for this model as for *in vivo* models.

As described earlier, research in developmental biology, regenerative medicine, disease modeling and drug discovery can benefit significantly from human *in vitro* models (→1.1.4). While a range of human adipocyte cell culture systems exist, the current *in vitro* models show limitations significantly reducing their utility. Ideally, cellular models should recapitulate *in vivo* development, function and pathology. In addition, they should be available in necessary quantities for rigorous study since most applications rely on efficient and robust cell culture systems.

Primary human adipose tissue, while easy to attain in large quantities, is very difficult to maintain and cannot be expanded in culture. Mesenchymal stem cells (MSC) have been established in cell culture systems derived from bone marrow and adipose tissue (ADSVC) and both cell types can be differentiated into adipocytes[245, 246]. However, the utility of these cells is impaired by their limited proliferative potential and low differentiation efficiency, especially in older lineages. Furthermore, they are difficult to alter through homologous recombination and exhibit a high variability between lines[247].

In addition to these limitations, the desire to examine the early steps of adipocyte differentiation led to the development of several hPSC-based adipocyte differentiation

protocols[193]. While these protocols successfully demonstrated the ability of hPSCs to differentiate into adipocytes, low differentiation efficiencies prevented a thorough characterization of the adipocytes[248-251]. In addition to the low efficiency, a high variability between lines has been observed in these approaches, rendering them suboptimal for many assays. To improve on these issues, an intermediate MSC state has been introduced by some groups with promising improvements in robustness. Yet efficiencies similar to primary MSC differentiation protocols could not be established, and the cells have not been characterized at a functional level[245, 247, 252, 253].

In summary, the existing human *in vitro* cell culture systems for adipocytes have several limitations such as low reproducibility of results, low differentiation efficiencies, limited proliferation potential, undetermined maturity, and high variability between cell lines. Given the potential advantages of human *in vitro* cell culture systems in drug discovery, disease modeling and regenerative medicine (→1.1.4), there is an urgent need for a suitable adipocyte differentiation technology. No protocol for the efficient generation of large numbers of hPSC-derived adipocytes is available, and a detailed phenotypic characterization that documents fidelity to primary cells has not been performed for any human adipocyte *in vitro* model.

1.2.2 Brown Adipose Tissue

The central function of white adipose tissue is energy storage. However, many mammals possess another form of adipose tissue called brown adipose tissue (BAT). The primary function of BAT is the regulation of body temperature, and it enables homeotherm animals to maintain their temperature in cold environments[254]. Due to their high surface area to volume ratio, especially small mammals depend on this so-called “non-shivering thermogenesis”. But even in larger mammals, hypothermia is a threat to newborn infants and a major death risk for premature neonates[255]. Furthermore, BAT seems to play an

important role in maintaining the core temperature of hibernating animals above a certain threshold[174].

1.2.2.1 Mechanism of Heat Generation

In most cells generating adenosine triphosphate (ATP) is a very efficient process in which a proton gradient across the inner mitochondrial membrane is tightly coupled with ATP production[256, 257]. In BAT this process can be uncoupled by the protein UCP1 (uncoupling protein 1, also known as thermogenin). UCP1 is a membrane protein of the mitochondrial carrier protein family located in the outer mitochondrial membrane. This protein can drive the protons into the mitochondrial matrix, thereby uncoupling the proton motive force from ATP production[258]. The resulting release of potential energy is dispersed as heat. A range of factors, for instance cold temperature or large food intake, can induce this response via the activation of the sympathetic nervous system (SNS). This activation is generally mediated by norepinephrine, which induces lipolysis in brown adipocytes resulting in an increased concentration of free fatty acids (FFA)[259]. This, in turn, induces the β-oxidation of the FFAs, which provides acetyl CoA for oxidation via the citric acid cycle and ultimately elevates the proton motive force across the mitochondrial membrane. Additionally, the FFAs themselves also induce thermogenesis through UCP1 activation[260].

1.2.2.2 Brown Adipose Tissue in Humans

BAT can make up to 5% of the body weight in human neonates, and this percentage is thought to progressively decrease with age[261]. Adult humans were long thought to be independent of thermoregulation through BAT, due to their lower surface area to volume ratio, ability to generate heat by shivering, ability to move away from cold exposures and increased thermal insulation of subcutaneous fat and body hair. Even if BAT still did exist in adult humans, its influence on the adult metabolism was thought to be negligible.

This view has shifted in the last decade with the reevaluation of results of unrelated pursuits in another scientific field. In nuclear medicine, one way to localize tumors is to test for cellular activity. Since tumors are often glycolytic, it is possible to trace tumor metastasis via fluorodeoxyglucose positron emission tomography (FDG-PET). To utilize this imaging technology, 2-[^{18}F]-fluoro-2-desoxy-glucose (FDG) is administered to the patient and readily transported into cells via a protein of the glucose transporter (GLUT) family. Desoxy-glucose cannot be further metabolized or easily leave the cell. Thus, FDG accumulates in high amounts within cells that take up a lot of glucose and can be used as an indicator for high cellular activity. The labeling of glucose with radioactive ^{18}F provides the possibility to visualize this accumulation within the body. Next to the known glucose sinks, like the brain and heart, in a subset of patients the supraclavicular and the neck region, as well as additional paravertebral, mediastinal and suprarenal regions showed a surprisingly high glucose uptake. Because tumor tissue is usually not symmetrically distributed, this phenomenon was largely considered to be a non-tumor related uptake and thought to be a result of muscle tension due to anxiety[262]. This was further supported by the fact that the anti-anxiety drug diazepam, which also suppresses the β-adrenergic response, was able to diminish the FG uptake in these regions[263]. Only after FDG-PET became routinely combined with computer tomography (CT), it became obvious that this explanation was insufficient. The density and composition of the observed area resembled adipose tissue much more than muscle tissue does, and as a result, brown adipose tissue was proposed as an alternative explanation for this phenomenon[264, 265]. Since then, a range of studies have further supported the identification of this tissue as BAT[262]. The evidence included many characteristics of BAT known from rodent biology: the ability to induce FDG uptake in this tissue by acute exposure to cold or sympathetical stimulation, a very high density of mitochondria, and an abundance of the protein UCP1. Based on this evidence, the presence of BAT in some adult humans has generally been considered to be scientifically established in the field of adipocyte biology[266]. Nonetheless, the extent to which BAT contributes to metabolism

and whether it is involved in obesity is still debated and hinges upon two factors: its prevalence in adult humans and the degree to which it affects the energy balance.

1.2.2.3 Prevalence of Brown Adipose Tissue in Adult Humans

It has been challenging to access the prevalence of BAT in adult humans based on FDG-PET. FDG uptake is remarkably variable and irreproducible. Initial studies reporting that 2-7% of adult subjects possess BAT is most definitely an underestimation[267-269]. This observed variability is most likely due to the fact that BAT activity needs to be stimulated in order to act as a glucose sink and thus be detectable via FDG-PET[270, 271]. A range of new studies that performed multiple measurements and/or induced the activity of BAT deduced the prevalence of BAT between 30-100%, depending on cohort, gender, age and means of activation[272-274]. Alongside the variability in prevalence in adult humans, the magnitude of uptake as an indicator of BAT mass varied significantly. The prevalence and amounts of BAT was found to be higher in women than in men and increased in cohorts consisting of subjects living in cold climates when compared to subject living in more temperate climates. Intriguingly, these studies further found that BAT mass is inversely correlated with the BMI, the body fat percentage, blood glucose levels and the age of an individual and positively correlated with the resting metabolic rate[272, 273, 275].

1.2.2.4 Evidence of the Effect of Brown Adipose Tissue on Metabolism

The diminution of brown adipose tissue with increasing age was immediately proposed as a possible explanation for middle-age obesity[257, 275]. Furthermore, the inverse correlation of BAT activity with the BMI offered an explanation for the high variability in susceptibility of individuals to obesity. The underlying argument is that the chronic imbalance of energy intake and expenditure, which is central to the development of obesity, can be counteracted by the increased energy expenditure of BAT. Thus, maintaining the activity and abundance of BAT throughout life might be an important

protector against obesity[257, 274, 276]. Further support for this theory was presented by a genetic association study that identified a polymorphism in the UCP1 promoter, which leads to reduced UCP1 expression, associated with significantly higher BMI and abdominal obesity[277]. While this line of reasoning is compelling, the evidence in humans is largely based on association and correlation studies, which requires assumptions about cause and effect, and may also erroneously neglect factors affecting BAT and obesity simultaneously.

For research with rodents, on the other hand, it is feasible to design tightly controlled experiments. Over the decades this enabled the creation of a wealth of experimental results, altogether confirming the important role of BAT in diet-induced obesity in these animals. $UCP1^{-/-}$ mice are sensitive to cold and late-onset obesity. Rodents with selective ablation of BAT exhibited excess weight gain and are highly sensitive to high-fat diet (HFD) induced obesity[278-282]. The chronic treatment of rats with a β-3-adrenoceptor agonist was able to increase thermogenesis, brown adipocyte mass and resting metabolic rate. In addition, the development of HFD-induced obesity in young rats was delayed and long term treatment was able to reverse the obese phenotype[283, 284].

The aforementioned association of reduced BAT mass with increased age in human subjects was consistent with experiments in rodents that found significantly reduced non-shivering thermogenesis in old animals compared to young animals. Along with the possible effect on middle-aged obesity, this may hint at the involvement of BAT in another age related issue, the predisposition of the elderly to hypothermia[275].

While these findings are promising hints that BAT may also be involved in the development of or resistance to obesity in humans, there is no direct evidence of its metabolic contribution so far. No study has been able to demonstrate that human BAT is as highly metabolically active as its rodent counterpart. Also lacking are studies connecting human BAT to alterations in thermogenic capacity and activity. Furthermore,

given the interest in it as a potential cure for obesity, no studies have been able to demonstrate the effect of human BAT on weight loss or gain[274].

1.2.2.5 Feasibility of Brown Adipose Tissue in Treatment of Obesity

Hand in hand with the question of whether and to what extent BAT contributes to human metabolism is the question of its feasibility as a treatment for obesity. Two main strategies have been initially proposed: the increase of brown adipocyte mass and the increase of the thermogenic capacity of brown adipocytes.

Increasing brown adipocyte mass, while demonstrated in rodents, has a variety of hurdles to overcome to be a viable treatment option in humans. One way to increase the general BAT mass would be to increase the proliferation of brown adipocyte progenitor cells but this population of cells has not been defined nor selectively targeted in humans. The PPARG2 agonist thiazolidinedione darglitazone has been shown to induce proliferation of BAT in rats and monkeys but also caused several severe side effects[285]. Potentially, a more selective and potent PPARG2 agonist could achieve this goal, but research on even the most basic effects of any chemical is hindered by the absence of a suitable human cell culture system. Exposure to bone morphogenic protein 7 (BMP7) or triiodothyronine 3 (T3) has been implicated to induce increased BAT mass, but there are major technical, efficacy and safety issues to overcome before these can be considered viable clinical options[255, 286-288].

Another way to increase brown adipocyte mass would be to convert cells into BAT through the over-expression of key transcription factors. The groundbreaking discovery of the ability of CEBPB and PRD1-BF1-RIZ1 homologous domain containing protein 16 (Prdm16) to convert mouse myoblasts into brown adipocytes hints at such a possibility. However, this approach had a very low efficiency in human cells, which so far has prevented a stringent molecular and functional characterization[289, 290].

The strategy to increase the activity of existing brown adipocytes and thus increase the overall energy expenditure may therefore be a more readily available treatment option. Brown adipose activity can be induced by cold exposure and caloric restriction, but, as behavioral changes, these are among the more difficult treatment options to establish and maintain. Since PPARG2 agonist not only increases BAT mass but also its activity and since thyroid hormone receptor agonists have shown to activate brown adipocytes in rats, these might be interesting pathways for drug development as well. Other existing pharmacological interventions in these pathways have caused severe side effects rendering them impractical for clinical use.

Another possible drug target to modulate BAT activity in humans is the β-adrenergic receptor. BAT activity is regulated through the sympathetic nervous system, likely through the release of norepinephrine[291, 292]. Propranolol, a β-adrenergic antagonist has been shown to reduce PET-FDG uptake in brown adipocytes. Nonetheless, there have still been no studies to test the effect of β-adrenergic agonist on human BAT[293-295].

The effects of propranolol on BAT point to a possible issue regarding drug discovery in general and the study of BAT in particular. Propranolol acts mainly through the inhibition of the β1-adrenergic receptor and is much less effective on the β3-adrenergic receptor, the principle regulator of BAT activity in rodents[266]. This difference illuminates the necessity for a human-based brown adipocyte cell culture system to avoid species-specific differences in the development of drugs tailored to human metabolism.

1.2.2.6 Developmental Origin and Transcriptional Control of Brown Adipocytes

Adipocytes, muscle, bone and cartilage are believed to be predominantly mesodermal in origin. Mesenchymal stem cells (MSC) have been successfully differentiated into white and brown adipocytes as well as myoblasts, osteoblasts, chondrocytes and connective tissue[255]. It has long been thought that BAT and WAT both derive from one pre-

adipocyte cell population based on the fact that both are dependent on the expression of PPARG2, the so-called master regulator of the adipocyte lineage[296, 297]. Since PPARG2 by itself was not able to differentiate cells into the brown adipocyte lineage, the presence of additional factors seemed to be necessary to induce brown adipocyte fate. In an elegant study performed in 2008, Prdm16 was identified as that factor[290]. Surprisingly, the genetic fate mapping preformed in this study further found that brown adipocytes and skeletal muscle but not white adipocytes are derived from Myf-5 expressing myogenic precursors, indicating a separate pre-adipocyte stem cell population for brown and white adipocytes[290]. Further research identified that Prdm16, by forming a transcriptional complex which includes the CAAT Binding Protein beta (CEBPB), acts as a bi-directional switch between brown adipocyte and skeletal muscle fate[289]. The majority of this insight is based on work in mouse *in vivo* and *in vitro* models. The combined expression of PRDM16/CEBPB in human fibroblasts has only yielded brown adipocytes with low efficiency, which has prevented a thorough evaluation of human BAT thus far. Whether human brown adipocytes are similar in transcriptional regulation and function to mouse brown adipocytes is still an open question. Moreover, a suitable human cell culture system in which to study, characterize and utilize brown adipocytes is still needed.

1.2.2.7 "Brite" and "Beige" Adipocytes

In addition to the classical brown adipocyte depots, particular cells within the white adipose tissue sometimes exhibit brown adipocyte characteristics. These adipocytes are often referred to as "brite" (brown-in-white) adipocytes[274]. While initially thought to be an admixture of BAT in WAT depots, there is reason to believe that these cells are not of the same lineage as the cells found in classical brown adipocyte depots[298]. They share a range of common characteristics with BAT, like increased UCP1 expression, but lack others, as they do not seem to show an increase in glucose uptake after stimulation. These "brite" adipocytes have not been investigated in detail and their contribution to

metabolism and thermogenesis is unknown[274]. Nonetheless, they might emerge as a potential therapeutical target once a better characterization has been achieved.

Remarkably is the observation that under certain conditions classical brown adipocyte properties can be induced in white adipocytes. The first evidence of this phenomenon was found by a study identifying the expression of UCP1 mRNA in WAT depots after treatment with thiazolidinedione darglitazone[299]. These cells are generally called "beige" adipocytes, and the process of white adipocytes adopting brown adipocyte characteristics is termed "browning"[300]. In the last decade, several ways to induce browning to some extent have been identified, including prolonged cold exposure, exposure to the myokine irisine or through the induction of cyclooxygenase-2 (COX2) expression[241, 300, 301]. Given the relative mass of WAT, the induction of browning is thought to have an immense effect on energy homeostasis and thus provide another potential therapeutic target in the fight against obesity. To achieve this goal, it will be necessary to perform a thorough characterization of human BAT in general, which in turn requires a suitable human adipocyte cell culture system, whether it be white or brown.

1.2.3 Comparison of White and Brown Adipose Tissue

Endogenous human BAT is difficult to obtain and has yet to be studied thoroughly. Nonetheless, based on work in mice, BAT and WAT can be distinguished by an assortment of histological, molecular and functional characteristics. At a histological level, mature white adipocytes contain a single large predominant lipid droplet, while brown adipocytes contain multiple small lipid droplets. While white adipocytes are relatively large (50-100μm), individual brown adipocytes are smaller in size (25-40μm)[302, 303]. The cytoplasm of brown adipocytes is densely packed with mitochondria, which are on average larger (>0.5μm) than mitochondria in white adipocytes (<0.3μm)[304]. The iron contained in mitochondria is thought to be the source of the characteristic brown

color that gave these adipocytes their name. At the tissue level, BAT appears to be highly vascularized to provide adequate nutrients while WAT is more diffusely vascularized, often with a single capillary providing support[255].

At a molecular level, WAT and BAT show a few differences in gene expression. A high expression of mitochondrial markers is more characteristic of BAT than WAT, including genes such as PGC1a, cytochrome c-1 (CYC1) or cytochrome c oxidase subunit VIIIA (COX8A). Also, as discussed earlier, UCP1, PRDM16 and CEBPB are more highly expressed in BAT than WAT as well as additional genes like deiodinase iodothyronine type 2 (DIO2) of very long chain fatty acid like-3 (ELOVL3). Conversely, genes with increased expression in WAT include fatty acid binding protein 4 (FABP4), hormone sensitive lipase (HSL) and adiponectin (ADIPOQ)[305].

At a functional level, BAT has a higher basal and maximal metabolic activity, as identified by increased oxygen consumption, an increased proton production rate and increased glucose uptake. Distinctively, mature functional WAT should be able to release free fatty acids and adipokines like leptin upon being stimulated. Importantly, since many of the mentioned characteristics of BAT are also found in WAT and the differences are often in degrees rather than in absolute terms, a thorough evaluation of brown adipocytes can only be achieved if they are directly compared to WAT.

2. Aims:

The utility of hPSC as a general tool in research is dependent on the availability of efficient and robust culture platforms and the ability to genetically manipulate the cells with relative ease. Additionally, most experiments rely on the ability to differentiate these cells into a homogeneous population of functional mature cells. For the purpose of adipocyte biology, this amounts to the establishment of an hPSC-derived adipocyte differentiation protocol that is efficient, robust and capable of recapitulating *in vivo* development, function and pathology.

Aim 1: Establish a Methodology to Efficiently Culture and Genetically Manipulate Human Pluripotent Stem Cells

When I first started working with hPSCs, the cells were cultured on mouse embryonic feeder cells and in media containing animal sera. The maintenance of the cells was challenging and laborious, since hPSCs grown in these conditions are fragile, prone to spontaneous differentiation and difficult to passage. The successful genetic alteration of an hPSC line was more the exception than the rule. The subsequent introduction of a feeder-free culture system as well as the possibility to use defined media conditions improved the stability of the culture conditions but compared to other non-hPSC *in vitro* cell culture systems work was still arduous. We hypothesize that many of the encountered issues are due to suboptimal culture conditions and can be avoided with a suitable hPSC culture methodology. We further hypothesize that the use of a combination of feeder-free Geltrex coating, TeSR defined media, Accutase to dissociate and detach the cells and Rock-Inhibitor to stabilize the intermediate single cell state, represent such a culture system. We plan to characterize the suitability of this culture system for hPSCs thoroughly and to compare it to the most common feeder-free culture platform currently

in use. This enhanced culture platform would significantly facilitate the utilization of hPSCs in research.

Aim 2: Differentiation of Human Pluripotent Stem Cells into White Adipocytes

The current methods to generate human white adipocytes in an *in vitro* setting possess a range of limitations. Adipocytes can only be differentiated with low efficiencies and in limited quantities, resulting in a lack of reproducibility and adequate characterization. Furthermore, the adipocyte models often fail to recapitulate normal and disease states, rendering them useless for many applications. We hypothesize that the overexpression of the lineage specific transcription factor PPARG2 can differentiate hPSCs into white adipocytes with high efficiency and reproducibility. We plan to investigate the differentiated cells for morphological, molecular and functional characteristics in detail and compare them to existing *in vitro* systems and mature adipocytes. We will further evaluate their utility as a model system for adipose biology. The successful establishment could provide a renewable source of white adipocytes in culture and may thus assist the fight against obesity and other adipose disorders.

Aim 3: Differentiation of Human Pluripotent Stem Cells into Brown Adipocytes

The discovery that most, if not all, adult humans have distinct functional brown adipose depots and that the relative mass of these depots is negatively correlated with the body mass index has fueled considerable interest in the therapeutic potential of brown adipocytes. However, research on human brown adipocytes has been substantially hindered by the inaccessibility of primary tissue and the lack of suitable human *in vitro* models. We hypothesize that a transient ectopic expression of a combination of the

transcription factors PPARG2, CEBPB and PRDM16 is able to generate human brown adipocytes with high efficiency and reproducibility. We further plan to perform a thorough investigation of the morphological, molecular and functional characteristics of the cells and compare them to human white adipocytes. A detailed analysis of human brown adipocytes, the first of its kind, would further illuminate the potential utility of brown adipocytes in the treatment of obesity.

Aim 4: In vivo Characterization of hPSC-derived White and Brown Adipocytes

The most stringent test for any hPSC-derived cell type is their ability to form a functional tissue *in vivo*. In the case of the hPSC-based adipocyte models, this can be tested by the transplantation of the cells into immunodeficient mice. We hypothesize that the introduction of brown and white adipocytes, respectively, yields transplants with the morphological and functional characteristic of the associated primary tissue. If successful, these experiments would provide the most compelling proof of the successful differentiation of hPSC-derived adipocytes to date.

3. List of publications

1) Efficient Culturing and Genetic Manipulation of Human Pluripotent Stem Cells

Robert T. Schinzel, Tim Ahfeldt, Frank H. Lau, Youn-Kyoung Lee, Alicia Cowley, Tony Shen, Derek Peters, David H. Lum, Chad A. Cowan

PLoS One. 2011;6(12):e27495.

The original article can be found attached to the end of this thesis.

2) Programming Human Pluripotent Stem Cells into White and Brown Adipocytes

Tim Ahfeldt*, **Robert T. Schinzel***, Youn-Kyoung Lee*, David Hendrickson, Adam Kaplan, David H. Lum, Ray Camahort, Fang Xia, Jennifer Shay, Eugene P. Rhee, Clary B. Clish, Rahul C. Deo, Tony Shen, Frank H. Lau, Alicia Cowley, Greg Mowrer, Heba Al-Siddiqi, Matthias Nahrendorf, Kiran Musunuru, Robert E. Gerszten, John L. Rinn, Chad A. Cowan

Nature Cell Biology. 2012 Jan 15;14(2):209-19

*These authors contributed equally to this work

The original article will be available online at:
http://www.nature.com/ncb/journal/v14/n2/full/ncb2411.html

4. Erklärung über Eigenanteil

Die vorliegende Doktorarbeit besteht aus zwei wissenschaftlichen Veröffentlichungen. An beiden Publikationen bin ich entweder alleiniger oder geteilter Erstautor. Der von mir zu diesen Manuskripten beigetragene experimentelle Eigenanteil wird im Folgenden dargelegt.

Die initialen Ideen und Grundkonzepte an der Publikation ”**Efficient Culturing and Genetic Manipulation of Human Pluripotent Stem Cells**” wurden von mir zusammen mit meinem Betreuer Chad A. Cowan entwickelt. Die Planung des experimentellen Aufbaus, die Auswertung der Ergebnisse und die Verfassung des Manuskriptes erfolgten durch meine Person und ich war an allen experimentellen Aspekten beteiligt. Der Teratoma Formations Assay wurde mit Hilfe von Frank H. Lau durchgeführt wurde. Youn-Kyoung Lee unterstützte mich bei der Lenti-Virus Titer-Bestimmung, Derek Peters bei der FACS Analyse der Elektroporationseffizienz.

Der Schwerpunkt meiner Arbeit an der Publikation **“Programming Human Pluripotent Stem Cells into White and Brown Adipocytes”** lag bei der Differenzierung von humanen pluripotenten Stammzellen zu braunem Fett. In Zusammenarbeit mit meinem Betreuer Chad A. Cowan wurden die grundlegenden Konzepte und Strategien zur Herstellung der Zellen, entwickelt. Ich war für die Etablierung der Kulturbedingungen und der Identifizierung der notwendigen Transkriptionsfaktoren zuständig, Zudem wurden alle Charakterisierungen und der Vergleich von braunen zu weißen Adipozyten von mir durchgeführt. Youn-Kyoung Lee half bei der Durchführung des Glycerol Assay, den *in vivo* Transplantationen und dem Glucose Aufnahme Assay. Tim Ahfeldt unterstützte mich bei den den *in vivo* Transplantationen und den Microarrays der braunen Adipocyten.

Tim Ahfeldt entwickelte die grundlegenden Konzepte und Strategien für die Generierung von MPCs, und die Differenzierung von MPCs zu weißen Adipozyten. An den

Charakterisierungen der MPCs und der weißen Adipozyten war ich unter seiner Anleitung beteiligt. Das Lipid Profiling wurde von Eugene Rhee, die Microarray Analyse vom weißen Fett wurde von Tim Ahfeldt, Frank Lau, Rahul Deo und mir durchgeführt. Das Manuskript wurde in Zusammenarbeit mit Tim Ahfeldt und Chad A. Cowan geschrieben.

Teile der Publikation „Efficient Culturing and Genetic Manipulation of Human Pluripotent Stem Cells“ wurden zuvor für ein weiteres Dissertationverfahren genutzt. Die Dissertation „**Switching Human Cell Fate: Reprogramming via pluripotency to adipogenesis**“, eingereicht von Dipl. Biochem. Tim Ahfeldt an der Universität Hamburg, beinhaltet Daten, die auch in dieser Dissertation verwendet und diskutiert wurden.

In Detail betrifft dies folgende Resultate: Die CD73-FACS Charakterisierung der MPCs (Figure 1B), die PPARG2 Lenti-Virus Expressions Analyse (Figure 1C), die Bestimmung der Differenzierung Effizienz der weißen Adipozyten (Figure 2B) und deren Charakterisierung mittels Immunofluoreszenz-Färbung (Figure 2B, 2C) und RT-PCR (Figure 3A). Außerdem wurden qPCR Daten, welche weißes Fett hergestellt aus MPCs beziehungsweise ADSVCs vergleicht, genutzt, (Supplementary Figure S8A) und die Langzeitkultivierung der Zellen besprochen (Supplementary Figure S4E). Schließlich waren die funktionelle Charakterisierung der weißen Adipozyten mittels Glycerol und Adiponectin Assay sowie das Lipid Profiling (Figure 5C) teil des anderen Dissertationsverfahren.

5. Discussion

Human pluripotent stem cells (hPSCs) harbor potential to become an important tool in studying developmental biology, and one day may revolutionize medicine. However, standing in the way of hPSCs being used to their full potential, a variety of technical obstacles must be overcome. hPSCs are fragile, prone to spontaneous differentiation, difficult to culture as single cells, and poorly amenable to genetic manipulation. Nearly three decades following the first discovery of human embryonic stem cells, an efficient and robust hPSC culture methodology has yet to be developed.

An additional challenge for the utility of hPSC use in research is the absence of suitable methods to direct differentiation into homogenous populations of functionally mature cells. With regard to the study of adipocyte biology, this translates into a lack of highly efficient and reproducible differentiation protocols directed towards the generation of functionally mature adipocytes. Correspondingly, this has delayed advancement in our understanding of adipocyte biology in both normal and diseased states, as well as progress towards the faithful modeling of associated human disease.

The methods and data detailed in this thesis make relevant contribution towards several of these aforementioned issues. By providing a robust and efficient hPSC methodology, it contributes substantially to the implementation of hPSCs as an important scientific tool. Utilizing this enhanced culture platform, we demonstrate the efficient passage of hPSCs as single cells, stabilization of the pluripotent state against spontaneous differentiation, increased expansion speed, and facilitated genetic manipulation (→5.1).

Additionally, we have introduced a highly efficient and reliable hPSC-based adipocyte differentiation system. Utilizing efforts aimed at recapitulating the native *in vivo* developmental environment, combined with the ectopic expression of lineage-associated transcription factors, we were able to generate both brown and white adipocytes. Both,

brown and white adipocytes possess their distinct morphological, molecular and functional feature corresponding to their tissue type *in vivo*. Furthermore, the cells are capable of forming tissues with functional and morphological qualities similar to primary white and brown adipose tissue in a mouse *in vivo* model. These experiments confirm the identity and maturity of the two distinct adipocyte types. This hPSC-based *in vitro* system permits the study of adipocytes in their normal and disease states, capable of providing insight towards understanding their influence on energy balance. Furthermore, this system is an important step towards establishing an experimental setting to faithfully model human disease (→5.2).

5.1 Efficient Culture and Manipulation of Human Pluripotent Stem Cells

Recent advances in stem cell research have enabled the culture of hPSCs under closely defined media conditions and on a feeder-free protein matrix. This important improvement has reduced experimental condition variability by eliminating differences in animal sera and mouse embryonic feeder cells. Nonetheless, major obstacles have remained which delay the extension of hPSC as a widely used experimental tool. These have included persistently high degrees of spontaneous differentiation, the inability to passage the hPSCs as single cells, problems related to genetic manipulation, and the slow expansion speed.

The full realization of the potential of hPSC technology necessitates an efficient and robust hPSC culture methodology, which is presently not available. We address this need by introducing a novel hPSC cell culture platform, which we have termed enhanced culture platform (ECP), and which utilizes Geltrex coating, TeSR defined media, Accutase Enzyme to dissociate and detach the cells, and Rho-associated protein kinase (ROCK)-inhibitor[122, 123, 125, 127]. We have extensively evaluated and compared this

platform to the standard culture platform (SCP) of hPSC culture in feeder free conditions, which consists of a combination of Geltrex, TeSR and Dispase. Accutase is a mixture of proteolytic and collagenolytic enzymes that has been shown to allow gentle dissociation of various cell types such as pancreatic cells, neural stem cells and hESCs on MEFs[127, 306, 307]. Enzymatic dissociation of hPSCs with Accutase yields a transient single cell state which we subsequently stabilized by the addition of Rock-Inhibitor[125].

There are several key features an hPSC culture system should possess: most importantly, the cell should maintain their pluripotency over time while remaining karyotypically stable. Ideally, the methodology should further provide robust conditions, with relative ease in handling of the cells and limit the overall time in culture through efficient cell expansion.

5.1.1 Maintenance of Pluripotency and Karyotypical Integrity

We first examined if the ECP is suitable for the long-term culture of hPSCs by evaluating the ability of the system to maintain pluripotency and sustain the genetic integrity of the cells over time. hPSCs were cultured for an extended period of time and examined for morphological changes, expression of pluripotency associated markers, and the ability to form teratomas (Supplementary Figure 1A-C). These characteristics were maintained following 15 consecutive passages. Particularly, the cells were able to form teratomas, a tumor comprised of all three germ layers, thus passing the most stringent test and gold-standard for the demonstration of pluripotency in hPSCs. Based on this data it can be concluded that hPSCs cultured with ECP retain their pluripotency over time.

The occurrence of karyotypical abnormalities in hPSC culture is a well-known phenomenon since the first derivation of hESCs. Single-cell culture has been implicated most in this regard, with an increased amount of karyotypical instabilities noted over time[128-130]. As the dissociation of hPSCs using the ECP involves a single cell state, we tested if the ECP is able to maintain cells with their correct karyotype. Two hPSC lines

were passaged an extensive number of times, following which no abnormalities were observed (Supplementary Figure 1D). Further upscaling aimed at investigating the basic mutation rate with statistical significance would require a substantial increase in the number of lines and setups tested. Thus, it is premature to exclude the possibility that the utilization of ECP may cause an increased amount of karyotypical instabilities when compared to alternative culture systems, and caution in regards to karyotypical stability should be sustained. Nonetheless, the absence of any changes in two hPSC lines suggest that the ECP is at least to some degree suitable for culturing hPSCs.

5.1.2 Viability after Passaging and Freeze/Thaw

We continued by comparing the ECP against well-established culture methodology, which we termed standard culture platform (SCP). Like the ECP, The SCP is used to grow and passage cells under defined media conditions on a feeder-free protein matrix as well, yet the SCP detaches hPSC colonies with the enzyme Dispase. Following digestion, colonies are mechanically detached from the plate followed by disruption via pipetting. We started by investigating the cell viability in both systems by assessing the amount of cells reattached after each passage (often referred to as replating efficiency) and found a statistical significant increase of viability in the ECP compared to the SCP (Figure 1A). Similar improvements in viabilities were observed when the cells are frozen and thawed with the ECP (Figure 1B). As a result, use of the ECP permitted us to significantly improve the rate of cell expansion, reduce the preparation time for experiments and the total time that cells spend in culture, as well as to minimize the passage number.

Several reasons might be responsible for the comparably higher amount of cell death when Dispase is used to dissociate hPSC colonies. First, the enzyme digest itself might cause greater cellular stress reactions and induce apoptosis. This explanation could further explain the massive increase in cell death after overexposure to Dispase, a phenomenon not as apparent with the use of Accutase. Additionally, the mechanical stress of the break-up of hPSC colonies is much higher with the use of SCP. Finally,

retained attachments to dying or dead cells may lead to cell death in a clump as part of a broader cell death response, despite some of the cells perhaps surviving the initial procedure[308].

5.1.3 Clonogenic Potential

Growth of hPSCs as single cells has various advantages. It allows the derivation of cell populations through clonal expansion, facilitates the viability after flow cytometry sorting (FACS), and improves experimental procedures by providing an increased cell surface area available to interact with exogenous components. The ECP significantly improves the clonogenic potential of hPSCs in comparison to the SCP, allowing around 70% of cells to form colonies from single cells. ROCK-Inhibitor was introduced to the ECP as it has been shown to improvement single cell viability of hPSCs, and a series of experiments were performed to determine the effect of ROCK-Inhibitor in the ECP[125]. ROCK-Inhibitor treatment was able to improve the viability after replating and freeze/thawing as well as increase the cloning efficiency of hPSCs. However, the majority of the observed improvement is independent of the presence of ROCK-Inhibitor, and can therefore be attributed to the introduction of the ECP (Supplementary Figure 2).

5.1.4 Colony Size Distribution and Spontaneous Differentiation

With the routine usage of the ECP, we observed another beneficial effect of the single cell culture on hPSC culture. Utilizing the enhanced system resulted in very uniform colony sizes when compared to the SCP. We were able to quantify and confirm this observation by measuring the cell surface area of the hPSC colonies (Figure 1C). The dissociation into single cells and the subsequent formation of the colony by a single or just a few cells in comparison to colonies randomly broken up by mechanical forces in SCP, most likely explains that effect. A homogeneous colony size distribution may aid further standardization of experimental setups and should improve the precision and reproducibility of experiments.

The occurrence of spontaneously arising non-pluripotent cells in culture is a prominent problem which can interfere with the reproducibility and standardization of experimental conditions. Newly generated hiPSC and early hESC lines seem especially prone to the spread of these cells in culture, and without proper reduction techniques, can quickly become the predominant cell type in culture. We have observed a reduction of differentiated cells over time in the hPSC culture when utilizing the ECP. To quantify this phenomenon, we admixed two hPSC cell lines: one with a relatively high percentage, and the other with a low percentage of spontaneously differentiated cells. The ratio of cells positive for the pluripotency marker TRA-1-60, before and after 3 passages using either the ECP or the SCP, supported our hypothesis of lesser differentiation using the ECP. However, it is known that under circumstances hPSCs fluctuate in their expression of cell surface marker including TRA-1-60, so the relative expression may not directly correspond to the actual numbers and ratio between pluripotent and non-pluripotent cells. This phenomenon may be further assessed with additional pluripotency markers and an extended period of time in culture.

hPSCs possess one of the highest proliferation rates of any human cells in culture with a doubling time of roughly 24h[35]. One possible explanation for the observed lesser degree of non-pluripotent cells is that hPSC outcompete non-pluripotent cells by their higher relative rate of proliferation. Non-pluripotent cells, by contrast, appear better able to survive a passage performed through digest with Trypsin or Dispase. By increasing hPSC viability through usage of the ECP, it is possible that any comparative disadvantage of pluripotent cells might be reduced and hPSCs are thereby able to become and remain the dominant cell type in culture. Currently, this idea remains speculative before confirmation by additional experiments. Nonetheless, if true, this mechanism may further reduce variability and improve standardization and reproducibility of experiments with hPSCs.

5.1.5 Lenti Viral Transduction

One of the essential techniques to establish hPSCs as a widely used research tool are methods for efficient genetic manipulation. For example, the introduction of overexpression and knockdown constructs via viral transduction enables the investigation of molecular mechanisms regulating development as well as the repair of genetic mutations in patient-specific iPSC[137]. Lenti-viral transduction has been successfully achieved in hPSCs with the usage of ultra-concentrated virus yet the efficiency remained relatively low (70%-80%) compared to other cell types in which routinely efficiencies of 100% can be achieved[135, 136]. For this reason, in addition to ultra-concentration of the virus, clonal expansion and selection strategies are often required to establish a homogeneous population of cells.

We have previously observed that cells at the border of the hPSC colonies are more likely to be successfully transduced than cells in the center of the colonies (Figure 2A). This implied that the cell surface area is an important factor influencing the transduction efficiency, since cells at the outside are exposed to a greater degree than cells in the inside of the colony. The culture of hPSCs with the ECP includes an intermediate single cell step and we reasoned that the increased surface to volume area, compared to chunks of cells using SCP, may improve the efficiency of this method. We transduced hPSCs cultured using either platform, with lentiviral particles carrying a green fluorescence protein (GFP) reporter construct to investigate this possibility. As predicted, GFP-positive cells were found predominantly at the border of colonies in the SCP setting, while with the use of ECP, GFP positive cells were more evenly distributed throughout the colony. To quantify the overall efficiency we evaluated the percentage of GFP positive cells via flow cytometry. The ECP transduction efficiencies exceeded routinely 95% without the use of ultra-centrifugated virus. This represented a significantly higher efficiency as measured in the SCP setups and should enable the routine establishment of

hPSC populations that homogenously express a factor without the need for selection strategies.

5.1.6 Homologous Recombination

The successful generation of targeted genetic manipulation via homologous recombination (HR) is crucially dependant on constructs spanning large regions of homologous sequence. Due to the size limitation of the Lenti-Viral packaging system[309], electroporation is the method of choice to introduce these gene targeting constructs. Unfortunately, commonly used protocols for electroporation of hPSCs produce a low viability of the electroporated cells and yield low efficiencies in the delivery of the targeting construct.

Based on the realization that the ECP significantly increases cell viability after passaging and freeze/thawing, we investigated if the platform might also improve the viability of cells after electroporation. Furthermore, we reasoned that if the increase of efficiency in lentiviral transduction is due to the increased surface to volume ratio of single cells, it may potentially benefit the electroporation efficiency of hPSCs as well. Consistent with our hypothesis, we detected a significantly improved viability and electroporation efficiency after electroporation of hPSCs using the ECP as compared to the SCP settings (Figure 2D). The combination of both effects yielded greater than a 20-fold increase in the total number of viable cells with each electroporation. To establish the utility of this method for HR we duplicated the previously reported targeting of the ISL1 locus in a proof of principle experiment[139] (Figure 2F, 2G). The frequency of HR at the locus was comparable to the previously reported frequency, which led us to conclude that the recombination frequency at the locus is not altered with the change of culture conditions. In summary, the use of the ECP allows for highly efficient target construct delivery in hPSCs, and by increasing the number of colonies available for screening, should facilitate gene targeting.

Nonetheless, gene targeting in hPSCs via HR has remained challenging. The low recombination frequency makes it necessary to screen a high amount of potential colonies for each targeting and some gene loci seem to resist HR altogether. Recent publications have introduced alternative strategies to improve the recombination frequency; for instance, these have employed the usage of zinc finger nucleases or TALENs[147]. The combination of the improved delivery through the ECP and the increased recombination frequency of these techniques promise to facilitate gene targeting in general and may render it to a routine procedure in hPSCs similar to the gene targeting in mouse ESC today.

5.1.7 "Mouse–ESC" like Human Pluripotent Stem Cells

Mouse and human PSCs differ significantly in their appearance (→1.1.2.4). mPSC colonies are more domelike and refractile than their human counterpart, while hPSC colonies appear flat in a petri dish[39]. Additionally, hPSCs are dependent upon the growth-factor basic fibroblast growth factor (bFGF) to maintain their pluripotency while mPSCs depend on leukemia inhibitory factor (LIF). Based on these observations, the existence of two distinct pluripotency states was suggested with the traditional hPSC state referred to as "classical" state and the alternative state is termed "mouse-ESC" like. Recently, various authors were able to convert "classical" hPSCs into a "mouse-ESC"-like hPSCs and thus confirm the existence of two discrete and independent state of pluripotency in culture[32, 34, 35].

This realization harbors practical implication, as the culture of hPSCs has been challenging when compared to mESC culture. In comparison to mESC, hPSCs are less robust, resistant to single-cell dissociation, prone to spontaneous differentiation, and harbor reduced karyotypical stability[23, 24, 36-38]. It is hoped that conversion of hPSCs into the alternative state may facilitate the culture of human pluripotent stem cells significantly (→1.1.5.3).

Maintaining the mESC-like state requires the ectopic expression of one or a combination of transcriptions factors such as Klf4, Oct4 or Klf2. The resulting cells are highly similar to those of mESCs in their growth properties, X-chromosome activation state (XaXa), gene expression profile, and LIF-signaling pathway dependence. The existence of such a state is a valuable insight and the ability to induce and test these cells may lead to a deeper understanding of development and pluripotency. Yet based on the result of experiments introduced in this chapter, it is more likely that the challenges of hPSC culture and genetic manipulation are due to the culture of the cells in sub-optimal conditions rather than an inherent incapability of hPSCs. Certainly, additional test are necessary to illuminate this with any degree of certainty, comparing both pluripotency states in humans and mice in detail, to exclude minor differences in their basic properties. At a practical level, the combination of various recent hPSC culture improvements have culminated in a powerful enhanced culture platform which permits their efficient culture and genetic manipulation without inducing an alternative pluripotency state.

5.1.8 Conclusion

Some of the most basic technical challenges in the usage of hPSCs are difficulties regarding culture and handling of the cells. Before hPSC lines are capable of becoming a widely used experimental tool, a variety of issues must be overcome. Here, we introduce an enhanced culture platform (ECP) which allows an efficient and robust culture of hPSCs. The ECP was able to address a range of key issues of hPSCs cultured in *in vitro* conditions, such as an improvement in growth, maintenance, and genetic manipulation of hPSCs. The ECP should therefore significantly facilitate the ease of utilizing hPSCs and thus ultimately facilitate many applications of hPSC research.

5.2 Programming Human Pluripotent Cells into White and Brown Adipocytes

Obesity is rapidly becoming a worldwide epidemic with severe consequences for health, longevity, and the general living standard[194, 195]. Understanding the role that adipocytes play under both normal and disease-related conditions may be of critical importance in efforts to avoid the worst effects of this epidemic[207, 208]. There are two basic types of adipose tissue: brown and white. White adipocytes are involved in energy storage, metabolism, inflammation, and the immune response, and are thought to play a central role in the development of obesity and its detrimental effects[199]. The primary function of brown adipose tissues is the regulation of body temperature, a process associated with vast amounts of energy expenditure. Recently, it has become clear that adults may possess brown adipose tissue in significant amounts and that the mass of the tissue found in individuals negatively correlates to their BMI, a realization which has sparked strong interest towards investigating any possible utility of its use in combating obesity.

The culture of human adipocytes *in vitro* may yield important contributions towards a variety of important research areas. Beyond characterization of the basic properties of healthy and diseased cells, human adipocyte culture may lead to the identification of biomarkers, the establishment of *in vitro* disease-specific models, drug discovery, or cell replacement and regeneration technologies. However, the existing human *in vitro* models have significant limitations, including low differentiation efficiencies, poor reproducibility, inadequate characterization, and limited proliferation potential. To our knowledge, currently, no suitable human *in vitro* model to study brown adipocyte function exists (→1.2.1.6).

5.2.1 Experimental Strategy

A suitable cellular *in vitro* system should possess several important features, including the capacity to generate large quantities of cells, accurately reflect associated *in vivo* sates of maturity and functionality, and recapitulate cellular disease phenotypes and disease progression. Furthermore, protocols ideally should be able to derive the cells with highly efficient to enable the investigation of a homogenous cell population.

With regard to above features, existing cellular *in vitro* systems for the culture of human adipocytes have harbored limitations in one or more features, substantially limiting their utility. Primary human adipocytes are difficult to maintain and do not expand in culture. Alternative cellular systems have involved the generation of fibroblast-like adult stem cell lines derived from the adipose stromal-vascular fraction (ADSVC) or from the bonemarrow (MSC)[245, 246]. While these cells are able to be readily differentiated into adipocytes, they possess a limited proliferative potential, exhibit decreased differentiation with continued passage, show highly variable differentiation potentials between lines, and are highly likely to be limited to only a few distinct types of adipocytes (→1.2.1.6)[247].

hPSC-based systems may provide major advantages for adipocyte culture in that they can generate large quantities of cells and differentiate into various cell subtypes including both brown and white adipocytes. White adipocytes have been previously derived from hPSCs by various groups, verifying the inherent ability of hPSCs to generate adipocytes. However, these prior efforts have been plagued by poor differentiation efficiencies, thereby preventing detailed characterization of the generated cells[248-251]. While efforts by our laboratory to reproduce these results did confirm the derivation of adipocytes, we experienced a high degree of variability amongst attempts and a low degree of overall conversion.

The *in vivo* development of adipocytes involves an intermediate mesenchymal progenitor state. Thus, we hypothesized that the initial derivation of a homogeneous population of

hPSC-derived mesenchymal progenitor cells (MPC) might improve the efficiency and reliability of adipocyte differentiation[252, 253]. This approach subsequently reduced the previously noted variability between setups, yet failed to significantly improve the overall differentiation efficiency.

In their landmark paper, Yamanaka and colleagues we able change the fate of terminally differentiated cells through the ectopic expression of several transcription factors[40]. This sparked a multitude of successful attempts utilizing a similar strategy to alter the cell fate from one differentiated cell to another, an approach termed "lineage conversion." In the spirit of these experimental approaches and experimental observations in fibroblastic cells, we hypothesized that brief overexpression of PPARG2, the key transcription factor involved in adipogenesis, may be beneficial in improving the efficiency of white adipocyte derivation[191]. We termed this approach "programming" of the cells.

The results presented in (→5.1.5) demonstrate high lentiviral transduction efficiency in hPSCs. However, we observed a progressive silencing of ectopic expression with time, the degree of which associates with reductions in differentiation potential and culminates in negligible measurable expression in terminally differentiated cells. Since the progressive silencing may interfere with the overall differentiation efficiency, we have opted to perform lentiviral transduction at a later stage, introducing the factor in MPCs rather than hPSCs.

There are legitimate reasons to question the validity of cells differentiated through an ectopic expression of transcription factor (TF). Firstly, in the case of constitutive expression, any differentiation state may not be independent of forced expression and rather represent an artificial metastable state. Secondly, the differentiation may not result from a primary function of the introduced factor, but rather be result from secondary causes such as competition for TF co-factors. In our studies, we have tried to reduce any potential confounding by these principles via way of utilizing a doxycycline-inducible expression system. The inducible nature also permits testing the differentiated cells for

their dependency on ectopic TF expression by systematically altering the timing of expression across experiments. Another potential concern regards the integration of the lentiviral constructs in the genome, a process which has potential to create abnormal phenotypes through random mutagenesis. However, as adipocytes in our protocols are not derived from a clonal expansion of one transduced cell but rather by transducing large amounts of MPCs at a time, the statistical chance that any phenotypes observed in the majority of cells is due to integrative mutagenesis can be considered to be very low.

Other potential concerns include a potential masking of disease phenotypes, especially in developmental diseases, by the programming of the cells, as well as the general doubt regarding if any development observed in an *in vitro* setting represents the actual in vivo development. While these are viable concern reflecting real uncertainties, especially in the study of early developmental decisions, the overall advantages of a system which allows an efficiently and robust derivation of adipocyte from hPSCs are immense. Keeping these potential caveats in mind, an hPSC-based adipocyte culture system still has high potential towards make important contributions to research.

The central transcriptional regulation of brown adipocyte fate was for a long time unknown. Recently, PRDM16 and CEBPB have been identified as key components in maintenance and development of BAT in mice[289]. Based on our scheme to generate white adipocytes through ectopic expression of PPARG2, we devised a similar experimental scheme for the derivation of BAT. By introducing combinations of the TF PPARG2, CEBPB and PRDM16 we screened for potential factors which may yield brown adipocytes. Based on the observations and reasoning introduced in this chapter, we established an experimental scheme to derive white and brown adipocytes (Figure 1A).

5.2.2 Differentiating Human Pluripotent Stem Cells into Mesenchymal Progenitor Cells

As the first step, we generated MPCs from hPSCs and subsequently performed a detailed characterization of the MPCs. Several established protocols allow the generation of hPSC-derived MPCs, which, under the right stimuli, have been shown to differentiate into cartilage, osteoblast, myocytes, and adipocytes. These protocols often involve elaborate strategies, as illustrated by one such co-culture system with OP9 cell combined with a flow cytometry sorting strategy for the antigen CD73[247]. While successful in generating MPC lines, the inherent processes have been laborious and inefficient. We aimed at facilitating this process by developing an alternative method, one that is independent of co-culture or sorting strategies (Supplementary Figure S1A, S1B). We then characterized the resulting cells throughout the differentiation process by recording gene expression levels associated with both pluripotency and mesoderm development, and observed a progressive loss of the former and increase of the ladder (Supplementary Figure S1C). After roughly 3 passages, the derived cells consisted of a homogeneous population with fibroblast-like morphology which was both replicative and capable of expansion for up to 15 passages. The large majority (~96%) of the cells were found to be positive for a range of mesodermal, and negative for blood lineage and pluripotency cell surface markers (Figure 1B and Supplementary Figure S2A-D). Furthermore, global transcriptional analysis indicated a high degree of similarity to a variety of established MSC lines, and we were able to successfully differentiate these MPCs into osteoblasts, chondrocytes, and adipocytes (Supplementary Figure S3, Supplementary Figure S1D, S1E). Based on these results, we conclude that we have developed a simplified protocol to generate MPCs from hPSC.

5.2.3 Differentiation of Mesenchymal Progenitor Cells into White Adipocytes

After establishing this method to generate hPSC-derived MPCs, we tested their ability to generate mature adipocytes upon exposure to a media containing a combination of known inducers of adipogenesis. We compared the effect of the stimuli on MPCs to ADSVCs and found, in both experimental settings, multilocular lipid droplets resembling immature white adipocytes (Figure 2B, Supplementary Figure S4A-D). The tested ADSVC lines showed a considerable variability in differentiation efficiency, ranging from 10-70%, depending on their donor origin. hPSC-derived MPCs generated generally fewer adipocytes (3-10%) and exhibited a similar variation in efficiency. We further observed that, similar to their replicative capacity, the ability of ADSVCs and MPCs to differentiate into adipocytes decreased with increased passage number.

5.2.4 Programming White Adipocytes

The low efficiency and high variability of our aforementioned results led us to explore alternative approaches aimed towards improving the differentiation efficiency. For this, we analyzed the effect of temporary controlled ectopic expression of specific transcription factors, a strategy we termed "programming" of cell fate. To differentiate white adipocytes we selected PPARG2, the key regulatory TF of adipogenesis (→1.2.1.1). We maintained the programmed MPCs under the same adipogenic stimuli as before, but induced expression of the transgene by adding doxycycline for the first 16 days. By using a doxycycline-inducible promoter driving PPARG2, we were able to achieve a controlled expression of the introduced transgenes. After an additional 5 days of doxycycline withdrawal, we considered the cells fully differentiated and transgene-independent.

This strategy was able to efficiently differentiate MPCs into cells that possess the typical morphological features of a mature white adipocyte: a single large, well-defined lipid droplet. In contrast, unprogrammed MPCs differentiate in lower quantities and exhibited

an immature multilocular phenotype (Figure 2A-C). To quantify the amount of differentiation, we used a co-staining strategy utilizing the nuclear dye HOECHST and the adipocyte marker CEBPA. Both stains target the cell nucleus, permitting determination of the ratio of CEBPA to HOECHST stained nuclei with image analysis software. Utilizing this automated method of quantification, we observed a statistical significant increase of adipocyte differentiation efficiency from an average of 9% from unprogrammed differentiated MPCs to an average of 88% in those cells differentiated from PPARG2-programmed MPCs. The ability to consistently convert the cells into white adipocytes with percentages >80%, led us to conclude that this differentiation approach is both robust and efficient.

5.2.5 Molecular Characterization of Human Pluripotent Stem Cell Derived White Adipocytes

For our adipocytes to be most useful as a research tool, they optimally would possess a range of molecular and functional characteristics that are similar to primary white adipocytes. In assessing this degree of similarity, we designed several experiments to assay the maturity of the differentiated cells. We started by testing the programmed white adipocytes for white adipocyte specific markers, such as fatty-acid-binding protein 4 (FABP4), CCAAT/enhancer-binding protein alpha (CEBPA), PPARG2, or the lipid dye Bodipy by staining (Figure 2B, 2C). All these markers were abundantly present in the programmed adipocytes. We then tested the gene expression level of a panel of genes via RT-qPCR. The panel included important adipocytes markers such as lipoprotein lipase (LPL), hormone sensitive lipase (HSL), adiponectin, FABP4, CEBPA, and PPARG2. All of these genes are known to be involved in lipid turnover or lipid metabolism, as well as transcriptional control of mature adipocytes. With the exception of PPARG2, all markers were statistically significantly enriched in the programmed white adipocytes compared to the controls (Figure 3A). PPARG2, by surprising contrast, was found to be endogenously expressed in significant levels in both differentiated and undifferentiated MPCs as well as

ADSVCs. Thus, we wondered why endogenously expressed PPARG2 is insufficient to commit the cells to an adipocyte fate, while ectopic expressed of PPARG2 seems readily capable of doing so? A plausible explanation relates to the idea that a differentiation state is subject to constant dynamic control by transcriptional regulators. A perturbation of the stoichiometry of these factors, such as the over-expression of a transgene, may alter a differentiation state, and in our case inducing the differentiation of white adipocytes from MPCs. In this view, the relative abundance of the regulating factors is the determining factor for cell-fate decisions rather than a mere presence of a TF. Future study will be important in determining the exact mechanisms through which high levels of PPARG2 cause commitment to the adipocyte lineage. In summary, we have established that programming of hPSC-derived MPC via transient ectopic PPARG2 expression consistently yields white adipocytes exhibit a range of morphologic and molecular characteristics which are consistent with a mature white adipocyte fate.

The next step in our investigation of the characteristics of programmed white adipocytes was the identification of the transcriptional signature on a genome-wide scale. We used the result of the assay to compare the cells to primary fat, undifferentiated MPCs and ADSVCs as well as PPARG2 programmed ADSVCs. Unsupervised clustering for all of the measured genes resulted in two primary clusters, with undifferentiated MPCs and ADSVCs in one and PPARG2 programmed MPCs and ADSVCs as well as primary white fat in the other cluster. We continued by comparing the programmed white adipocytes with undifferentiated MPCs for expression of a panel of known adipocyte-specific genes and found their highly enriched expression in the differentiated cells (Figure 4B). Closer inspection of the most significantly differentially expressed genes of these clusters yielded a set of genes which included annotations for the PPARG2 signaling pathway as well as the genesis and metabolism of fatty acids and lipids (Supplementary Table S2). When we investigated the genes with highest differential expression between primary adipocytes and PPARG2-programmed adipocytes, we found that many of the genes enriched in the primary white adipocytes could be attributed to the

various non-adipocyte cell types present in adipose tissue such as leukocytes, vascular endothelium and red blood cells (Supplementary Table S3). When taken together, these data suggest that programmed white adipocytes and primary white adipose tissue are strikingly similar on a genome-wide expression scale.

We continued by testing the ability of those cells to be cultures for extended periods of time by growing them for 28 additional days without doxycycline. Throughout the culture, the cells maintained mature white adipocyte morphology. After a total of 49 days in culture the cells were able to incorporate Bodipy and stained positive for the adipocyte marker CEBPA (Supplementary Figure S4E). These results implicate that programmed white adipocytes can be kept in culture for extended periods of time and that the acquired white adipocyte state is stable and independent of transgene expression.

5.2.6 Functional Properties of Programmed White Adipocytes

For hPSC-derived white adipocytes to provide a suitable *in vitro* model, it is crucial the cells, next to the molecular and morphological characteristics, also exhibit functional characteristics similar to primary adipocytes. For one, hPSC-derived adipocytes should to be able to synthesize fatty acids as well as perform lipolysis in response to a stimulus. Furthermore, they should take up glucose in response to insulin and if possible, secrete adipokines.

We started by characterizing de-novo fatty acid synthesis and storage using a tandem mass spectroscopy lipodomics platform. This platform measures the cellular lipid content including membrane lipids as well as triglycerides, and is capable of distinguishing various triglyceride species with respect to their fatty acid length and saturation status. Based on the relative abundance of the triglycerides, fatty acid de-novo synthesis and storage can be inferred. We compared programmed white adipocytes against undifferentiated, ADSVC-derived, and primary white adipocytes and found a strong correlation in the triglyceride distribution of programmed and primary adipocytes (Figure

5C). This result was in contrast to ADSVC-derived and undifferentiated adipocytes, both of which showed significantly less similarity to primary adipocytes (Supplementary Figure S7). Upon a closer assessment of the minor differences in triglyceride abundance of programmed and primary adipocytes, we observed increased levels of some short-chain fatty acid species and diacylglycerol in programmed white adipocytes compared to primary white adipocytes. This may be explained by either an increased de-novo synthesis or enhanced lipolysis, the former of which is consistent with results from global gene expression analyses discussed earlier. The lipid content did not show any significant variation concerning membrane lipids in all cell types analyzed. In summary, we found a remarkable degree of similarity in the lipid content between primary adipocytes and programmed adipocytes, especially in respect to triglycerides.

One of the primary functions of white adipocytes is the release of the stored energy in times of energy demand in the organism. In response to a stimulus, the cells break down triglycerides into free fatty acids and glycerol, a process termed lipolysis. The extent to which cells perform this task can be measured by the amount of glycerol released into the surrounding. We measured a significantly increased glycerol release after β-adrenergic stimulation in PPARG2-prgrammed MPCs and ADSVCs in comparison to that found in undifferentiated and unprogrammed controls (Figure 5A). We therefore conclude that the cells are able to exhibit a fully physiologic response, which, when taken together with the ability for de-novo lipid synthesis, strongly suggest that the examined cells represent functionally mature adipocytes.

White adipose tissue plays an important role in the regulation of satiety and metabolism. For this white adipocytes produce and secrete several hormones such as leptin and adiponectin (→1.2.1). We measured the concentration of these adipokines following stimulation and observed a high level of secreted adiponectin in all PPARG2-progrmmed cell lines. Adiponectin has been shown to play an important role in the regulation of

glucose and fatty acid catabolism[310]. Additionally, we were able to measure the release of low levels of Leptin into the media (Supplementary Figure S8C).

Another important role of white adipocytes is their involvement in the blood glucose homeostasis. In response to insulin, adipocytes rapidly clear the blood of excess glucose. An inability to do so strongly associates with the development of diabetes mellitus type 2. For PPARG2-programmed adipocytes to be useful tool in the study of insulin resistance, their ability to respond to insulin is crucial. One way to examine the insulin response in adipocytes is to measure protein levels of v-akt murine thymoma viral oncogene homolog (AKT) and phosporylated AKT (pAKT) respectively. In mature white adipocytes, while in the presence of insulin, AKT is phosphorylated, inducing the translocation of glucose transporter (GLUT) to the plasma membrane. We thus examined the relative abundance of AKT and pAKT before and after an insulin stimulus. Prior to an insulin stimulus, no pAKT was found in the programmed white adipocytes, while the administration of insulin robustly increased pAKT levels. When the experiment was repeated in the presence of free fatty acids, known contributors to insulin resistance in white adipocytes, we found decreased levels of pAKT (Figure 5D).

We continued by comparing the extent to which programmed and unprogrammed MPCs internalize glucose in response to insulin by utilizing T3HU2-desoxy-d-glucose. This radioactively labeled glucose is readily taken up by the cells through the GLUT transporter but cannot be metabolized and thereby accumulates intracellularly. We found that while the basal level of glucose uptake was similar between both cell populations, programmed adipocytes take up significantly higher amounts of labeled glucose after exposure to insulin. Taken together these experiments demonstrate that hPSC-derived PPARG2-programmed adipocytes can act as a model for insulin response and insulin resistance.

Taken together, these results confirm the establishment of a system which can facilitate the efficient differentiation of hPSCs into white adipocytes capable of performing

functional physiological responses characteristic of their associated mature cell type. This system may thus provide an ideal cellular source for a variety of *in vitro* studies of normal and diseased white adipocytes.

5.2.7 Programming Brown Adipocytes

Based on the successful differentiation of hPSC into white adipocytes, we devised a similar approach to generate human brown adipocytes. We chose to maintain the media conditions and starting cell population constant, and introduced a set of transcription factors implicated in brown adipogenesis: PPARG2, CEBPB, and PRDM16 in various combinations[289, 290]. We continued by screened for an increase in the expression of UCP1, a protein known to be enriched in BAT, to identify the TF combination capable of inducing brown adipocyte fate (Figure 1A) (→1.2.2.6). Of the various transcription factor combinations, three yielded statistically significant increases in expression of UCP1, including the CEBPB/PRDM16 combination as well as the combinations PPARG2/CEBPB and PPARG2/CEBPB/PRDM16 (Supplementary Figure S5E). We noted that when PPARG2 was introduced, the cells showed a much stronger UCP1 induction. A factor confounding our evaluation of brown adipocyte differentiation is that white adipocytes exhibit a low level of UCP1 expression as well, opening the possibility that any measured increase in expression might represent increased white adipocyte differentiation efficiency rather than the generation of genuine brown adipocytes. To avoid this potential error, we assayed for the expression of two white adipocyte markers against a PPARG2-only white adipocyte control: ADIPOQ and the cell-death-inducing DFFA-like effector c (CIDEC). All three transcription factor combinations which induced UCP1 expression yielded similar expression of the two white adipocyte markers, which were notably, much lower as found in the PPARG2 setting alone. Based on these results we chose the two transcription factor combinations with the highest increase in UCP1 expression, PPARG2/CEBPB and PPARG2/CEBPB/PRDM16, for further characterization.

The PPARG2/CEBPB- and PPARG2/CEBPB/PRDM16-programmed brown adipocytes possessed striking morphological differences compared to the PPARG2-programmed white adipocytes. The brown adipocytes had, with very few exceptions, many small lipid droplets (multilocular), while the programmed white adipocytes generally possessed one single large lipid droplet (monolocular) (Figure 2B, 2D). We continued by confirming the results of our screen at a protein level and found a strong staining of the cytoplasm of the brown adipocytes against UCP1. We further noticed a slight brown discoloration of the programmed brown adipocytes, which could potentially indicate a high abundance of mitochondria in these cells. Upon staining the cells with the mitochondrial marker MitoTracker, we observed a strong signal in the cytoplasm (Figure 2D). Based on these results, we concluded that we were able to generate an adipocyte population which is distinct to white adipocytes cell fate, and which shows characteristics consistent with a brown adipocyte cell fate.

5.2.8 Comparison of White and Brown Adipocytes

We next examined if the programmed white and brown adipocytes can be distinguished based on their expression signature. While both types of adipocytes possess a very similar gene expression pattern, a few genes are known to vary and can be used to distinguish white from brown adipocytes. However, as none of the known differentially expressed genes are exclusive to one adipocyte type, we compared multiple markers as part of a profile, identifying adipocytes in general as well as brown or white adipocyte specifically. All of the examined white adipocyte markers were statistically significantly enriched in PPARG2-programmed white adipocytes, including ADIPOQ, hormone sensitive lipase (HSL), and fatty acid binding protein 4 (FABP4). On the other hand, the brown specific markers, elongation of very long chain fatty acid like-3 (ELOVL3) as well as UCP1, were expressed in significantly greater amount only in PPARG/CEBPB and PPARG2/CEBPB/PRDM16 programmed cells. Additionally, the major regulator for mitochondrial biogenesis peroxisome proliferator-activated receptor γ1-α (PGC1α) and

the mitochondrial marker Cytochrome C1 (CYC1) were found to be significantly more highly expressed in programmed brown adipocytes, further pointing to a higher abundance of mitochondria in these cells. Expression for the general adipocyte markers lipoprotein lipase (LPL) and endogenous PPARG2 were similar through all tested conditions (Figure 3B). Since these results were measured following doxycycline withdrawal, we concluded that the expression was independent of the transgene expression and rather the result of an endogenous cellular program (Supplementary Figure S5C).

We continued by analyzing the programmed brown adipocytes at the transcriptome level. Transcription profiles of PPARG2/CEBPB- and PPARG2/CEBPB/PRDM16-programmed brown adipocytes were generated by microarray analysis under the same conditions as the earlier analysis of PPARG2-programmed white adipocyte, thus allowing a direct comparison. The hierarchical clustering revealed that all of the programmed adipocytes and the primary adipocytes formed a distinct cluster with the undifferentiated samples (Supplementary Figure S6E). Importantly, within the adipocyte cluster, primary white adipocyte clustered with programmed white adipocyte, in a fashion distinct from the programmed brown adipocytes. Next, we focused on a range of genes that have been associated with a brown or white adipocyte identity. Amongst other brown fat markers, the brown adipocytes tissue markers UCP1, ELOVL3, and CYC1 were enriched in the programmed brown adipocytes, confirming the RT-PCR results earlier reported. Additionally, genes associated with white fat identity were most strongly expressed in programmed and primary adipocytes (Figure 4C). The only outlier we detected was endogenous PRDM16, which unexpectedly showed the highest gene expression in primary and programmed white adipocytes. Based on these results, we concluded that programmed brown adipocytes and programmed white adipocytes have distinct signatures corresponding to their associated cellular identities.

The primary function of brown adipose tissue is thermogenesis. In this role, the cells uncouple the proton motive force and energy usually used for ATP-synthesis is dissipated in the form of heat. In response to stimuli such as a β-adrenergic activation, the brown adipocytes induce lipolysis which increases the intracellular FFA concentration. Other than in white adipocytes, which release the FFA into the bloodstream, brown adipocytes break down the FFA via β-oxidation to provide acetyl-CoA. Via the citric-acid cycle, Acetyl-CoA in turn is used to elevate the proton motive force over the mitochondrial membrane (→1.2.2.1). To investigate if programmed brown adipocytes are able to utilize this mechanism, we examined the ability of these cells to induce lipolysis in response to a stimulus. For this we measured the glycerol release before and after exposure to forskolin, a substance which activates lipolysis through increasing the intracellular cAMP concentration. We were able to detect a significant increased glycerol release in both brown and white adipocytes, a change not found in undifferentiated cells (Figure 6A, Supplementary Figure 8D). While this implicates the basic functional capacity of both cell types to perform lipolysis, this experiment cannot detect differences between white from brown adipocytes.

To distinguish between brown and white adipocytes at a functional level, we measured the metabolic activity of both cell types, as a high metabolic rate is characteristic of brown but not white adipocytes. We investigated the basal and maximal metabolic rate based on measurements of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAT). We found the programmed brown adipocytes to have a significantly higher maximal metabolic rate compared to programmed white adipocyte, which allowed a distinction between both cell types at a functional level (Figure 6B, 6C).

Based on these results, we conclude that we have successfully generated the first genuine *in vitro* model of human brown adipocytes. This model has already demonstrated the first direct evidence that human brown adipocyte have a significantly increased metabolic activity compared to white adipocyte.

5.2.9 *In Vivo* Transplantation of Human Pluripotent Stem Cell Derived Adipocytes

As a final test for our experimental system, we examined if hPSC-derived adipocytes are capable of establishing adipose tissues *in vivo*. In this effort, we transplanted the programmed white and brown adipocytes subcutaneously into immunocompromised mice. The introduced cells soon formed a fat pad close to the injection side and after about 6 week we collected and histological examined these transplants. The fat pads either generated by human white or human brown adipocytes had the morphological characteristics of their associated primary adipose tissues. To avoid contamination with host adipocytes, we confirmed their extracorporeal origin by immunohistochemical staining against human-specific antibody MAB1281 (Figure 7A, Supplementary Figure S8E). In addition to the morphological similarities, the programmed white adipocytes stained positive for the adipocyte marker CEBPA while the hPSC-derived brown adipocyte stained positive for the brown adipocyte marker UCP1 (Figure 7B, Supplementary Figure S8E). Intriguingly, the transplanted brown adipose tissue was abundantly innervated by MAB1281-negative cell nuclei, in a pattern often indicative of strong vascularization, an important characteristic of brown adipose tissue *in vivo*. However, to make definitive statement confirming this hypothesis, further experiments will be necessary. In summary, we successfully transplanted human brown and white adipocytes into mice and the cells were able to form tissue with the morphological and molecular characteristics of their associated primary tissues.

Due to their high metabolic activity, brown adipose tissue can act as a "glucose sink" when activated. In fact, results in nuclear medicine using fluorodeoxyglucose positron emission tomography (FDG-PET) were the first to imply the existence of brown adipose tissue in human adults[262]. To test the functionality of our programmed brown adipocytes *in vivo*, we injected radioactively labeled glucose into mice carrying the human adipocyte transplants. We subsequently performed a FDG-PET scan and found our transplanted

hPSC-derived brown adipocytes to be highly FDG-avid and to exhibit a clear PET signal compared to transplanted white adipocytes (Figure 7C). These results are consistent with earlier discussed results regarding a high metabolic activity and functional maturity of the programmed brown adipocytes. To our knowledge this is further the first direct prove that human brown adipocytes are highly metabolic active and can act as a glucose sink *in vivo*.

Surprisingly, our studies found no significant difference between PPARG2/CEBPB- and PPARG2/CEBPB/PRDM16-programmed brown adipocytes. For us, the ectopic expression of PRDM16 was not necessary to induce brown adipocyte fate, a result that stands in contrast to earlier studies in rodents[289, 290]. One possible explanation for the noted independence of ectopic PRDM16 transgene expression would be if PRDM16 were expressed in undifferentiated hPSC-derived MPCs. We therefore examined the endogenous expression levels of PRDM16 throughout differentiation and were able to detect low levels of the gene transcripts in all stages (Supplementary Figure S8F). Whether this low level of expression is sufficient to support brown adipocyte differentiation, or if, in contrast to mouse brown adipose tissue, human brown adipocyte differentiation is independent of PRDM16 remains to be seen. Another intriguing possibility is the existence of an alternative developmental origin for brown adipocytes other than Myf-5 expressing myogenic precursors. Further studies of human brown adipose tissue will hopefully add clarification to these questions.

5.2.10 Conclusion

We have successfully established an *in vitro* system which allows consistent and efficient differentiation of human pluripotent stem cells into brown and white adipocytes through the transient expression of lineage associated transcription factors. Both generated types of adipocytes possess distinct morphological features and gene expression profiles and demonstrate functional properties characteristic of their corresponding tissue type *in vivo*. Upon transplantation into mice, these cells are capable of forming tissues with functional

and morphological qualities similar to primary white and brown adipose tissue. In summary, the results of these experiments confirm the identity and maturity of the generated adipocytes. By providing a homogeneous and replenishable source for adipocytes, this system promises to be highly useful towards the faithful modeling of human disease, as well as in the study of human adipocyte metabolism in both normal and diseased states.

5.3 Perspectives

The introduction of an efficient method to generate white and brown adipocytes from hPSCs has sparked a wealth of additional projects. In collaboration with the Laboratory of Robert E. Gerszten, we have been able to contribute to the identification of a novel PGC1α-dependant myokine. The small metabolite β-aminoisobutyric acid (BAIBA) is released by skeletal muscles after exercise. We were able to expand on results generated in mice by demonstrating that exposing hPSC to the small molecule myokine BAIBA during differentiation into white adipocytes induces brown-adipocyte-specific gene expression and an increase in basal and insulin stimulated glucose uptake as well as oxygen consumption. Together with other compelling evidence generated by the Gerszten laboratory, we concluded that BAIBA is involved in the exercise-induced protection from metabolic disease. We are currently in the process of initiating studies aimed at elucidating the mechanism underlying this effect and extending our investigation to the effect of BAIBA on hPSC-derived liver cells. The ability of this substance to induce increased metabolic activity and thus energy expenditure in human white adipocytes may function as, or lead to future therapeutic identification, of an anti-obesity drug.

Recently, interest in utilizing BAT as a potential cure for obesity has strongly increased. However, no studies have been able to demonstrate the effect of human BAT on weight

loss or gain. With the ability to transplant hPSC-derived BAT into the mouse model, and the subsequent development of the cells into functional brown adipose tissue, studying the effect human BAT on weight loss or gain, has *in vivo*. We are currently planning the transplantation of multiple human BAT pads into immunocompromised mice and expose the mice to HFD. If an increased human BAT mass is able to protect the animals from HFD-induced obesity, it would strongly support the claim that human brown adipocytes is a suitable target in the fight against obesity.

We were also able to successfully established two hiPSC lines from patients suffering from congenital lipodystrophy caused by mutations in LMNA. The analysis of these cells identified significant differences in morphology, differentiation efficiency, and insulin-response compared to control lines. We are currently in the process of correcting the mutation in the patient-derived hiPSC lines, as well as creating genetically modified hPSC lines with mutation implicated in the development of lipodystrophy. These promising initial results further give support to our assessment that programmed hPSC-derived adipocytes are suitable to faithfully model human diseases.

In addition, we were able to identify a cellular phenotype in programmed white adipocyte in a sortilin 1 (SORT1) knock out study. The genetic modification was performed with the help of TALENs and the hPSC methodology introduced in chapter (→5.1). Using a similar approach we were able to target a suite of important genes involved in metabolic diseases and diabetes mellitus type 2. These include hPSC lines carrying genetic alterations in the genes AKT2, or and we are in the process of screening for cellular phenotypes.

Furthermore we are currently developing a screen for substances which aim at increasing the metabolic rate in programmed white adipocytes. Based on the idea of “browning” white adipocytes, we plan to introduce a reporter construct into hPSC lines, which drives GFP expression under the UCP1 promoter (→1.2.2.7). After the cells are programmed into white and brown adipocytes, various compounds will be screened for their effect on

UCP1 expression. This project was developed in close collaboration with ROCHE pharmaceuticals, which is currently developing a high throughput screens involving large compound libraries based on our protocols.

The delivery of the transcription factors for programming via lenti-virus transduction involves the integration of the construct into the hPSC genome. This random integration can potentially interrupt important genes and render the cells unsuitable for clinical cell therapy. Currently a method to introduce transcription factors via modified RNA promises to circumvent this problem[74]. The strategy is based on a RNA-based approach recently developed to generate integration-free hiPSC. Normally, introducing single stranded RNA into mammalian cells induces a response involving interferon and NF-κB immune pathways which initiate the cell to undergo apoptosis. However, this immune response can be avoided by modifying the single stranded RNA to mimic endogenous mRNA or by directly suppressing the innate immune response. We are also currently optimizing the delivery of PPARG2 and CEBPB into hPSC-derived MPCs, with the goal of establishing an adipocyte differentiation protocol without any genomic alteration. This would provide the first step towards development of suitable cell replacement system for clinical therapy and might at some point even enable the conversion of human tissue into white or brown adipocytes an *in vivo*, thus avoiding any intermediate cell culture at all.

6. Summary:

Human pluripotent stem cells have the potential to make substantial contributions to developmental biology and revolutionize medical research. However, their utility thus far has been limited by a variety of basic technical challenges. The existing standard culture methodology is laborious, inefficient, and inconsistent. Additionally, many applications are hindered by a dearth of suitable differentiation methods, rendering many important cell types inaccessible. For instance, the field of adipocyte biology, central in the fight against the emerging obesity epidemic, lacks an efficient and robust protocol to generate brown and white adipocytes.

This thesis consists of two parts, the first of which concerns the development of an enhanced hPSC culture platform. Here, it is demonstrated that the newly developed enhanced culture platform permits the efficient and robust culture of hPSCs. The platform was able to address, and improve upon, key limitations of the most commonly used standard culture platform. The growth and maintenance of hPSCs is thereby significantly improved and genetic manipulation substantially facilitated.

The second part of this thesis focuses upon the development of differentiation techniques deriving brown and white adipocytes from hPSCs. Herein, we have developed a strategy to induce the ectopic expression of lineage specific transcription factors, thereby guiding the differentiation into these respective cell types. Based on molecular and morphological markers, we conclude that the transient overexpression of PPARG2 can differentiate hPSC-derived MPCs robustly and efficiently into mature white adipocytes. The functional capabilities of the resulting cells were evaluated, and revealed abilities to perform lipolysis and de-novo fatty acid synthesis, internalize glucose in response to insulin, and release adipokines upon appropriate stimulation. Based upon their functional repertoire, we conclude that we have successfully established an *in vitro* hPSC-based system to generate mature white adipocytes. Furthermore, these results indicate that

hPSC-derived white adipocytes are suitable to model human disorders, such as obesity, lipodystrophy, or diabetes mellitus type 2.

Recently, the discovery of brown adipose tissue in adult humans, a tissue known to be highly metabolically active in rodents, has sparked extensive interest in investigating its potential manipulation and use as an anti-obesity tool. However, very little is known about the tissue in humans and no *in-vitro* cell model exist. In an effort aimed at establishing such a model, we screened the transcription factors PPARG2, CEBPB and PRDM16 for their potential to induce brown adipocyte fate from hPSCs. Based on morphological and molecular characterization, we were able to identify the transcription factor combinations PPARG2/CEBPB and PPARG2/CEBPB/PRDM16 as potential candidates. We confirmed a brown adipocyte identity through extensive characterization of the molecular and functional properties of the cells and directly comparing them to programmed white adipocytes. Importantly, these cells feature an increased oxygen consumption and extracellular acidification rate, as well as greater cellular glucose uptake when compared to white adipocytes, all strong indicators associated with a substantially increased metabolic rate.

Lastly, we reintroduced the hPSC-derived cells into immunodeficient mice to access their ability to generate adipose tissue *in-vivo*. After 6 weeks the transplanted white and brown adipocytes had formed fat pads which resembled the associated primary tissue in morphological and molecular characteristics. Human brown adipocytes take up large quantities of glucose when stimulated. In fact, this characteristic enabled the first identification of brown adipose tissue in adults by measuring radioactively labeled glucose via PET-CT. Utilizing a similar approach, we were able to demonstrate that hPSC-derived programmed brown adipocytes have the capability to act as a glucose sink *in-vivo.* While newly transplanted human brown adipocytes were soon enriched in radioactively labeled glucose, the control white adipocyte transplants refrain from accumulating significant amounts.

Obesity is on the verge of becoming a world-wide epidemic with deleterious consequences for the living standard, health, and longevity of a large part of the population. Currently, only few interventions are known to mitigate the most severe health consequences, let alone induce long-term weight reduction in patients. The findings presented in this thesis make important contributions in the ongoing search for remedies to these problems. The introduction of a highly efficient system to differentiate the cells into mature brown and white adipocyte provides an attractive model to investigate human adipose metabolism in both normal physiologic and disease conditions, and may ultimately help to understand the role adipocytes play in human disease.

7. Zusammenfassung

Humane pluripotente Stammzellen (hPSC) haben das Potential die biologische und medizinische Forschung zu revolutionieren. Zuvor müssen jedoch eine Reihe grundlegender Probleme gelöst werden. So sind die vorhandenen Kulturbedingungen ineffizient, unzuverlässig und aufwendig. Für viele wichtige Fragestellungen fehlen zudem geeignete Differenzierungsprotokolle, wodurch wichtige Zelltypen für Experimente nicht zur Verfügung stehen. Beispielsweise mangelt es an effektiven und robusten Strategien um Adipozyten in Zellkulturen herzustellen und zu kultivieren.

Fettleibigkeit hat in den letzten Jahrzehnten epidemische Ausmaße in weiten Teilen der Bevölkerung erreicht, mit negativen Auswirkungen auf Gesundheit und Lebensqualität vieler Betroffenen. Ein wichtiger Faktor, für die mit Übergewicht assoziierten Stoffwechselerkrankungen, ist der Zustand von Adipozyten. Eine Methode die es ermöglicht funktionelle Adipozyten effizient *in vitro* zu kultivieren, würde die Untersuchung dieser Zellen im gesunden und pathologischen Zustand ermöglichen und könnte dadurch den Kampf gegen die Folgen der Fettleibigkeit unterstützen.

Diese Doktorarbeit besteht aus zwei Teilen; der erste Teil bezieht sich auf die Entwicklung eines verbesserten Kultursystems von hPSCs, während der zweite Teil eine Methode zur Generierung von weißen und braunen Adipozyten aus Stammzellen vorstellt. Im ersten Teil der Arbeit demonstrieren wir, dass die sogenannte “enhanced culture platform” (ECP) geeignet für die Kultur von humanen Stammzellen ist. Zudem ermöglicht die Nutzung dieser Plattform Unzulänglichkeiten vorhandender Zellkultursysteme erheblich zu verbessern. Zum Beispiel wurden durch Optimierung der Kulturbedingungen, die Zellkultur sowohl effizienter gemacht und stabilisiert, als auch genetische Manipulation deutlich vereinfacht.

Der zweite Teil der Arbeit hat die Entwicklung eines experimentellen Systems zur Differenzierung von hPSC in braune und weiße Adipozyten zum Thema. Dafür

entwickelten wir eine Strategie die Zellen, mittels Überexpression ausgewählter Transkriptionsfaktoren, in bestimmte Zelltypen lenkt. Anhand einer Reihe von Untersuchungen auf morphologischer und molekularer Ebene folgerten wir, dass die Überexpression des adipozyten-spezifischen Transkriptionsfaktors PPARG2 ausreicht, um aus hPSC hergestellten mesodermalen Stammzellen effizient und robust vollständig ausgereifte weiße Fettzellen zu differenzieren. Die so generierten Adipozyten besitzen zudem für Fettzellen spezifische funktionelle Fähigkeiten: Sie können Lipolyse von Fettsäuren durchführen, neue Fettsäuren herstellen, Glukose nach Insulin-Stimulus aufnehmen und Adipokine freisetzen. Zusammenfassend bestätigten diese Ergebnisse die Identität und Funktionalität der Zellen und wir folgerten, dass wir erfolgreich ein *in vitro* System zur Herstellung von weißen Adipozyten entwickelt haben. Diese Resultate deuten zudem auf eine Eignung des Zellkultursystems für die Untersuchung des Einflusses von Adipozyten auf Krankheiten, wie zum Beispiel Fettleibigkeit, Lipodystrophie oder Diabetes mellitus Typ 2, hin.

Braunes Fettgewebe ist in Säuglingen und kleinen Säugetieren zur Generierung von Körperwärme zuständig. Mit der Wärmeproduktion ist die Freisetzung großer Mengen metabolischer Energie verbunden. Aufgrund des möglicherweise erheblichen Einflusses auf den Energiehaushalt eines Lebewesens, hat die Entdeckung, dass auch erwachsene Menschen Depots aus braunem Fett besitzen, starkes Interesse ausgelöst. Als potenzieller Ansatzpunkt zur Bekämpfung von Adipositas könnte das Gewebe die Entwicklung neuartiger Medikamente ermöglichen. Für die Untersuchung von menschlichen braunen Adipozyten stand bisher jedoch kein *in vitro* Zellkultur Modell zur Verfügung und wenig ist bisher über diese Zellen im Menschen bekannt.

In dieser Arbeit haben wir die Fähigkeit der Transkriptionsfaktoren PPARG2, CEBPB und PRDM16 untersucht, hPSCs in braune Adipozyten umzuwandeln. Wir konnten zeigen, dass die Kombinationen PPARG2/CEBPB und PPARG2/CEBPB/PRDM16 fähig waren, Zellen mit morphologische und molekulare Charakteristika von braunem

Fettgewebe zu generieren. Wir analysierten die braunen Adipozyten und konnten zeigen, dass die Zellen gegenüber den programmierten weißen Adipozyten einen erhöhten Sauerstoffverbrauch besitzen, verstärkt extrazelluläre Acidität verursachen und vermehrt Glucose aufnehmen. Diese Ergebnisse deuten auf einen deutlich erhöhten Stoffwechsel der programmierten braunen Adipozyten hin, eine wichtige funktionelle Eigenschaft von braunem Fettgewebe *in vivo*.

Um zu untersuchen, ob die aus hPSC generierten weißen und braunen Adipozyten die Fähigkeit haben, ihre entsprechenden Gewebetypen *in vivo* zu erzeugen, haben wir die Zellen in immun-kompromittierte Mäuse transplantiert. Nach 6 Wochen bildete sich aus den injizierten Zellen ein Fettpolster welches morphologische und molekulare Charakteristika der entsprechenden primären Zelltypen aufwies. Zudem sammelten sich in den transplantierten braunen Adipozyten, große Mengen an radioaktiv markierter Glukose an, ein weiterer Hinweis auf die erhöhte Stoffwechselrate der Zellen und ein eindeutiger Hinweis, dass es sich bei diesen Zellen um authentische braune Adipozyten handelt.

Derzeit gibt es nur wenige wirksame Möglichkeiten, der Epidemie von Adipositas und den damit verbundenen Folgeerkrankungen, Einhalt gebieten zu können. Diese Doktorarbeit hat, durch die Einführung von Transkriptionsfaktor vermittelter Differenzierung von weißen und braunen Adipozyten und deren detaillierte Charakterisierung, einen wichtigen Beitrag zu dem Verständnis von Adipozyten geleistet. Die hier vorgestellten Methoden werden die Untersuchung der Rolle von Adipozyten in normalen und pathologischen Prozessen ermöglichen und können so die Entwicklung von Therapien gegen Fettsucht und damit assoziierten Folgeerkrankungen wie Diabetes mellitus, Schlaganfällen oder Herz-Kreislauf-Erkrankungen unterstützen.

8. Abbreviation list:

ADIPOQ – adiponectin

ADSVC – adipose-derived stromal-vascular cell

AKT – v-akt murine thymoma viral oncogene homolog

ATP – adenosine triphosphate

ALS – amyoltrophic lateral sklerosis

Ascl1 – achaete-scute complex homolog 1

BAIBA – β-aminoisobutyric acid

BAT – brown adipose tissue

bFGF – basic fibroblast growth factor

BMI – body mass index

BMP7 – bone morphogenic protein 7

Brn2 – brain-2

CEBPA – CCAAT/enhancer-binding protein alpha

CEBPB – CCAAT/enhancer-binding protein beta

CEBPG – CCAAT/enhancer-binding protein gamma

CIDEC – cell-death-inducing DFFA-like effector c

c-Myc –v-myc myelocytomatosis viral oncogene homolog

COX2 – cyclooxygenase-2

COX8A – cytochrome c oxidase subunit VIIIA

CT – computer tomography

CYC1 – cytochrome c-1

DIO2 – deiodinase iodothyronine type 2

ECAT – extracellular acidification rate

ECC – embryonic carcinoma cell

ECP – enhanced culture platform

ELOVL3 – very long chain fatty acid like-3

EpiSC – epiblast-derived stem cell

ESC – embryonic stem cell

FABP4 – fatty acid binding protein 4

FACS – flow cytometry sorting

FBS – fetal bovine serum

FDG – fluorodeoxyglucose

FDG-PET – fluorodeoxyglucose positron emission tomography

FFA – free fatty acids

FPLD – familiar partial lipodystrophy

FPLD2 – familiar partial lipodystrophy type 2

FPLD3 – familiar partial lipodystrophy type 3

GFP – green fluorescence protein

GLUT – glucose transporter

HDL – high-density lipoprotein

HFD – high-fat diet

hiPSC – human induced pluripotent stem cell

hPSC – human pluripotent stem cell

HR – homologous recombination

HSL – hormone sensitive lipase

ICM – inner cell mass

IL-6 – interleukin-6

iPSC – induced pluripotent stem cell

KLF4 – kruppel-like factor 4

LIF – leukemia inhibitory factor

LPL – lipoprotein lipase

mESC – mouse embryonic stem cell

MafA – v-maf musculoaponeurotic fibrosarcoma oncogene homolog A

MEF – mouse embryonic fibroblast

MPC – mesenchymal precursor cells

MSC – mesenchymal stem cell

MyoD – myogenic differentiation 1

Myt1l – myelin transcription factor 1-like

Ngn3 – Neurogenin 3

OCR – oxygen consumption rate

OCT4 – octamer-binding transcription factor 4

pAKT – phosphorylated v-akt murine thymoma viral oncogene homolog

Pdx1 – pancreatic and duodenal homeobox 1

PGC1α – peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

PGD – preimplantation genetic diagnostics

PPARG2 – peroxisome proliferator-activated receptor gamma 2

PRDM16 – PRD1-BF1-RIZ1 homologous domain containing protein 16

PSC – pluripotent stem cell

ROCK – Rho-associated protein kinase

RT-PCR – reverse transcription - polymerase chain reaction

T3 – triiodothyronine 3

TALEN – transcription activator-like effector nuclease

SCNT – somatic-cell nuclear transfer

SCP – standard culture platform

SMA – spinal muscular atrophy

SNN – sympathetic nervous system

SORT1 – sortilin

SOX2 – sex-determining region Y-box2

SVC – stromal-vascular cell

TF – transcription factor

TNF-α – tumor necrosis factor-α

UCP1 – uncoupling protein 1

WAT – white adipose tissue

WHR – waist to hip ratio

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10. Eidesstattliche Erklärung

Ich versichere hiermit, dass die vorliegende Dissertation selbstständig verfasst, die für diese Arbeit benutzten Hilfsmittel genannt und die Ergebnisse anderer klar gekennzeichnet habe.

Teile der Arbeit wurden für ein weiteres Prüfungsverfahren genutzt. Die Dissertation „Switching Human Cell Fate: Reprogramming via pluripotency to adipogenesis“, eingereicht von Dipl. Biochem. Tim Ahfeldt an der Universität Hamburg, beinhaltet Daten, die auch für diesehier vorliegende Arbeit verwendet wurden. Detaillierte Angaben über die gemeinsamen Resultate kann der Erklärung über den Eigenanteil entnommen werden.

Ich bin damit einverstanden, dass ein Exemplar meiner Dissertation in der Bibliothek ausgeliehen werden kann.

Robert Schinzel,

Cambridge, den 19. Juni 2012

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Efficient Culturing and Genetic Manipulation of Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSC) hold great promise as models for understanding disease and as a source of cells for transplantation therapies. However, the lack of simple, robust and efficient culture methods remains a significant obstacle for realizing the utility of hPSCs. Here we describe a platform for the culture of hPSCs that 1) allows for dissociation and replating of single cells, 2) significantly increases viability and replating efficiency, 3) improves freeze/thaw viability 4) improves cloning efficiency and 5) colony size variation. When combined with standard methodologies for genetic manipulation, we found that the enhanced culture platform allowed for lentiviral transduction rates of up to 95% and electroporation efficiencies of up to 25%, with a significant increase in the total number of antibiotic-selected colonies for screening for homologous recombination. We further demonstrated the utility of the enhanced culture platform by successfully targeting the *ISL1* locus. We conclude that many of the difficulties associated with culturing and genetic manipulation of hPSCs can be addressed with optimized culture conditions, and we suggest that the use of the enhanced culture platform could greatly improve the ease of handling and general utility of hPSCs.

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Introduction

Since the derivation of human embryonic stem cells [1], their growth and maintenance in culture have remained challenging. When compared to mouse pluripotent stem cells (mPSCs), the human counterparts (hPSCs) are less robust, more prone to spontaneous differentiation, difficult to culture as single cells, and less amenable to genetic manipulation. With the generation of human induced pluripotent stem cells [2–4], there has been increased interest in the use of hPSCs for a variety of applications. Recently, the introduction of defined media conditions, feeder-free culture systems, and chemicals to facilitate survival of hPSCs as single cells [5–7] have led to significant improvements, yet an efficient and robust culture methodology has been lacking.

We used a combination of recently published hPSC culture protocols and their further optimization to develop a protocol that we term the enhanced culture platform (ECP). We extensively evaluated this platform and compared it to one of the more widely used culture method, here termed the standard culture platform (SCP). We developed multiple lines of evidence that culturing hPSCs using the ECP significantly facilitates their handling and genetic manipulation. Use of the ECP maintained the pluripotency and genetic integrity of hPSCs over long-term culturing and passaging. The ECP improved replating efficiencies and viability of single-cells when passaging hPSCs. This culture platform also increased the viability of hPSCS after freezing and thawing. Importantly, the ECP yielded higher clonogenic efficiency, increased transduction by lentiviral vectors, and improved electroporation efficiencies of hPSCs. Finally, we were readily able to perform homologous recombination using the ECP. Thus, the use of the ECP for growth, maintenance, and manipulation of hPSCs provides a robust and efficient culture methodology that promises to improve the utility of hPSCs.

Results and Discussion

The ECP was the combination of a feeder free culture system utilizing Geltrex [5], TeSR defined media [7], Accutase [8] to dissociate and detach cells and Rock-Inhibitor (Y-27632) [6] to stabilize the subsequent intermediate single cell state. This culture platform was extensively evaluated and compared to the standard culture platform (SCP) of hPSCs in feeder free conditions, consisting of a combination of Geltrex, TeSR and Dispase.

In order to establish that the ECP was capable of maintaining the pluripotency and genetic integrity of hPSCs over extended culturing, we passaged HUES9 [9] and BJ-RiPSC [10] cells over 15 times using the ECP. Throughout this culture period, the cells maintained well-defined, phase-bright borders, a high nucleus-to-

cytoplasma ratio, and prominent nucleoli. We further evaluated the cells immunohistochemically for markers of pluripotency including OCT4, SOX2, NANOG and TRA-1-81 (Figure S1A) and found them to be positive for each of the markers. We also confirmed high expression of two master regulators of pluripotency, *OCT4* and *NANOG*, in cells grown using the ECP by quantitative reverse transcription followed by polymerase chain reaction (qRT-PCR) amplification (Figure S1B). We also found that cells of both lines grown using the ECP for more than 15 passages formed teratomas comprising all three embryonic germ layers (Figure S1C) and remained karyotypically normal (Figure S1D).

We next compared the ECP to a well-established culture methodology (SCP) with respect to the ease of handling and maintaining hPSCs in culture. We first determined the percentage of cells reattaching after passage (often referred to as the replating efficiency) using three human embryonic stem cell (hESC) lines and two human induced pluripotent stem cell (hiPSC) lines and found a significant increase ($p = 2\times10^{-6}$) from an average 23% (SCP) to 85% (ECP) of cells that replated and remained viable (Figure 1A). As a consequence, we were able to dilute the standard split ratio of 1:4 with the SCP to as much as a 1:15 ratio with the ECP, allowing for more rapid expansion of hPSCs. We next sought to determine if the ECP might also improve the viability of hPSCs after freezing and thawing (Figure 1B). In all 5 hPSC lines, the percentage of viable cells recovered after freezing and thawing significantly improved ($p = 2\times10^{-6}$) from an average of 10% (SCP) to an average of 65% (ECP). The ability to quickly expand hPSCs in culture using the ECP as well as quickly create large stocks of viable frozen cells should thereby reduce the time necessary to set up experiments, as well as reduce the total time in culture for hPSCs as they are prepared for experiments.

To investigate the clonogenic potential of hPSCs grown with the ECP, the cloning efficiency in three hPSC lines was determined by limiting dilution (Figure 1C). We found that an average of 4% of plated single cells grown in SCP were able to form clonogenic colonies in comparison to almost 70% grown using the ECP ($p = 0.002$). As the Rho Associated Kinase (ROCK) Inhibitor (Y-27632) has been previously shown to stabilize the hESC single-cell state [6,11] and is a component of the ECP, a series of experiments were performed to determine the effect of ROCK-Inhibitor in the ECP (Figure S2). While inclusion of ROCK-Inhibitor improved the replating, freeze/thaw, and cloning efficiency of hPSCs, most of the improvement observed with the ECP appeared to be due to the combination of the newly introduced culture techniques rather than the addition of the chemical.

With routine use of the ECP, we made two additional observations. First, dissociation of hPSCs to single cells resulted in more uniform colony sizes. We quantified the colony size distribution by measuring the surface area of 150 colonies three days after passage with either the ECP or the SCP and found a reduction in variability with the ECP (Figure 1D). Second, we observed an increase in the proportion of Tra-1-60 positive cells in culture over three passages (Figure 1E). We quantified this observation by measuring TRA-1-60 via flow cytometry using two hPSC lines that had a relatively high percentage of TRA-1-60 negative cells admixed in culture (42% in HUES1 and 47% in BJ RiPSC). After repeated passaging using the SCP, both hPSC lines showed a marked decrease in the amount of TRA1-60-positive cells [HUES1: 58% in passage 0 (p0) to 40% in passage 3 (p3); BJ RiPSC: 53% in p0 to 12% in p3]. In contrast, hPSCs cultured using the ECP increased the percentage of TRA-1-60-positive cells over time (HUES1: 58% in p0 to 66% in p3; BJ RiPSC: 53% in p0 to 58% in p3). As hPSCs are known to fluctuate in their expression of cell surface markers such as TRA-1-60, this observation may not correspond to an increase in the number of pluripotent cells when cultured with the ECP. In order to determine if the ECP does in fact reduce the proportion of non-pluripotent cells over time additional markers of pluripotency would need to be assessed over a much longer period of time in culture (i.e. 10 passages or more). The increased proportion of TRA-1-60 positive hPSCs together with the reduced colony size variation, may further reduce variability and improve standardization and reproducibility of experiments with hPSCs.

We next sought to evaluate the ECP using two standard methods of genetic manipulation, lentiviral transduction and gene targeting. Lentiviral transduction has been previously reported in hPSCs using ultra-concentrated virus [12], although the reported efficiency in hPSCs remained relatively low (70%–80%) as compared to several other cell types in which transduction efficiencies of 100% can be routinely achieved without ultra-concentrating the virus. Upon transduction of hPSCs grown using the SCP with lentiviral particles designed to constitutively express green fluorescent protein (LV-GFP), GFP-positive cells were found predominantly at the borders of hPSC colonies. In contrast, hPSCs grown in the ECP and subsequently transduced with LV-GFP displayed GFP-positive cells throughout the hPSC colonies (Figure 2A). To quantify the overall efficiency of lentiviral transduction, four hPSC lines grown with either the SCP or the ECP were transduced with LV-GFP using a range of viral particle concentrations, and the percentage of GFP-positive cells was evaluated via flow cytometry (Figure 2B). A significant increase ($p = 0.03$) in transduction efficiency was observed in hPSCs grown with the ECP (96%) as compared to cells grown with the SCP (54%). In all of the four hPSC lines tested, the transduction efficiencies exceeded 95% with the use of ECP and without the need for ultra-concentration of the virus. The introduction of overexpression and knockdown constructs via lentivirus with greater than 95% efficiency should allow for the routine establishment of hPSC populations that homogenously express the desired factors without the need for selection strategies.

Finally, we investigated the utility of the ECP for the delivery of plasmids into hPSCs via electroporation for homologous recombination (HR). We first determined the viability of cells electroporated using either the ECP or the SCP and found a significantly increased ($p = 0.002$) viability of the cells electroporated with the ECP, with an average of 69% surviving the procedure (Figure 2D top). Additionally, the overall percentage of cells that transiently expressed GFP from an electroporated plasmid significantly improved ($p = 0.004$) from an average of 7.4% (SCP) to 20% (ECP) (Figure 2D middle), thus allowing for a more than 20-fold increase of the total number of viable GFP-positive cells from 0.64% to 13.91% of the initial number of cells. We then attempted to duplicate the previously reported targeting by HR of the *ISL1* locus [13]. After a single electroporation of 1×10^6 cells, 3,320 colonies of HUES9 cells and 2,750 colonies of BJ RiPSC cells were obtained after antibiotic selection (Figure 2F). We evaluated 85 of the BJ RiPSC colonies for HR via long-range PCR and found one successful event (Figure 2G). The efficiency of HR at this locus with the ECP (1.17%) was comparable to what had been previously reported (1.42%). Thus, the recombination frequency at the locus does not appear to change with alteration of culture conditions, but use of the ECP allows for highly efficient target construct delivery and, given the increase in the number of colonies available for screening, should thereby facilitate gene targeting.

In conclusion, the ECP allows for dissociation and replating of single hPSCs, significantly increases viability and replating

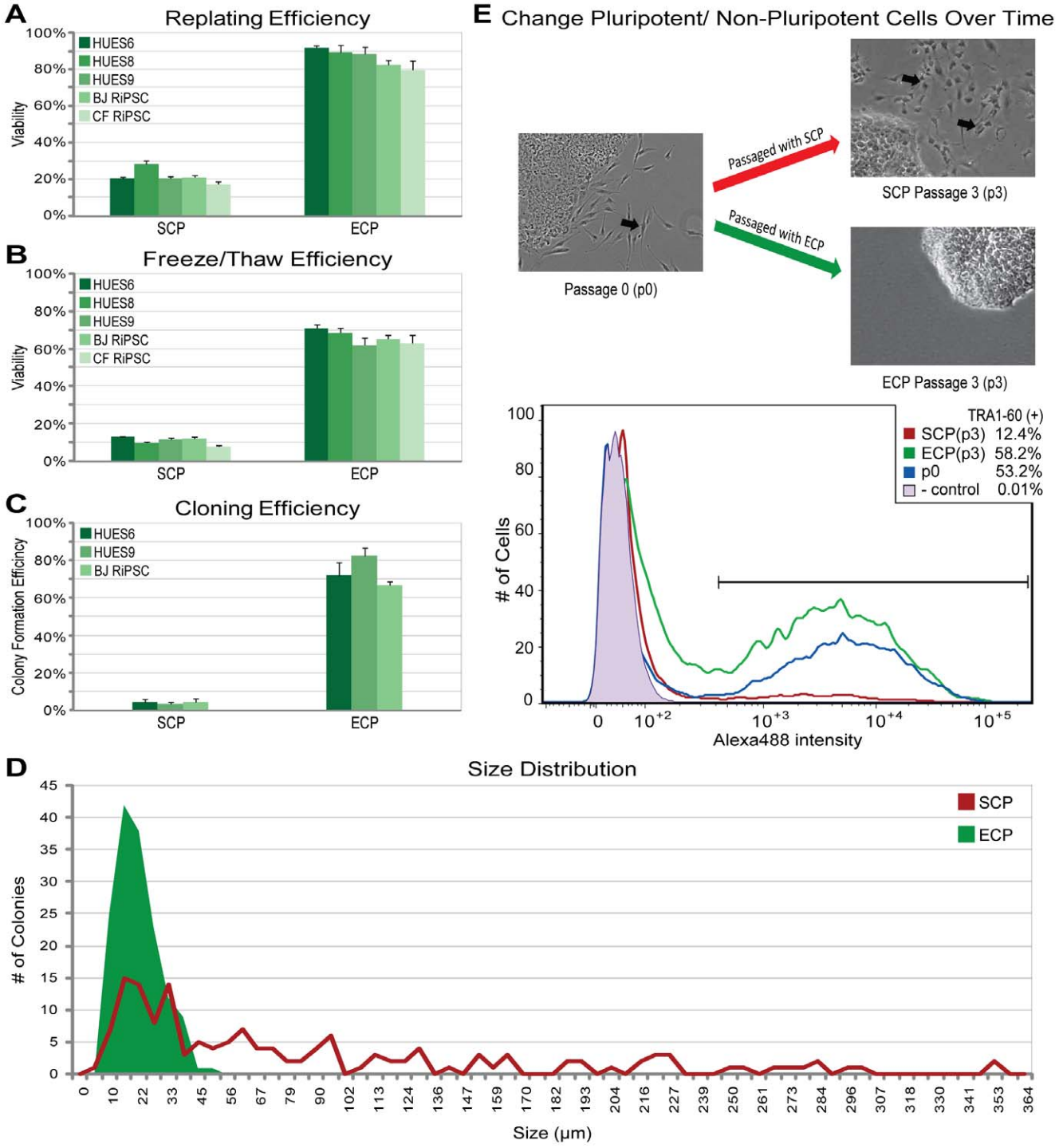

Figure 1. Comparing cell culture of hPSCs using the standard (SCP) or the enhanced culture platform (ECP). A) Quantification of cells reattached 2 h after passage in a total of 5 cell lines either using the SCP or ECP to passage the cells (n = 3 per cell line, standard error, $p = 1.68^{-06}$). **B**) Quantification of cells reattached after being frozen in liquid nitrogen for 7–10 d and subsequently thawed (n = 3 per cell line, standard error, $p = 1.69^{-06}$). **C**) To determine the clonogenic potential, a limiting dilution assay on a total of 3 cell lines was performed (96 cell plated per cell line, standard error, p = 0.002). Counted were only colonies with positive immunoflourescence staining for the pluripotency markers OCT3/4 and NANOG. **D**) Colony size distribution in μm, 3 days after passage with either the SCP or ECP (n = 150 colonies each). **E**) Representative images of BJ RiPSC colonies before and after being passaged 3 times with SCP or ECP respectively (black arrows indicates differentiated, non-pluripotent cells) and quantification of the change via flow cytometry utilizing TRA1-60 as a pluripotency marker.
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efficiency, and improves freeze/thaw viability and cloning efficiency of hPSCs. The growth of hPSCs with the ECP also reduced colony size variation and might further reduce the proportion of spontaneously arising non-pluripotent cells. When combined with standard methodologies for genetic manipulation, we found that the enhanced culture platform allowed for lentiviral

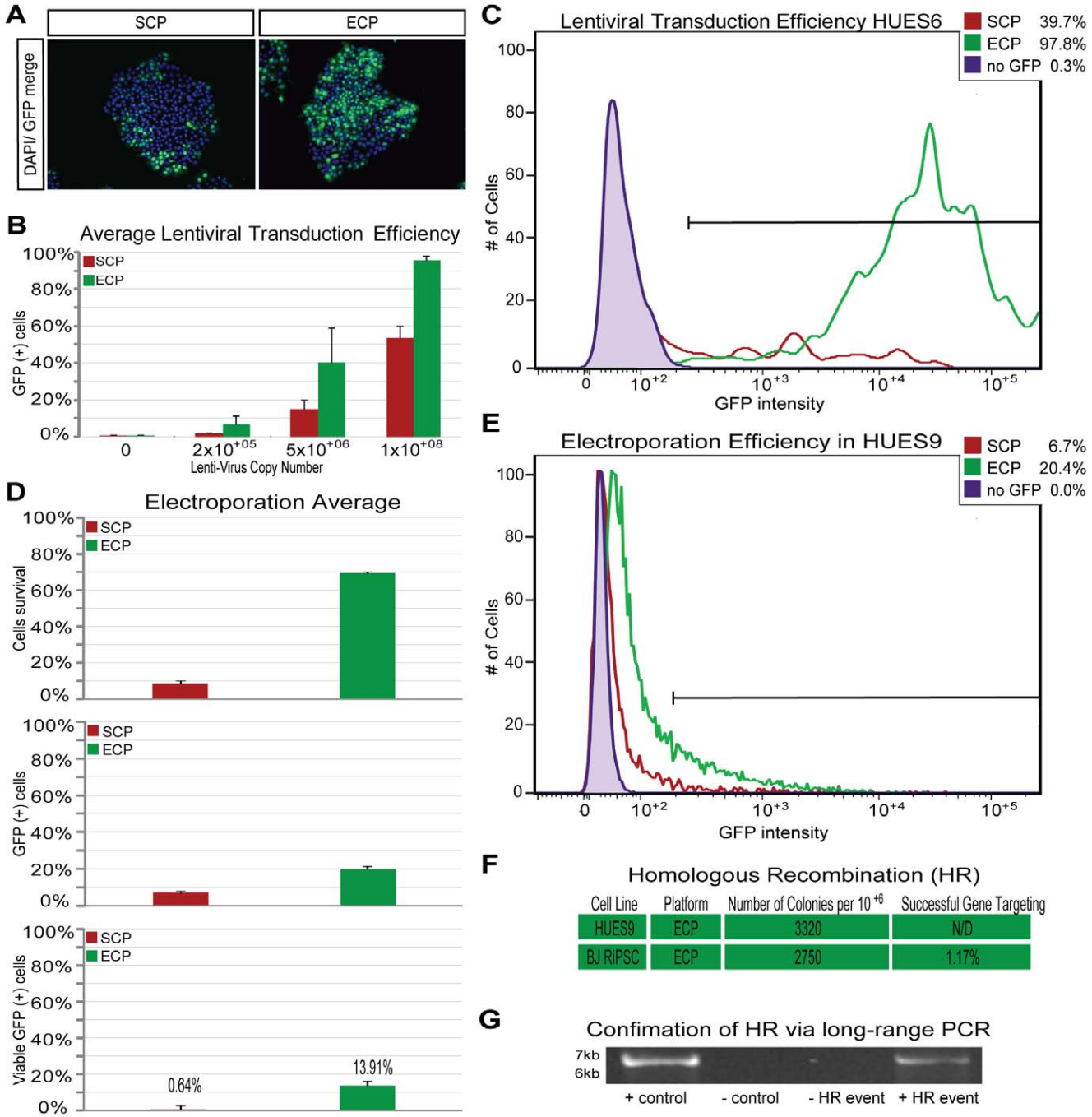

Figure 2. Comparing genetic manipulation efficiencies of hPSCs using either the standard (SCP) or the enhanced culture platform (ECP). A) Fluorescence image of Lentiviral transduction with an LV-GFP reporter indicate differences in efficiency and distribution of GFP+ cells (lentivirus copy number $5\times10^{+06}$ per 30.000cells). **B**) Quantification of lentiviral transduction efficiency via flow cytometry towards the LV-GFP reporter with various lentivirus copy numbers (30.000 cells per setup) in 4 different cell lines (standard error, $p = 0.03$; cell lines: HUES6, HUES9, CF RiPSC and BJ RiPSC). **C**) Representative flow cytometry plot for HUES6 lentiviral transduction efficiency. **D**) Average efficiency of an EP-GFP reporter plasmid delivered into the HUES9 line via electroporation. The top bars indicate the amount of cells surviving the procedure, the middle bars the percentage of cells that transiently express GFP measured via flow cytometry, and the bottom bar indicate the total percentage of viable GFP positive cell in regards to the initial cell number ($n = 3$ per platform, standard error). **E**) Representative flow cytometry plot for HUES9 cells expressing GFP after electroporation. **F**) The table indicates the amount of colonies available for screening after a single electroporation per 10^6 cells and subsequent antibiotic selection. **G**) Of these colonies, 85 were screened for successful gene targeting through homologous recombination via long range PCR.
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transduction rates of up to 95% and electroporation efficiencies of up to 25%, with a significant increase in the total number of antibiotic-resistant colonies. Thus, the growth, maintenance, and manipulation of hPSCs with the ECP significantly facilitates the ease of utilizing hPSCs and should ultimately facilitate many applications of hPSC research.

Methods

Ethics statement

All animal procedures were approved by the Massachusetts General Hospital subcommittee on research animal care under animal protocols 2009N000050. Mice were maintained at a barrier facility at the Centre for Regenerative Medicine, Massachusetts General Hospital under a 12 hr light/12 hr dark cycle at constant temperature (22°C) with free access to food and water. All efforts were made to minimize suffering.

Maintenance of pluripotent cells

hESCs and hiPSCs were cultured feeder free on Geltrex (Invitrogen) in the chemically defined medium mTESR1 (Stem Cell Technologies). ECP passage: hPSCs were dissociated with 20% Accutase (StemPro®) in PBS for 5–10 minutes at 37°C until the cells could easily be detached with pipetting. Accutase was removed and cells were replated in mTESR1 +0.4 μM ROCK-Inhibitor (Y-27632 Cayman Chemical). ROCK-inhibitor was removed 3–6 h after replating. SCP passage: hPSCs were dissociated by adding Dispase StemCell Tech, 15–20 mg/10 cm^2 plate and were incubated at 37°C for 3–5 minutes until colony borders started to detach. After repeated washing with DMEM cells were lifted with a cell scraper and the colonies manually disrupted by pipetting several times.

Quantifcation of the cell viability

To measure the cell viability of the replating efficiency, freeze/thawing and electroporation experiments, the cells were detached with Accutase 2–3 h after being replating. The cells were counted via Hausser Bright-Line Hemacytometer. All counting was done in triplets.

Immunocytochemistry

The following antibodies were used: Immunostaining- α-SOX2 (Abcam ab15830, 1/200), α-TRA1-81 (Millipore, MAB4381, 1/350), NANOG (Millipore, AB9220, 1/300), OCT3/4 (Abcam, ab19857, 1/300), and Alexa Fluor secondary antibodies (Invitrogen). DAPI stain was used in a 1:5000 dilution to mark cell nuclei. The Images were made either with a Nikon Digital Sight camera mounted to a Nikon Eclipse Ti-S microscope or a Olympus DP72 camera mounted to a Olympus 1×71 microscope. The software packages NIS-Elements and Olympus DP2-BSW were used for image analysis.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

hPSCs were lysed in buffer RLT (Qiagen) with 1% β-Mercaptoethanol and the total RNA purified via the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA yield was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and 500 ng of total RNA was further converted to cDNA using the Superscript First-Strand Kit (Invitrogen). Quantitative RT-PCR was performed using Quantifast-SYBR Green PCR mix (Qiagen) and the Realplex Mastercycler (Eppendorf) with 25 ng of total RNA per reaction. The reaction took place in the presence of Origene STAR qPCR primer pairs against OCT3/4 (HP206340), NANOG (HP215086) or NESTIN (HP209533).

Teratoma formation

For the teratoma assay, 5×10^6 cells of either HUES9 or BJ RiPSC were harvested, spun down, and all excess media was removed. In a 20-week old female SCID mouse, the capsule of the right kidney was gently elevated, and one droplet of concentrated cells was inserted under the capsule. At week 6, when adequate tumor size was observed, the tumor was harvested, fixed in 4% Paraformaldehyde, run through an ethanol gradient, and stored in 70% ethanol. Specimens were sectioned and H&E staining. Slides were imaged with a Leica light microscope.

Karyotyping

Cells were submitted to Cell Line Genetics for analysis.

Freeze/thawing

Cells were dissociated either through Accutase digest (ECP) or by Dispase digest (SCP), transferred to mFreSR® (Stem Cell Technologies) and immediately frozen down. After 2–3weeks in liquid N2 the cells were thawed and plated on Geltrex coated plates in either mTESR1 with 0.4 μM ROCK-Inhibitor (ECP) or mTESR1 (SCP).

Limiting dilution assay

The various cell lines were diluted to a concentration of 0.5cell/96well and two full 96well plates of each setup was prepared. After 7–10days the cells were fixated with 4% Paraformaldehyde, analyzed via Immunocytochemistry and screened for colonies expressing the pluripotency marker NANOG and OCT3/4.

Flow cytometry analysis

Cells were detached via Accutase digestion, and fixed for 20 minutes at 4°C in 2% Paraformaldehyde. The cells which were subjected immunocytochemistry were initially counted and 1×106 cells were transferred into polypropylene tubes. Staining for the TRA1-60 antigen was performed using an antibody conjugated to alexa fluor 488 (Biolegend, 330613) in 200 μl PBS with 5% FBS. The cells were counted with a FACSCalibur flow cytometer (BD Biosciences) and the data analyzed using the FlowJo software package (Treestar).

Colony size measurement and variability

Images of random parts of the plate were made either with a Nikon Digital Sight camera mounted to a Nikon Eclipse Ti-S microscope. Three days after replating of single cells, 20 pictures of random areas of the plates were taken and the colony sizes of 150 colonies of each platform were measured with the NIS elements BR 3.10 software.

Production of lentivirus and transduction

We used a third-generation, Tat-free packaging system [14] to produce recombinant lentivirus. The system consists of two packaging plasmids—pMDL, pREV—, the plasmid coding for VSV-G envelope and a vector for constitutive GFP under the CMV promoter (LV-GFP). All four plasmids were transfected into HEK293FT cells using calcium chlorate as previously reported [15] and the viral supernatant was collected 48 and 72 hours later. The supernatant was filtered through a 0.45 μm filter and the RNA copy numbers per ml determined using the Lenti-X qRT-PCR titration kit. For the transduction hPSCs were dissociated via ECP/SCP and replated. Immediately after reattaching, approximately 2 h after the replating, the lentivirus was added to the plate and incubated for 2 hours at 37°C. The viral supernatant was then removed, and cells were washed with DMEM and mTESR1 was added. 48 hours after transduction the cells were collected, fixed and analyzed for GFP expression via flow cytometry.

Electroporation

The Cells were detached with either Accutase or Dispase filtered through a 40 μm cell strainer (BD Falcon) and counted. 40 μg of linearized DNA (EP-GFP) were mixed with 3×10^6 cells in 800 μl PBS. An electric pulse was given in a 0.4 cm cuvette (Bio-Rad Gene Pulser®) with 320 V/200 μF [16]. The cells were recovered and plated on Geltrex coated plates in TeSR and 0.4 μM/ml ROCK-Inhibitor. Viability and efficiency was determined 24 h after electroporation via cell counting and flow cytometry.

Cell-lines

The following human embryonic stem cells lines were used for the experiments: HUES1, HUES2, HUES6, HUES8 and HUES9 all first described in Cowan et al., 2004. The following human induced pluripotent stem cell lines were used for the experiments: BJ RiPSC and CF RiPSC first described by Warren et al., 2010. The HEK293FT cell line used for Lenti-Virus production is distributed Invitrogen (R700-07).

Supporting Information

Figure S1 Long term culture of hPSCs with ECP dosn't effect pluripotency and Karyotype. HUES9 and BJ RiPSC cells were cultured 15 consecutive times with the ECP and analyzed for pluripotency via **A**) Immunohistochemistry with the indicated antibodies; nuclei were visualized with DAPI stain, via **B**) quantitive reverse transcription real time PCR for OCT3/4, NANOG and the negative control NESTIN a neural progenitor cell marker (normalized to HPRT)(n = 3), via **C**) Teratomaformation of HUES9 hESCs in immunodeficient SCID mice (black arrow top left indicates neural rosette; black arrow top right - pigmented epithelia, grey arrow top right gut-like epithelia; bottom left- muscle-like tissue; black arrow bottom right cartilage). **D**) Cell Karyotype of HUES9 hESCs after 19× passages.
(TIF)

Figure S2 The influence of ROCK-Inhibitor. A) The amount of cells reattached were determined 2 h after passage in a total of 5 cell lines either using the SCP or ECP each with and without 0.4 μM of ROCK-Inhibitor (n = 3 per cell line, standard error). **B**) Quantification of cells reattached after being frozen in liquid nitrogen for 7–10 d and subsequently thawed in the presence or absence of 0.4 μM of ROCK-Inhibitor (n = 3 per cell line, standard error). **C**) Determination of the clonogenic potential, through a limiting dilution assay on a total of 3 cell lines was performed with and without the addition of 0.4 μM of ROCK-Inhibitor (96 cell plated per cell line, standard error). Counted were only colonies with positive immune-fluorescence staining for the pluripotency markers OCT3/4 and NANOG.
(TIF)

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Author Contributions

Conceived and designed the experiments: RTS TA CAC. Performed the experiments: RTS TA YKL FHL AC TS DP. Analyzed the data: RTS CAC. Contributed reagents/materials/analysis tools: RTS. Wrote the paper: RTS TA CAC. Lenti-Virus transduction, Non-pluripotent cell abundance and size distribution: TA. Teratoma formation assay: FHL. Lenti-viral transduction efficiency: YKL. Immuno-staining pluripotency, QPCR pluripotency: AC. Size distribution, Lenti viral transduction setup figure 2a: TS. Elctroporation efficiency: DP. Plasmid design and vectors: DHL. Fluorescence pictures, Lenti-viral transduction: TS. qPCR pluripotency, immunofluorescence pictures pluripotency: AC. Determination of the Lenti viral titer: YKL. Evaluation of Lenit-viral transduction and non-pluripotent cell abundance FACS data: TA. Efficiency of electroporation: DP. For GFP-reporter constructs: DHL.

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