Switching Human Cell Fate:

Reprogramming via pluripotency to adipogenesis

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology at the

University of Hamburg (UHH)

Dissertation

Zur Erlangung der Würde des Doktors der Naturwissenschaften

des Fachbereichs Biologie, der Fakultät für Mathematik, Informatik und Naturwissenschaften,

der Universität Hamburg

---

by

Dipl. Biochem. Tim Ahfeldt

from Berlin

Hamburg 2011

Genehmigt vom Fachbereich Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Frau Prof. Dr. U. BEISIEGEL
Weiterer Gutachter der Dissertation:
Prof. Dr. T. BURMESTER
Tag der Disputation: 10. Dezember 2010

Hamburg, den 25. November 2010

Universität Hamburg
64
Fakultät

Professor Dr. Axel Temming
Leiter des Fachbereichs Biologie

Contents

1 Introduction

1.1 Mammalian Development

Development begins with the fertilization of an egg. During the development of an organism, specialized cells are formed by a one-way process (Gurdon and Melton, 2008). The developmental potential of any cell decreases while it becomes more committed to its fate. In the early mammalian embryo, totipotent cells are present and have the potential to differentiate and give rise to specialized cells that eventually form an entire organism. These totipotent cells also form extra-embryonic tissues and germ cells. In humans the early embryo takes the form of a blastocyst five days post fertilization. The blastocyst consists of an outer layer of cells called the trophoblast and the cells of the inner cell mass (ICM). The cells of the inner cell mass are able to give rise to cells from each of the three germ layers, the endoderm ectoderm and mesoderm.

These germ layers produce all specialized tissues of the body, made up of somatic cells. The ectoderm layer gives rise to the nervous system and many tissues such as the epidermis, hair, teeth and nails. The endoderm develops into the gastrointestinal tract, the respiratory tract, the liver and the pancreas. The mesoderm forms the heart, the skeletal muscles, connective tissue, the urogenital and the circulatory systems as well as blood and bones. Together, the three germ layers give rise to all cell types of the body. Because the inner cell mass cells give rise to the germ layers, and thus can become any cell type in the body as well as divide indefinitely, these cells are considered pluripotent.

1.2 Embryonic Stem Cells

1.2.1 The Derivation of embryonic stem cells

Mouse embryonic stem cells (mESC) are derived from the ICM of the pre-implantation mouse blastocyst (Evans and Kaufman, 1981). The successful derivation of human embryonic stem cells (hESC) by several groups (Akutsu et al., 2006; Cowan et al.,

2004) has permitted the use of hESCs as a tool to study human development at the cellular and molecular levels.

Because ESCs remain able to differentiate into any cell lineage, they allow the study of cell-specific diseases *in vitro*. Pre-implantation genetic diagnosis (PGD), using techniques like polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH), can identify monogenic disorders and chromosomal abnormalities in embryos generated by *in vitro* fertilization (IVF). Embryos possessing abnormalities are unsuitable for implantation and are generally discarded.

Several groups have derived disease-specific hESC lines from genetically abnormal embryos that were identified by PGD. For instance, Verlinsky et al. reported the creation of 18 hESC lines that represent diseases including adrenoleukodystrophy, Duchenne and Becker muscular dystrophy, Fanconi anaemia, Huntington disease, neurofibromatosis, and Marfan syndrome (Verlinsky et al., 2005). Pickering et al. reported the generation of a hESC disease line encoding the cystic fibrosis mutation ΔF508 (Pickering et al., 2005). At present only a handful of monogenic disorders can be identified by PGD. The genes associated with the vast majority of polygenic disorders remain unknown, preventing the identification of genetically abnormal embryos and thus the creation of hESC lines for these diseases.

1.2.2 Pluripotency and self-renewal of ESCs

Two key features of ESCs are pluripotency and self-renewal. Pluripotency is the ability to generate all cell types of the organism proper, and to divide indefinitely (Solter, 2006). One of the defining characteristics of an ESC line is the ability to differentiate into any cell type of the three germ layers. In practice, this is typically shown by the *in vitro* differentiation of the tested line into many different cell types. *In vivo,* this is shown by injecting the cells under the kidney capsule of immunocompromised mice, observing the formation of a teratoma, histologically analyzing the teratoma for tissues from all three germ layers. Another *in vivo* test available for mouse cells but not human cells is the injection of potential mESC into a blastocyst; a successful outcome of this test is the formation of a chimeric animal with the tested cells giving rise to tissues of all three

germ layers. By definition, ESCs should divide indefinitely this self-renewal is maintained in culture by preventing differentiation and promoting proliferation (Boyer et al., 2006; Niwa, 2007).

1.2.3 The transcriptional network governing pluripotency

Recently, the core network of transcription factors (TFs) that maintain ESC pluripotency was identified Three transcription factors, Oct3/4, Sox2, and Nanog, form this network. These factors do not exist in concert in any differentiated cell type, as such; the same TFs are also markers of pluripotent cells (Babaie et al., 2007; Mitsui et al., 2003; Nichols et al., 1998).

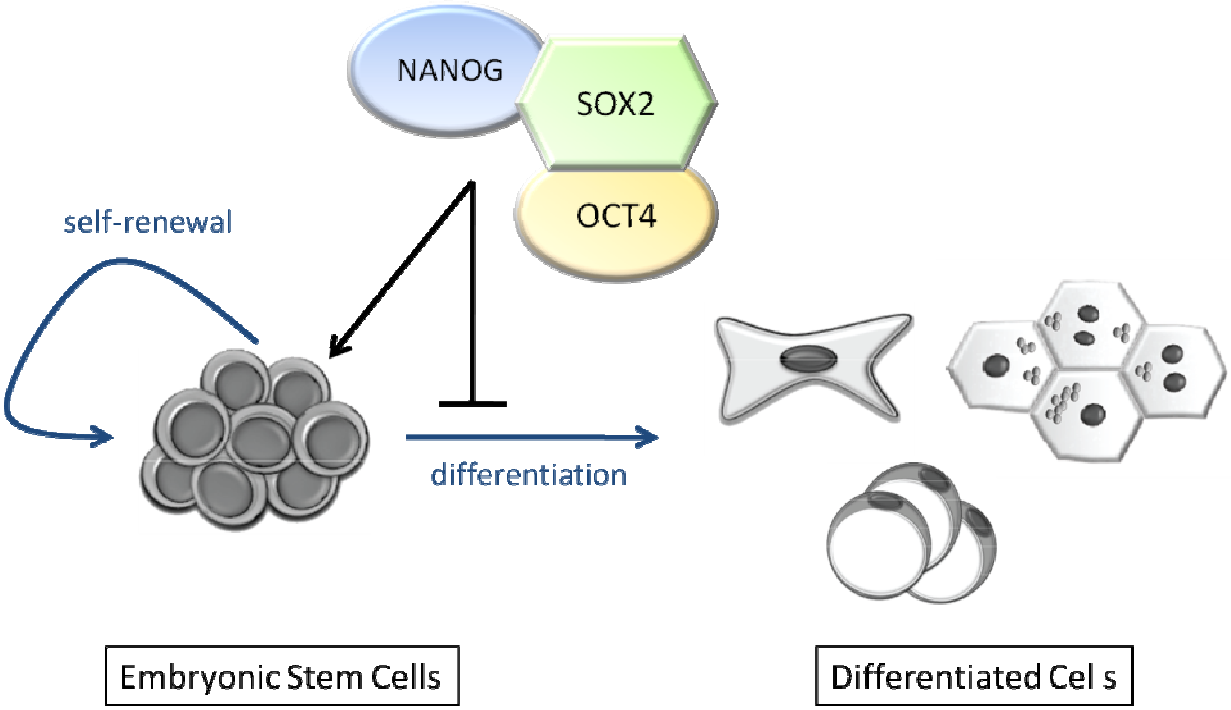


Figure 1-1. The embryonic stem cell switch. The three transcriptions factors Nanog, Sox2, and Oct4 act as a switch between pluripotent and differentiated states by maintaining self-renewal and inhibiting differentiation.

OCT4, SOX2, and NANOG have a unique expression pattern in ES cells and they play essential roles as master regulators of transcriptional control during early embryonic development (Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003).

1.2.4 Genetic modification of hESCs

Because hESC in principal can be differentiated towards the disease affected cell types, patient specific ESC could be extremely valuable for understanding human disease. In addition to the use of discarded IVF embryos it is possible to engineer disease gene containing ES cells. The first example of disease modeling hESCs were developed to study Lesch-Nyhan syndrome (Urbach et al., 2004). The pathophysiology of disease involves mutations in *HPRT1*, which is a key enzyme in the metabolism of purine bases. With nonfunctional or absent HPRT, these bases are degraded and excreted as uric acid, but the excess of uric acid leads to gout, urinary stones, and neurological disorders.

Using homologous recombination, a mutated *HPRT1* gene was introduced into hESCs. These cells displayed an overproduction and accumulation of uric acid as seen in Lesch-Nyhan patients. Of note, mouse strains carrying the *HPRT* mutations fail to display Lesch-Nyhan phenotypes or uric acid accumulation (Urbach et al., 2004), underscoring the need for human cell-based models of disease to accurately recapitulate human disease.

A different approach was taken to model Parkinson's disease in hESC-derived dopaminergic neurons. Capowsky and colleagues (Capowski et al., 2007) constitutively overexpressed a rare autosomal dominant mutation in α-synuclein. The mutant α-synuclein induced acute cytotoxicity and reduced the number of cells expressing markers of dopaminergic neurons such as tyrosine hydroxylase and gamma-aminobutyric acid (GABA). The observed cytotoxicity and the reduced marker expression recapitulate the events seen during the progression of Parkinson's disease.

A new approach to target hESCs is homologous recombination facilitated through target-specific zinc-finger nucleases (ZFNs). Engineered ZFNs that induce a sequence-specific double-strand break in a target DNA sequence increased homologous

recombination at that site by >1400-fold (Zou et al., 2009). This new technology has broad implications for gene therapy strategies as well as for disease modeling, and it will enable protocols by which researchers can use ZFNs to efficiently create or correct specific mutations at their genes of interest in hESCs. Nonetheless, at the present time it remains technically challenging to alter hESCs by homologous. This difficulty and the complexity of many diseases underscore the need to develop alternative means of obtaining disease-specific human pluripotent stem cell models.

1.3 Reprogramming Cells to Pluripotency

Prior to the 1950s, development was viewed as a unidirectional process. For totipotent and pluripotent cells to transform into differentiated cells, some unknown process caused them to lose their potential for becoming other cell types. This process was thought to be irreversible.

In the 1950s, amphibian studies overturned this idea by providing proof for the nuclear equivalence of cells. These studies showed that adult nuclei retain the capacity to generate cells of an entire organism after exposure to factors present in an oocyte (Briggs and King, 1952; Gurdon, 1962; Gurdon and Uehlinger, 1966). These experiments raised the possibility that any differentiated cell could be restored to a pluripotent state which, in turn, could re-differentiate into any cell type.

1.3.1 Reprogramming by somatic cell nuclear transfer

In somatic cell nuclear transfer (SCNT), a donor somatic nucleus is introduced into an oocyte that has been enucleated. The enucleated oocyte cytoplasm reprograms the somatic nuclei to an embryonic state. If this reprogrammed cell is allowed to divide it will develop into an embryo which will be a genetic clone of the original donor somatic cell. If the embryo is transplanted to a uterus and survives until birth this is reproductive cloning. The production of an embryo through SCNT and its use in the derivation of embryonic stem cells is termed therapeutic cloning. Neither reproductive nor therapeutic cloning has occurred in humans, but both are common and used in a variety of powerful tools in mice.

To show that the cells generated by SCNT are totipotent, this technique has been used to clone animals such as Dolly the sheep, demonstrating that the epigenetic state of the somatic cells can be restored to an embryonic state and that the resulting cells are totipotent (Wilmut et al., 1997). Mouse ESC lines have been derived using SCNT, but the technique has yet to be successfully applied to human cells.

While the characteristics of these cells makes them wildly useful in developing tools for understanding disease and biology several factors still limit the use of human SCNT. These include low efficiency, ethical concerns raised by the necessity for human eggs, the health risks tied to harvesting eggs, the necessity and health risks for human surrogates, and the paucity of available starting material.

1.3.2 Reprogramming by cell fusion

Oocyte cytoplasm has the capability to reprogram a somatic nucleus. Human eggs are in short supply. The egg, when fertilized with sperm, is totipotent, and embryonic stem cells are pluripotent. The hypothesis that ES cells could reprogram a somatic nucleus soon offered a potential route around the roadblocks of human SCNT. In fact, cell fusion experiments showed ESCs have the ability to reprogram somatic cells to a pluripotent state (Cowan et al., 2005; Tada et al., 1997).. Upon the fusion of an embryonic stem cell with a somatic cell, the somatic nucleus undergoes reprogramming to an undifferentiated state, resulting in the activation of silent pluripotency genes like *OCT4* and *NANOG* and the reactivation of inactive X-chromosomes The hybrid hESCs produced by cell fusion are one more tool that may allow for biochemical and genetic studies aimed at understanding nuclear reprogramming. However, the tetraploid DNA content of these cells makes them unsuitable for either disease modeling or customized cell therapy.

1.3.3 The discovery of induced pluripotent stem cells

Before the experiments of Gurdon it was thought that an irreversible change occurred in cells as they became more specialized. Indeed, there is currently no evidence in developmental biology of development moving backwards, or cells acquiring potential for becoming other cell types. Through the creation and characterization of embryonic

stem cells through SCNT and cell fusion, scientists were able to better understand the changes that occur in the nucleus upon reversal of potency. It was now understood that there existed a core transcriptional network and master regulatory factors for that network that, when perturbed, may be sufficient in reprogramming a differentiated cell to a pluripotent state. Further, the great hope for embryonic stem cells was that they could ultimately be used directly in therapeutics; however host versus graft disease remained a huge complicating factor. To be able to induce a patient's own cells to become pluripotent would have immense research and therapeutic potential. In 2006, Takahashi and Yamanaka achieved a breakthrough in the field of nuclear reprogramming (Takahashi and Yamanaka, 2006). Mouse embryonic fibroblasts (MEFs) were reprogrammed to a pluripotent state by the viral transduction of four transcription factors Oct4, Sox2, c-Myc, and Klf-4 (Maherali et al., 2007a; Okita et al., 2007; Wernig et al., 2007). Cells reprogrammed in this way are termed induced pluripotent stem (iPS) cells. A series of studies have shown that iPS cells can be generated from human somatic cells (Park et al., 2008c; Takahashi et al., 2007; Yu et al., 2007a), that they have a normal complement of chromosomes, and that they are phenotypically indistinguishable from ESCs derived from human embryos.

1.3.4 A secondary system to study reprogramming

The derivation of iPS cells remains a very inefficient process. In order to study the mechanisms of reprogramming, a more efficient system would be useful. Accordingly, we have developed a system to generate primary iPS cells using doxycyline inducible lentiviruses. The derived iPS cells can be differentiated into any cell type, and upon administration of doxycyline can be reprogrammed again to form secondary iPS cells at a much higher efficiency (Maherali et al., 2008). This allows for the study of reprogramming mechanism and kinetics in a much more defined way.

1.3.5 Advances in the derivation of induced pluripotent stem cells

Other studies have demonstrated that although the oncogene c-Myc increases the efficiency of iPS cell induction, it is not necessary for nuclear reprogramming (Nakagawa et al., 2008). There still exist several barriers to the use of human iPS cells

for cell replacement therapy. For instance, the introduction of Oct-4 poses a risk as it has been shown to act as a strong oncogene when expressed in somatic cells (Hochedlinger et al., 2005). Similarly, Klf4 and Lin28 have been linked with oncogenesis (Daley and Scadden, 2008). In addition, the viral integration of these transcription factors randomly into the genome may cause deleterious mutations, making these cells unsuitable for cell therapy.

Currently, efforts are directed at overcoming these obstacles, including the use of small molecules to replace the transcription factors. For instance, a small molecule known to be a TGF-β inhibitor was found in a chemical screen to replace the transcription factor SOX2 during reprogramming (Ichida et al., 2009).

Stadtfeld et al. demonstrated that genomic integration was not essential to successfully reprogram mouse fibroblasts, by using adenoviruses to supply cDNAs encoding reprogramming factors (Stadtfeld et al., 2008c); subsequently, this was demonstrated in human fibroblasts through the use of adenoviruses or Sendai viruses (Fusaki et al., 2009; Zhou and Freed, 2009). Kim et al. were able to deliver reprogramming factors with modified proteins that can cross the cell membrane (Kim et al., 2009b). However, this reprogramming procedure takes almost two months, rather than the one month typically required for a virus protocol. To date, the use of proteins or non-integrating viruses has served only as proof-of-principle experiments, since the efficiency of these approaches is extremely low and the techniques are technically challenging.

Other groups have successfully used nucleic acids that do not integrate into the recipient genome to make iPS cells. Yu et al. (Yu et al., 2009a) demonstrated that human iPS cells could be generated from a single nucleofection of three oriP/EBNA1 (Epstein-Barr nuclear antigen-1)–based episomal vectors, and other groups have shown that iPS cells can be generated by simple transient transfection of plasmid DNA encoding the reprogramming factors (Si-Tayeb et al., 2010). However, all transfection methods that utilize DNA carry a risk of random integration into the genome, and clones would need to be screened for sites of integration to assure safety before therapeutic use.

1.3.6 Reprogramming by serial transfection of RNA

Despite recent advances, integration-free iPS cell derivation remains difficult and in practice is rarely done as it is technically challenging, laborious and inefficient. Accordingly, we have developed a system to reprogram somatic cells to pluripotency through the use of modified RNAs in combination with interferon response inhibition. This technique has emerged as an efficient, easy and safe method to generate iPS cells and has the potential to develop into the standard method of iPSC derivation.

1.3.7 Disease modeling using induced pluripotent stem cells

Over the last two decades there has been significant progress toward a molecular understanding of human disease. Despite this progress, an understanding and ability to treat many of the most common human diseases has remained elusive. Genetic linkage studies have been successful in identifying loci contributing to single-gene Mendelian (monogenic) diseases. Over 95% of the disease genes identified to date contribute to Mendelian diseases (Botstein and Risch, 2003; Glazier et al., 2002). Unfortunately, many prevalent diseases such as Parkinson's disease, obesity, bipolar disorder, coronary artery disease and type 2 diabetes mellitus exhibit both polygenic and environmental components (Kingsmore et al., 2008; Martin et al., 1997). Only recently, with the advent of genome-wide association studies have the genes contributing to polygenic diseases begun to be identified. Due to interactions between multiple genes and the environment, it has been particularly difficult to develop accurate cellular models for these multigenic or complex forms of human disease. Further complicating studies, biopsied tissue containing the affected cell type is often extremely limited. Moreover, the developmental or pathological events leading to the disease usually occur long before diagnosis. The derivation of hESCs and iPS cells and the proposed medical benefits from utilizing these pluripotent cells has garnered enormous attention in academia and the public view.

Using readily available tissue from patients we and others have generated several cell lines of monogenic and complex disease (Park et al., 2008b). The list of cell lines generated from diseased patients include adenosine deaminase deficiency-related

severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne (DMD) and Becker muscular dystrophy (BMD), Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21, and the carrier state of Lesch-Nyhan syndrome. New iPS cell disease lines are being generated in rapid succession, and several groups have begun differentiating the cells into the cell type of disease relevance.

For example, amyotrophic lateral sclerosis (ALS) is a progressive, neurodegenerative disease in which motor neurons are lost, leading to loss of body muscle and death due to respiratory failure (Boillee et al., 2006). Eggan and colleagues have generated iPS cells from patients that suffer from ALS, followed by differentiation of the disease iPS cells into motor neurons (Dimos et al., 2008). However, the authors were not able to show a distinct phenotype in their derived motor neurons, perhaps not surprising given the long course of the disease in patients and contextual complexity *in vivo*.

More promising was the work of Lee et al in modeling the pathogenesis and potential treatment of familial dysautonomia (FD) (Lee et al., 2009). FD is a rare but fatal peripheral neuropathy caused by a point mutation in the gene *IKBKAP*. The authors demonstrated tissue-specific mis-splicing of *IKBKAP* in disease iPS cells *in vitro* and were further able to demonstrate defects in neurogenic differentiation and cellular behavior. The authors went on to show that drugs that have been reported to modulate splicing could be used to reverse the discovered phenotype, providing an important proof-of-principle that iPS cell technology can be useful in drug testing *in vitro*. Thus, using iPS cells as a tool represents a major step forward in disease modeling and has the potential to greatly facilitate both drug discovery and our understanding of the molecular and cellular basis of disease physiology.

1.3.8 iPS cells and the promise of regenerative medicine

In an effort to demonstrate the therapeutic utility of iPS cells, a proof-of-principle experiment was conducted to show the feasibility of gene therapy followed by cell replacement therapy (Hanna et al., 2007). Somatic fibroblasts from a mouse carrying a

mutant allele causing sickle cell anemia were used to create iPS cells. The genetic defect was repaired by homologous recombination, and the cells were differentiated into hematopoietic precursors. The donor mice were irradiated, and the genetically repaired hematopoietic precursors were transplanted. Transplanted cells were able to repopulate the mice with normal blood cells, thereby curing the animal of anemia. This approach to regenerative medicine may well represent a major future application of human iPS cells.

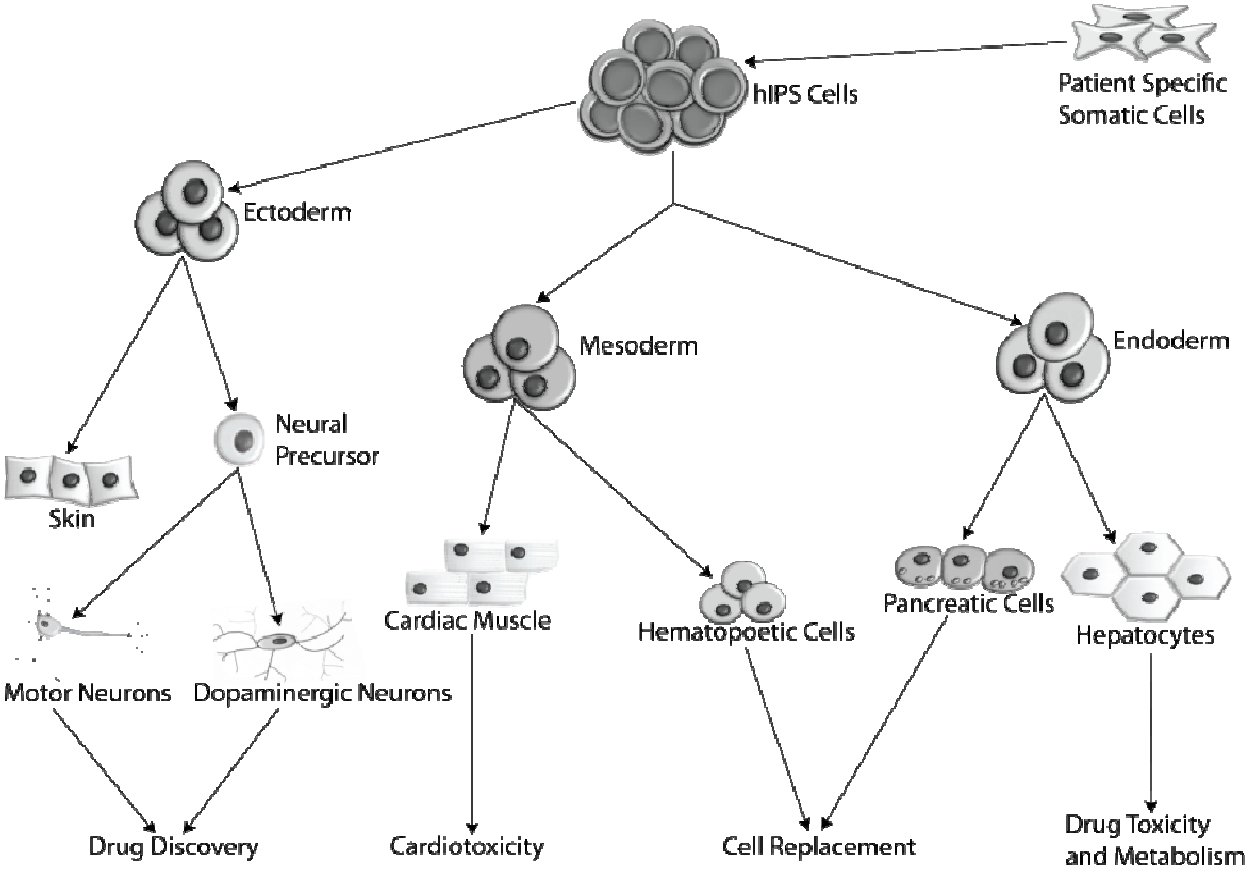


Figure 1-2. iPS cell differentiation in culture. Schematic drawing showing the reprogramming of patient-specific fibroblasts into human iPS cells. These iPS cells can be differentiated to all three germ layers, ectoderm, endoderm and mesoderm. Clinically relevant cell populations can be obtained from each germ layer. Ectoderm differentiation to skin, neural precursor cells and, subsequently, motor neurons and dopaminergic neurons is shown. Mesoderm can give rise to cardiac muscle and hematopoietic cells. From the endoderm germ layer, pancreatic β-cells and hepatocytes can be derived.

Finally, possible applications of these cells are outlined, namely their use in drug discovery, screening for cardiotoxicity, cell replacement therapy, and tests for drug toxicity and drug metabolism.

1.3.9 Ethical implications of using human pluripotent stem cells

The derivation of hESCs raises important ethical issues because it requires the destruction of human embryos. In contrast, human iPS cells are derived from available cell populations such as fibroblasts and keratinocytes from individuals, with the harms limited to those associated with skin-punch biopsies and hair pulls. Human iPS cells have been shown to be highly similar (though perhaps not identical) to human embryonic stem cells on a molecular level, and provide a compelling alternative for scientific research. Furthermore, the demonstrated creation of patient-specific iPSC lays the foundation for studying and directly treating human disease. In 2009 the United States government developed guidelines that ensure only embryonic stem cells derived from embryos that were “donated under ethically sound informed consent processes “ are approved for federal funding. We have chosen to work with the approved Harvard University embryonic stem cell (HUES) lines and a variety of human iPS cells for these reasons.

Primary human tissues, as well as the iPS cells and ADSV cells derived from these tissues, will come from anonymous patients with appropriate patient consent via a protocol approved by the MGH Institutional Review Board.

1.4 Differentiation of pluripotent cells into clinically relevant cell populations

In most cases, a disease-associated genotype will not manifest as a disease phenotype in cells in the pluripotent or multipotent state, but rather in specialized, differentiated cells that harbor a transcriptional and proteomic context in which the disease gene or genes impact the state of the cell. A very challenging task in the use of pluripotent cells is to direct them to become specialized terminally differentiated cells.

1.4.1 Differentiation of pluripotent cells by recapitulating *in vivo* events

By recapitulating *in vivo* development, researchers have been able to direct ESCs into a number of specialized cell types. Already, scientists have turned towards using these protocols to model development and disease in embryonic stem cells and more recently in iPSC. Despite this progress the vast majority of the greater than 200 known cell types in the human body have not been directly made in a dish.

One example of the utilization of hESC and iPS cell technology is differentiation into β-cells of the pancreas. The pancreas is a complex endocrine organ that is home to insulin-secreting β-cells located in the islets of Langerhans. In patients with type 1 diabetes mellitus, there is an autoimmune response that results in the destruction of β-cells, a lethal condition if not treated. This form of diabetes can be treated, albeit temporarily, by the transplantation of β-cell-containing islets harvested from donors (Lakey et al., 2006). However, the constant shortage of islets for transplantation demands an alternative source of β-cells. In 2006, D'Amourr et al. reported the successful differentiation of hESCs into endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin (D'Amour et al., 2006). The differentiation protocol mirrors major steps in *in vivo* development via a five-stage protocol. Each stage is defined by the addition of a unique combination of signaling molecules. Following differentiation, the cells release C-peptide in response to multiple secretory stimuli, and the insulin content of the derived cells is close to that of adult islet cells *in vivo*. In 2008, the same group reported the implantation of these hESC-derived pancreatic islet cells into mice (Kroon et al., 2008). These transplanted cells exhibited many properties of functional β-cells, such as the expression of insulin and β-cell transcription factors. This proof-of-principle experiment demonstrates that hESCs have the capacity to generate cells that can be functionally integrated into a living organism, and the experiments represent another important step towards cell replacement therapies, though obstacles remain before the cells can be used in clinical trials. An alternative therapeutic approach entails the use of these differentiation protocols in combination with patient-specific iPS cells to create cell models with which to study the degenerative processes in vitro and to search for

compounds to stop disease progression. However as noted before well-defined differentiation protocols that lead to significant yields of the desired cell population are uncommon.

1.5 Obesity

1.5.1 Relevance of Obesity

Obesity substantially increases the risk of developing a number of diseases such as cardiovascular disease and type 2 diabetes mellitus (Kopelman, 2000), and the American Heart Association (AHA) has classified obesity as a “major, modifiable risk factor” for coronary heart disease (Eckel et al., 2006). Obesity is a physical state in which natural energy reserves, stored in the adipose tissue of humans and other mammals, are increased to a point where they are associated with adverse health effects and mortality. Obesity is influenced by environmental, genetic, and behavioral components. It is the second leading cause of preventable death in the U.S. (Ogden et al., 2002). Adipose tissue secretes exocrine mediators that lead to endothelial dysfunction and atherosclerosis (Van Gaal et al., 2006). Increased triglycerides, decreased high density lipoproteins (HDL) levels and abnormal low density lipoproteins (LDL) composition characterize the primary dyslipidemia related to obesity and no doubt play a major role in the development of atherosclerosis and cardiovascular disease in obese individuals (Howard et al., 2003). An accurate understanding of adipose biology is necessary to more effectively prevent obesity and accompanying metabolic dysfunction.

Adipocytes are categorized as white and brown and they play distinct roles in the body. Brown adipose tissue (BAT) is a specialized tissue generating heat through non-shivering thermogenesis. These cells are rich in mitochondria, they exhibit multiloccular fat depots and in general contain much less lipids than white adipocytes (Lowell and Flier, 1997). White adipocytes contain fewer or a single large lipid filled organelle and White adipose tissue (WAT) in different body depots can have different functions. Variations in fat distribution in humans are correlated with metabolic disorders (Gesta et al., 2007). Transcriptional profiling of different human fat depots reveals transcriptional

differences that persist even after culture (Tchkonia et al., 2007).WAT plays a central role in energy homeostasis, and it act as an integrator of various physiological pathways (Rosen and Spiegelman, 2006). Adipocytes store and release energy and regulate the balance of nutrients in the blood. Through the release of adipokines such as leptin and adiponectin, adipose tissue communicates with other regulators of energy homeostasis like the central nervous system, pancreas, and liver (Kadowaki and Yamauchi, 2005; Pelleymounter et al., 1995; Rosen and Spiegelman, 2006). It is extremely difficult to dissect the role of one part of this complex system in an organism due to the number of players.

The storage of fat is the primary biological function of WAT, and excess of fat does not automatically lead to a disease state. It is mainly the dysregulation of multiple aspects of metabolism leading to the so-called “metabolic syndrome” that manifests itself in a disease phenotype (Despres and Lemieux, 2006).

In particular, obesity has been closely associated with insulin resistance and the development of type 2 diabetes mellitus (Kahn et al., 2006). High concentrations of circulating fatty acids and triglycerides, observed in both obesity and lipodystrophy, are thought to cause muscle insulin resistance and decreased glucose tolerance. This presents a significant obstacle for any treatment of obesity that involves the reduction of fat mass. Any treatment should result in the decrease of adipose tissue with an accompanying increase in energy expenditure to avoid the development of insulin resistance or type II diabetes mellitus.

As with other diseases, cellular models may provide a means to discover and investigate candidate drugs. The rational manipulation of adipose physiology and the use of *in vitro* derived adipocytes for drug screening is a promising avenue towards understanding obesity and, ultimately, developing therapies of associated pathological conditions such as cardiovascular disease and stroke.

1.5.2 Obesity as an in vitro model, current limitations

Ideally, cellular models of disease development should recapitulate the disease genotype and phenotype and should be available in necessary quantities to allow for

complete, rigorous studies. While human cell models and animal models have been powerful tools to study monogenic diseases, both have been limited in their ability to mimic polygenic diseases like the metabolic syndrome. Clearly, improved models of complex disease are required if biomedical research is to make progress towards the prevention and treatment of many of the most common human diseases.

Adipose tissue from patients is a readily obtained primary cell type but is very difficult to maintain in culture and impossible to expand with current methods. As a consequence, in vitro systems for understanding mature primary adipocyte function as it relates to obesity and metabolic disease do not exist. While mouse models for obesity exist, they represent rare single gene deletions that often do not adequately recapitulate human disease processes, in part due to differences between mouse and human metabolism.

With the establishment of the murine 3T3 cell culture system, adipocytes have become a popular model for the study of cell differentiation and transcriptional regulation (Green and Kehinde, 1975). The derivation of multipotent mesenchymal stem cells (MSCs) from bone marrow (Pittenger et al., 1999) and other tissues such as adipose tissue (Zuk et al., 2001) has enabled the differentiation of these cells into adipocytes, chondrocytes, osteoblasts, myoblasts, and connective tissue. The most basic characteristic marker shared by these lines is surface expression of CD73. Major limitations for the clinical translation of MSCs are the limited proliferative potential and the decreasing differentiation potential over time (Barberi and Studer, 2006), as well as the difficulty of modifying these cells by homologous recombination.

1.5.3 Using hESCs and iPS cells for differentiation into adipocytes

In the public view, the most anticipated use of hESCs and iPS cells is their application for cell replacement therapy and regenerative medicine. So far there have been no clinically approved uses of hESCs due to problems including: tissue histoincompatibility, the tumorigenic nature of hESCs, and the lack of differentiation protocols. Yet the use of hESCs as a tool to study human development at the cellular and molecular level can be undertaken immediately. hESCs and iPS cells self-renew and can be propagated indefinitely, and thus the necessary cell quantities for any kind of experiments can be

generated. They are pluripotent and so retain the potential to undergo differentiation into all cell lineages and any adult cell type and provide a unique opportunity to study cell type-specific development in vitro. Furthermore, patient- or disease-specific pluripotent cell lines have the potential to model the polygenic traits of complex diseases. Even though there exist published data on the differentiation of adipocytes from human pluripotent stem cells, these are largely proof of the ability of the pluripotent cells to convert into adipocytes in vitro; the protocols lack efficiency and the reports lack thorough characterization of the adipocytes (Dani, 1999; van Harmelen et al., 2007; Wolvetang and Hannan, 2008; Xiong et al., 2005).

The lack of efficiency is mainly caused by a lack of understanding of the developmental origins of adipocytes, which can be found in many different fat depots all through the human body and likely do not share the same developmental precursor cells. Adipose tissue is widely regarded as a mesodermal tissue (Gesta et al., 2007), but precise lineage-tracing studies have yet to be conducted and, in fact, ESC-derived neural crest cells of the ectodermal lineage can be differentiated into adipocytes. Other connective tissues and bone in the head have also been shown to be derived from the neural crest (Billon et al., 2007). To increase the efficiency of differentiation protocols, an alternative approach is the initial differentiation of pluripotent cells into multipotent mesenchymal precursors (MPCs), which can then be expanded to large numbers (Barberi et al., 2005; Olivier et al., 2006). When incubated in an adipogenic medium containing insulin, 3-Isobutyl-1-methylxanthin, and dexamethasone (Pittenger et al., 1999), these MPCs can be further differentiated into adipocyte-like cells, and several adipogenic markers including peroxisome proliferator-activated receptor γ2 (PPARγ2) can be detected at this stage. However, the efficiency of differentiation varies greatly between different MPC lines and falls short of the efficiency of differentiation of primary MSCs.

1.5.4 The transcriptional network that controls adipocyte identity

While the initial molecular events of adipogenesis remain unclear (Rosen and Spiegelman, 2000), it is widely accepted that the transcription factor PPARγ is the “master regulator” of terminal adipocyte differentiation. PPARγ and C/EBPα act in a self-regulatory feedback loop and activate the downstream expression genes involved in

terminal adipocyte differentiation. This transcriptional cascade has been studied and dissected extensively, mainly in 3T3-L1 cells (Rosen et al., 2002).

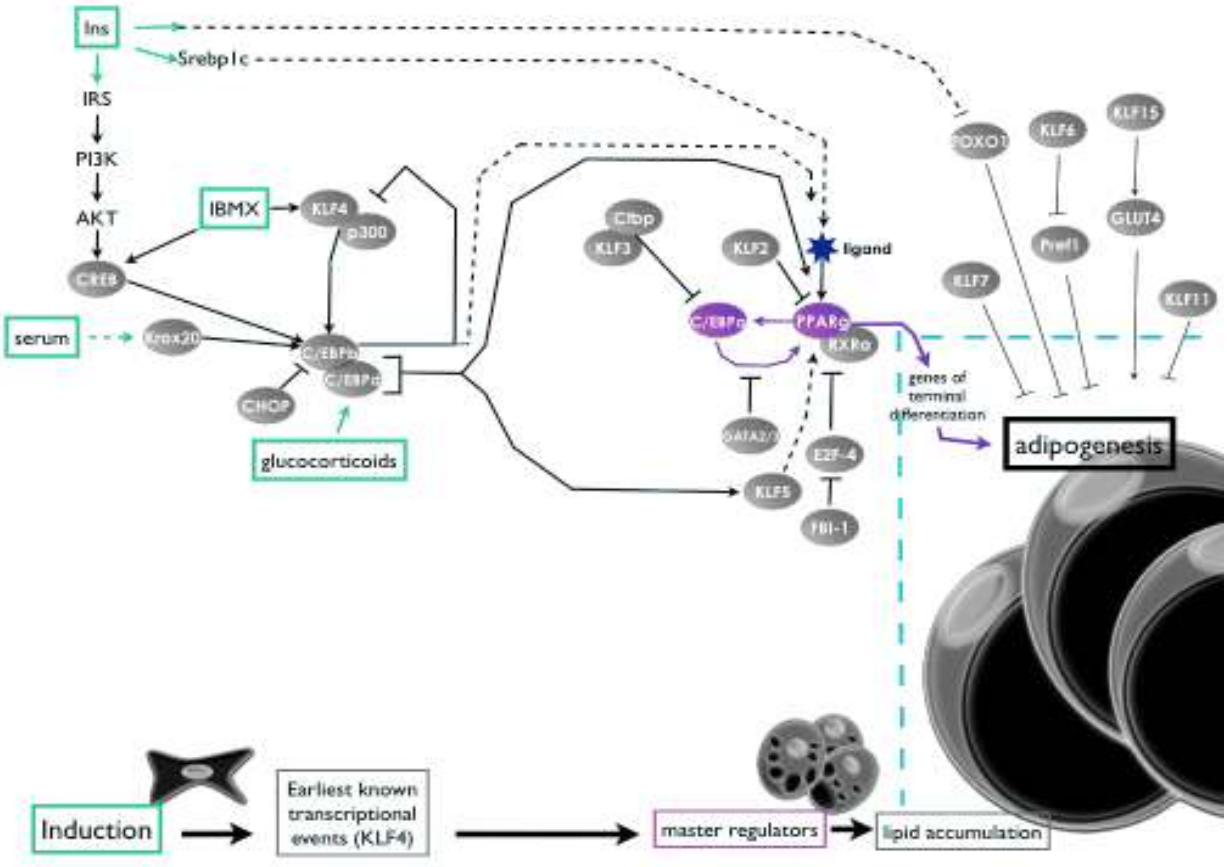


Figure 1-3. Core transcriptional control of adipogenesis. Cells treated with mitogens and hormones undergo a well-characterized series of transcriptional events. Several signals culminate in C/EBP*β* and C/EBP*δ* activation of PPAR*γ* and C/EBP*α* transcription. PPAR*γ* and C/EBP*α* then co-activate each other and a myriad of genes affecting processes of adipocyte differentiation and function. Figure taken from (Cook, 2009).

Many of these factors—PPARγ2, Krüppel-like factor 15 (KLF15), KROX20, cAMP response element-binding protein (CREB), sterol regulatory element binding protein-1c (SREBP1c), CCAAT/enhancer-binding protein alpha (C/EBPα), CCAAT/enhancer-binding protein beta (C/EBPβ), CCAAT/enhancer-binding protein delta (C/EBPδ), and

GATA binding protein 2 (GATA2)—are candidate regulators that may facilitate adipose differentiation when ectopically expressed in pluripotent cells.

1.6 Background information on techniques used in the study

1.6.1 Construction of Lentiviral vectors

The inducible virus system is based on the Tet-on system using reverse tetracycline-controlled transcriptional activator M2 (rtTA-M2). One vector expresses rtTA-M2 under the control of the ubiquitin promoter. All other cDNAs are expressed under the control of the tetracycline response element (TRE) promoter. Doxycycline administration activates the reverse Tet repressor, resulting in an activation of gene expression through the TRE promoter.

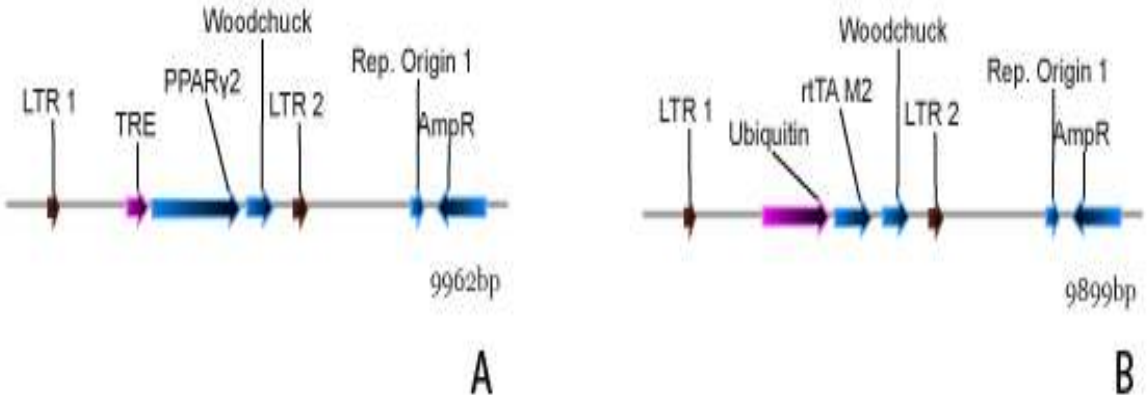


Figure 1-4. Vector maps of the lentiviral constructs. A: TRE-PPARγ2 based on FUGW vector; B: ubiquitin rtTA M2

The reprogramming vectors were constructed as previously described (Stadtfeld et al., 2008b), and PPARγ and other differentiation vectors were constructed by Gateway cloning after inserting a gateway recombination site into the TET-FUGW backbone.

Table 1-1. List of cloned lentiviral vectors

TET-O-cDNA	OCT4/POU5F1	SOX2	cMYC	KLF4	NANOG
	PPARy2	C/EBPa	C/EBPb	C/EBPd	SREBP1c
	KROX20	KLF15	CREB1	GATA2	
	eGFP	nuclear eGFP	nuclear mCherry		
Ubiquitin-cDNA	rtTA-M2				

2 Aims

The objective of this thesis is to create a functional and robust model that allows the investigation of the role of fat cells (adipocytes) in obesity. To that end I laid the foundation to study adipocytes from patients with distinct (disease) genotypes by creating reliable protocols for the derivation of hiPS cells. Using this protocol I sought to create a highly efficient differentiation protocol to generate adipocytes from pluripotent cells (hES/hiPS).

2.1 Aim 1: To generate human induced pluripotent stem cells from somatic cells.

I began working on reprogramming shortly before a series of papers was published showing that reprogramming of human somatic cells was possible using the 4 Yamanaka factors OCT4, SOX2, cMYC and KLF4 or very similar cocktails (Park et al., 2008c; Takahashi et al., 2007; Yu et al., 2007a). Viral silencing caused problems with efficiency in the protocols of that time, and further control over the length of time transgenes were expressed was impossible. I planned to overcome these obstacles by creating an inducible system for reprogramming. Doxycycline administration leads to transgene expression in my system and the transgene can be silenced by removal of doxycyline. I also hypothesized that reinduction of the transgenes through the administration of doxycycline in a differentiated primary hiPS cell would allow for the derivation of secondary iPS. This tool would allow for investigation of the mechanisms that facilitate reprogramming. We planned to analyze our derived cells with the most stringent tests available and compare them to hES cells.

2.2 Aim 2: To generate integration free human induced pluripotent stem cells using modified RNA

Despite advances in the field, many derived iPS lines exhibit incomplete silencing of the introduced transgenes. We hypothesized that modified RNA could be used to reprogram human cells to pluripotency and these cells would be completely free of residual transgene expression avoiding the risk associated with insertional mutagenesis

as well as the residual expression of oncogenes. Reprogramming is a very inefficient process and it is even more inefficient when viral free methods are employed. As it is easy to transfect human cells with RNA and the technology allows for a very precise titration of the individual reprogramming vectors we set up to generate a more efficient viral free protocol that has the potential to become widely used. This would be one important step before iPS cells could be considered candidates for cell replacement therapies.

2.3 Aim 3: To differentiate human pluripotent stem cells into adipocytes

As primary adipose tissue is difficult to maintain and cannot be expanded in culture conditions. Current *in vitro* models of obesity like 3T3-L1 cells do not adequately recapitulate normal or disease state adipocyte function or have other limitations like the senescence occurring in adipose derived stromal vascular cells

One of the critical problems that I faced when trying to differentiate pluripotent and multipotent human cells into adipocytes using standard methods was the extreme variability of the differentiation potential between lines. I hypothesized that that overexpressing transcription factors known to play a role in adipogenesis could differentiate pluripotent and multipotent cells into functional adipocytes with a high efficiency. This strategy would provide a renewable source of adipocytes and overcome the limitations of working with primary adipose tissue. I aimed to show that the derived cells become transgene independent. I further planned to analyze the cells rigorously using molecular, genetic, and cell biological analyses and hoped to develop a novel approach towards fighting obesity.

3 Results

In chapter 4 the results of the following three manuscripts will be presented along with additional data.

1.) **A high-efficiency system for the generation and study of human induced pluripotent stem cells.** Cell Stem Cell 3, 340-345.

Maherali, N.[*], **Ahfeldt, T.**[*], Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. (2008).

[*] These authors contributed equally as first authors

2.) **Highly efficient reprogramming to pluripotency and directed differentiation of human cells using synthetic modified mRNA.** Cell Stem Cell (in press)

Luigi Warren, Philip D. Manos, **Tim Ahfeldt**, Yuin-Han Loh, Hu Li, Frank Lau, Wataru Ebina, Zachary D. Smith, Alexander Meissner, George Q. Daley, Andrew S. Brack, James J. Collins, Chad Cowan, Thorsten M. Schlaeger, Derrick J. Rossi (2010)

3) **Programming human embryonic stem cells and human induced pluripotent stem cells into adipocytes.** Cell Stem Cell (Submission to Cell Stem Cells)

Tim Ahfeldt, Youn-Kyoung Lee, Adam Kaplan, Dave Lum, Robert Schinzel, Frank Lau, Rahul Deo, Jennifer Shay, Greg Mowrer, Eugene Rhee, Robert E. Gerszten, and Chad A. Cowan

3.1 To generate human induced pluripotent stem cells from somatic cells.

A high-efficiency system for the generation and study of human induced pluripotent stem cells.

3.1.1 Summary

Direct reprogramming of human fibroblasts to a pluripotent state has been achieved through ectopic expression of the transcription factors OCT4, SOX2, and either cMYC and KLF4 or NANOG and LIN28. Little is known, however, about the mechanisms by which reprogramming occurs, which is in part limited by the low efficiency of conversion. To this end, we sought to create a doxycycline-inducible lentiviral system to convert primary human fibroblasts and keratinocytes into human induced pluripotent stem cells (hiPSCs). hiPSCs generated with this system were molecularly and functionally similar to human embryonic stem cells (hESCs), demonstrated by gene expression profiles, DNA methylation status, and differentiation potential. While expression of the viral transgenes was required for several weeks in fibroblasts, we found that 10 days was sufficient for the reprogramming of keratinocytes. Using our inducible system, we developed a strategy to induce hiPSC formation at high frequency. Upon addition of doxycycline to hiPSC-derived differentiated cells, we obtained “secondary” hiPSCs at a frequency at least 100-fold greater than the initial conversion. The ability to reprogram cells at high efficiency provides a unique platform to dissect the underlying molecular and biochemical processes that accompany nuclear reprogramming.

3.1.2 Introduction

While human fibroblasts and a multitude of mouse somatic cell types can be reprogrammed to pluripotency by ectopic expression of transcription factors (Hanna et al., 2008; Maherali et al., 2007b; Okita et al., 2007; Stadtfeld et al., 2008a; Stadtfeld et al., 2008b; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007a), the conversion is highly inefficient (~0.01%), making it difficult to examine the underlying molecular events. Further, all hiPSCs to date have been generated with retroviruses and noninducible lentiviruses, both of which are inefficiently silenced and maintain transgene

expression. Another limitation in the generation of hiPSCs is the efficiency of viral gene transduction. While the fraction of cells infected by each individual factor and combinations of factors have not been addressed, only a small proportion of cells likely receive all factors. To overcome these limitations, we have established an inducible lentiviral system to generate “secondary” pluripotent cells, in which hiPSC clones are differentiated in vitro to yield fibroblast-like cells that harbor the inducible viral transgenes required for reprogramming. Because these cells maintain the same viral integrations that mediated the initial conversion to hiPSCs, this system bypasses the need for direct viral infection and produces a population of cells that can inducibly and homogeneously express the reprogramming factors. Such a system provides a powerful tool for mechanistic analysis, chemical and genetic screening for factors that enhance or block reprogramming, and the optimization of hiPSC derivation methods.

3.1.3 Results

cDNAs encoding human OCT4, SOX2, cMYC, KLF4, and NANOG were cloned into doxycycline (Dox)-inducible lentiviral vectors as previously described (Stadtfeld et al., 2008a). In addition, a reverse tetracycline transactivator (rtTA) driven by the ubiquitin promoter was cloned into a lentiviral vector. To generate hiPSCs, we infected neonatal foreskin fibroblasts (BJ) and keratinocytes with lentiviruses containing the rtTA and either four (OCT4, SOX2, cMYC, and KLF4; for fibroblasts only) or five reprogramming factors (4 + NANOG; both fibroblasts and keratinocytes) according to the scheme in Figure 4-1.

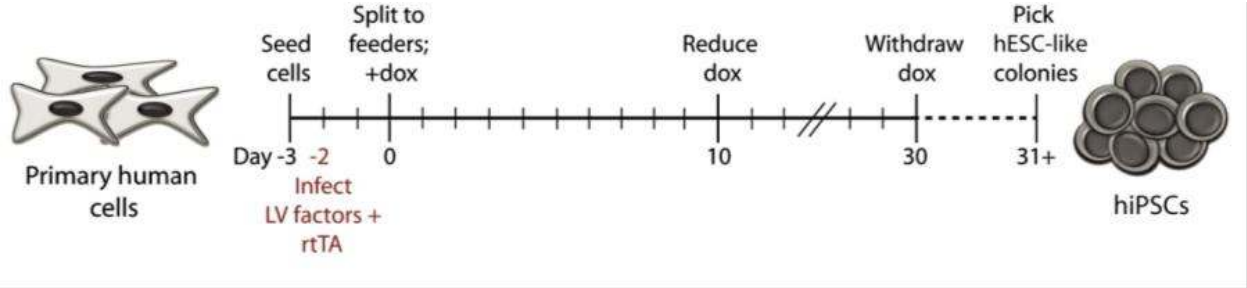


Figure 3-1 Experimental scheme for the generation of hiPSCs. Primary human fibroblasts and keratinocytes were infected with separate lentiviruses (LV) containing a constitutively active rtTA and Dox-inducible reprogramming factors. After infection, cells

were seeded to feeders, and Dox was applied for 30 days. hiPSC clones were picked based on hESC-like morphology and Dox-independent growth.

Following infection, cells were plated onto mouse embryonic fibroblast feeder cells (MEFs) under hESC conditions and induced with Dox. From the fibroblast cultures, non-hESC-like colonies emerged approximately 2 weeks after the addition of Dox, as previously observed (Takahashi et al., 2007). These colonies contained only Oct4 and Myc integrations and could not be expanded in the absence of Dox (data not shown). After 30 days of culture, colonies that resembled hESCs were observed, noted by a high nucleus-to-cytoplasmic ratio, prominent nucleoli, and well-defined phase-bright borders. All hESC-like colonies expressed the hESC-specific surface antigen Tra-1-81 (data not shown) and could be expanded in the absence of Dox. Colonies that did not resemble hESCs did not express Tra-1-81 and could not be passaged independent of Dox. Of ~2.5 × 10^5 infected fibroblasts seeded, four iPS colonies were obtained from each condition (four- or five-factor), representing a frequency of 0.002%. From the keratinocyte cultures, large non-ES-like colonies appeared within 1 week; within 3 weeks, hESC-like colonies appeared and could be passaged in the absence of Dox. Of ~ 3 × 10^5 cells seeded, seven colonies emerged, similar to the frequency observed for hiPSC derivation from fibroblasts (~0.002%).

hiPSC colonies stained positive for OCT4 protein and the hESC-specific surface antigen Tra-1-81 (Figure 4-2 and data not shown).

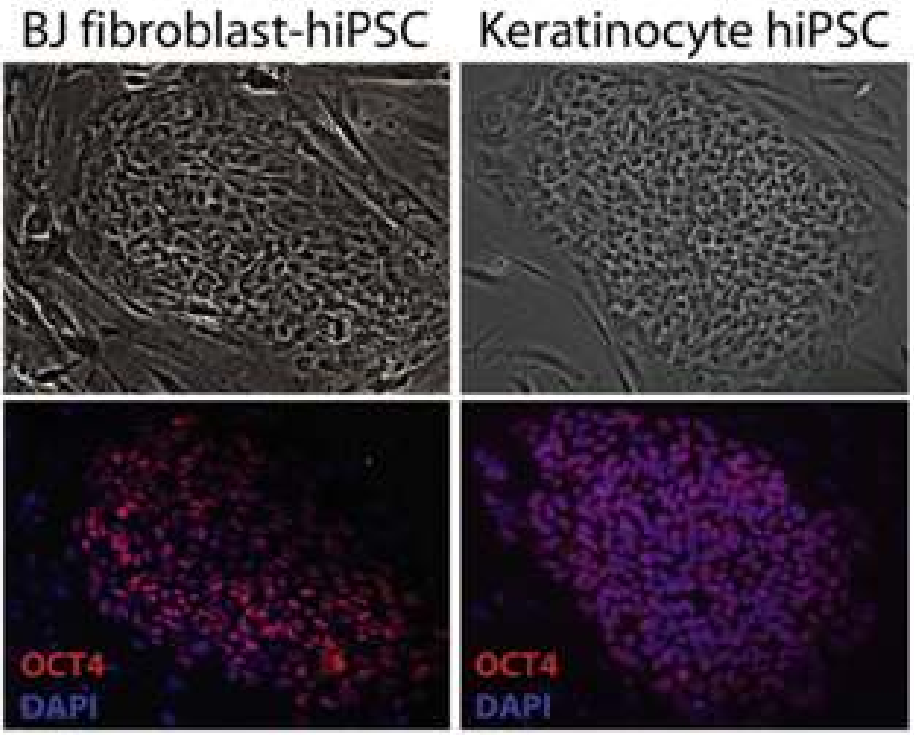


Figure 3-2 Morphology and marker expression in hiPSC colonies. Dox-independent fibroblast- and keratinocyte-derived hiPSCs express OCT4 protein.

Further, these cells showed expression of pluripotency genes from the endogenous loci and lacked expression of the viral transgenes (see Figure 4-3).

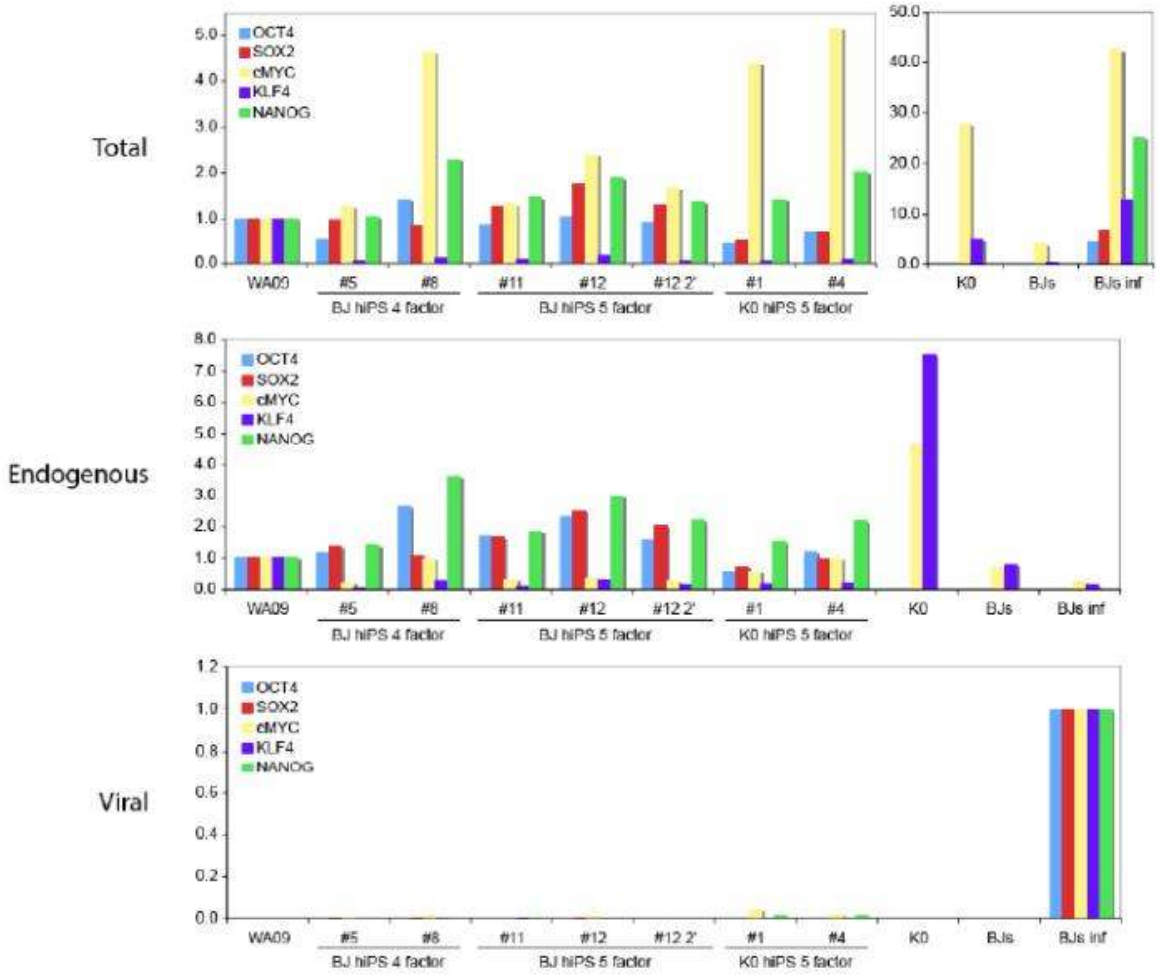


Figure 3-3 Quantitative RT-PCR analysis for total, endogenous, and viral gene expression of the reprogramming factors. WA09 hES cells, uninfected keratinocytes (K0) and BJ fibroblasts, and 5-factor-infected BJ fibroblasts (3 days +dox) served as controls. Values were standardized to GAPDH, then normalized to WA09 hESC (total and endogenous) or infected BJs (viral).

To assess whether hiPSCs were molecularly similar to hESCs, we examined promoter methylation and performed global transcriptional analysis. The NANOG and OCT4 promoters in fibroblast-derived hiPSCs were demethylated to a similar extent as in hESCs, in contrast to the highly methylated promoters in BJ fibroblasts (Figure 4-4), thus demonstrating epigenetic reprogramming in hiPSCs.

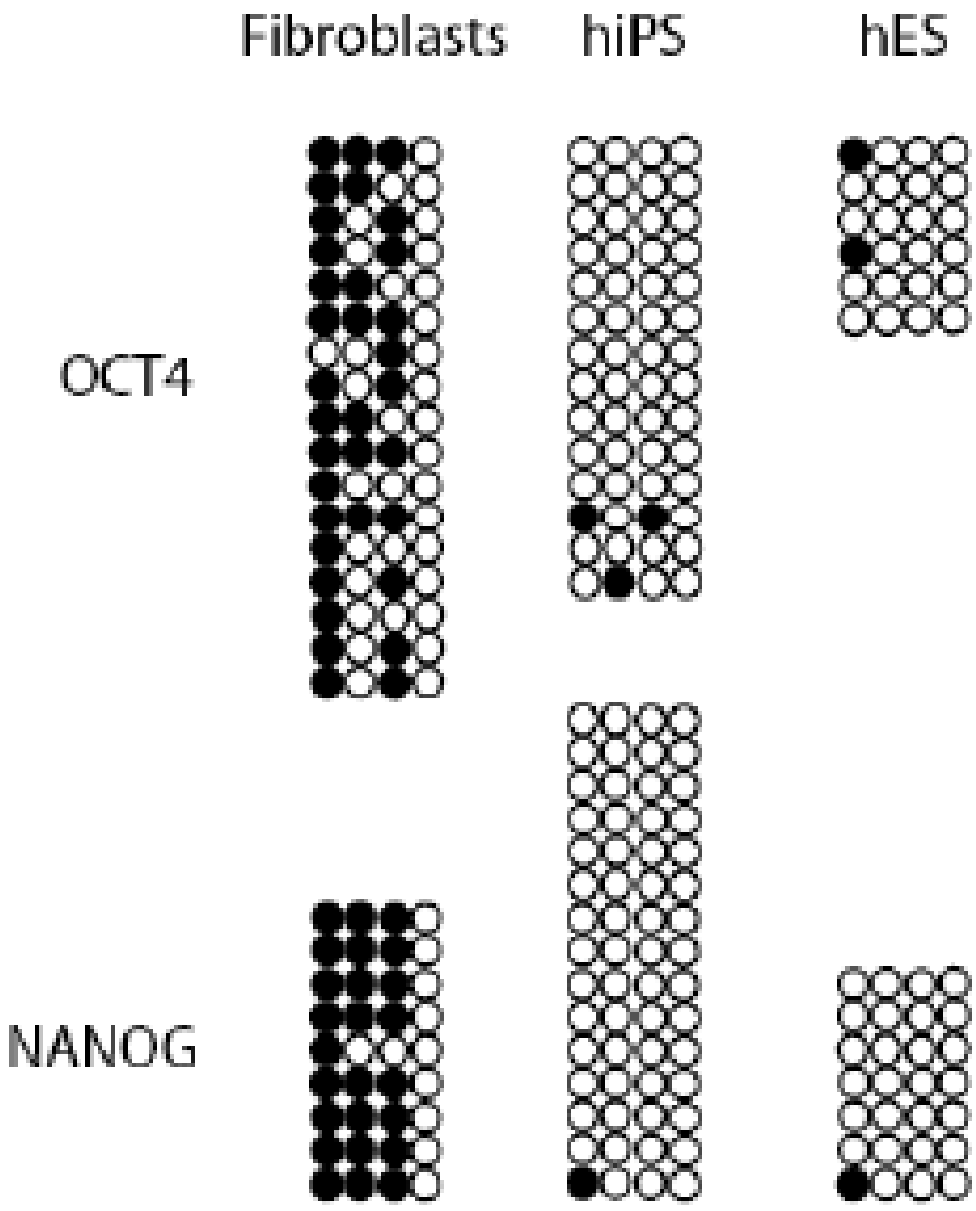


Figure 3-4 Bisulfite sequencing of the NANOG and the OCT4 promoter regions BJ fibroblasts, BJ fibroblast-derived hiPS, and WA09 hES cells. Promoter regions containing differentially methylated CpGs are shown. Open circles represent unmethylated CpGs; closed circles denote methylated CpGs.

Global analysis of gene expression in fibroblast-derived hiPSCs, hESCs, and fibroblasts was conducted by microarray through comparison of differentially expressed genes between fibroblasts and hESCs (Figure 4-5), indicating that hiPSCs had repressed the fibroblast program of gene expression and reactivated an embryonic program of transcription.

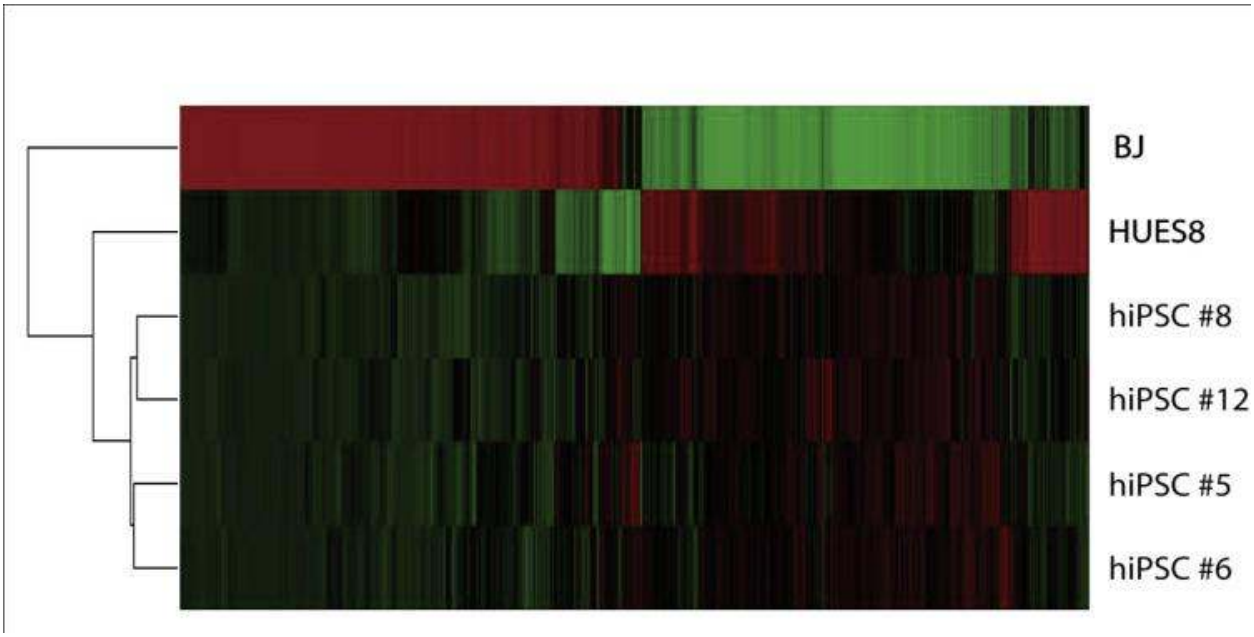


Figure 3-5 Microarray analysis of gene expression in hiPSCs. Genes with greater than 2-fold expression level between HUES8 hESCs and BJ fibroblasts were analyzed. Shown are BJ fibroblasts, HUES8 hESCs, and BJ fibroblast-derived hiPSC clones.

Pluripotency of both fibroblast- and keratinocyte-derived hiPSCs was examined in vitro through embryoid body (EB) formation. After 7 days in suspension culture, EBs were explanted and gave rise to well-defined neuronal outgrowths and beating cardiomyocyte structures (data not shown). Immunofluorescence analysis confirmed the presence of neurons, cardiomyocytes, skeletal muscle cells, and epithelial structures (Figure 4-6), thus demonstrating multilineage differentiation.

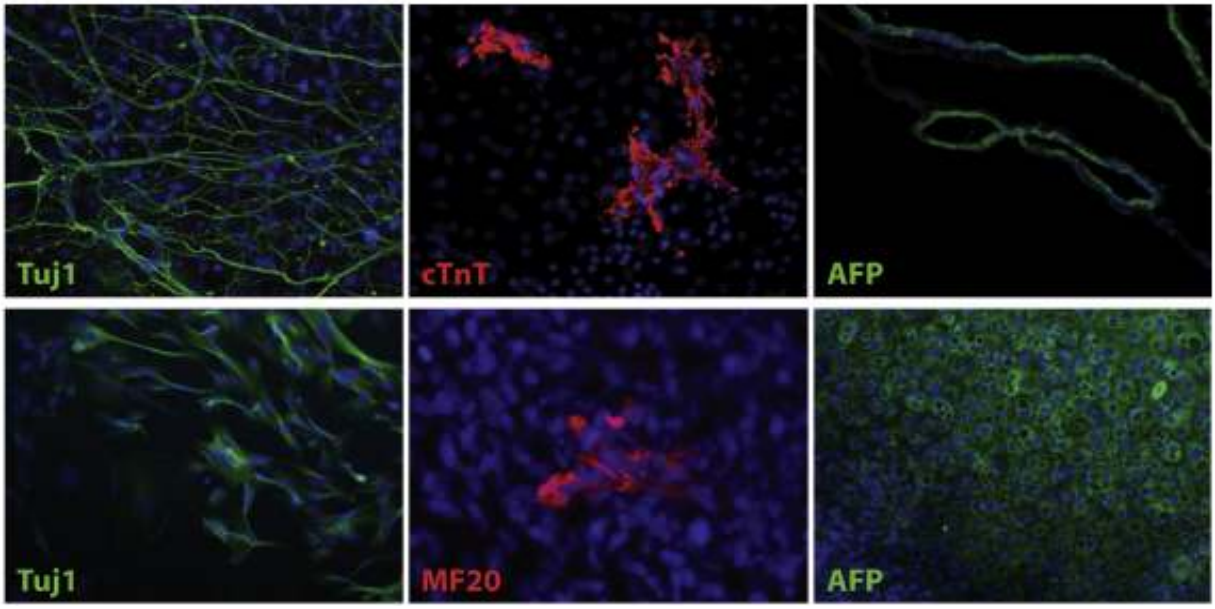


Figure 3-6 Immunonstainings of in vitro differentiated hIPSCs.
Upper panel: In vitro differentiation of fibroblast-derived hiPSCs into lineages from all three germ layers. Immunostaining for (i) Tuj1 (neuronal), (ii) cardiac troponin T (cTnT; cardiac muscle), and (iii) alpha-fetoprotein (AFP; epithelial, early endodermal). Botom Panel: In vitro differentiation of keratinocyte-derived hiPSCs into lineages from all three germ layers. Immunostaining for (i) Tuj1, (ii) skeletal muscle (MF20), and (iii) alpha-fetoprotein.

As a more stringent test of pluripotency, fibroblast-derived hiPSCs cells were injected either subcutaneously or under the kidney capsule of immunodeficient SCID mice to assay for teratoma formation. Tumors were recovered after 10 weeks and contained well-defined structures arising from all three embryonic germ layers, including pigmented cells, cartilage, skeletal muscle, and gut-like epithelium (Figure 4-7).

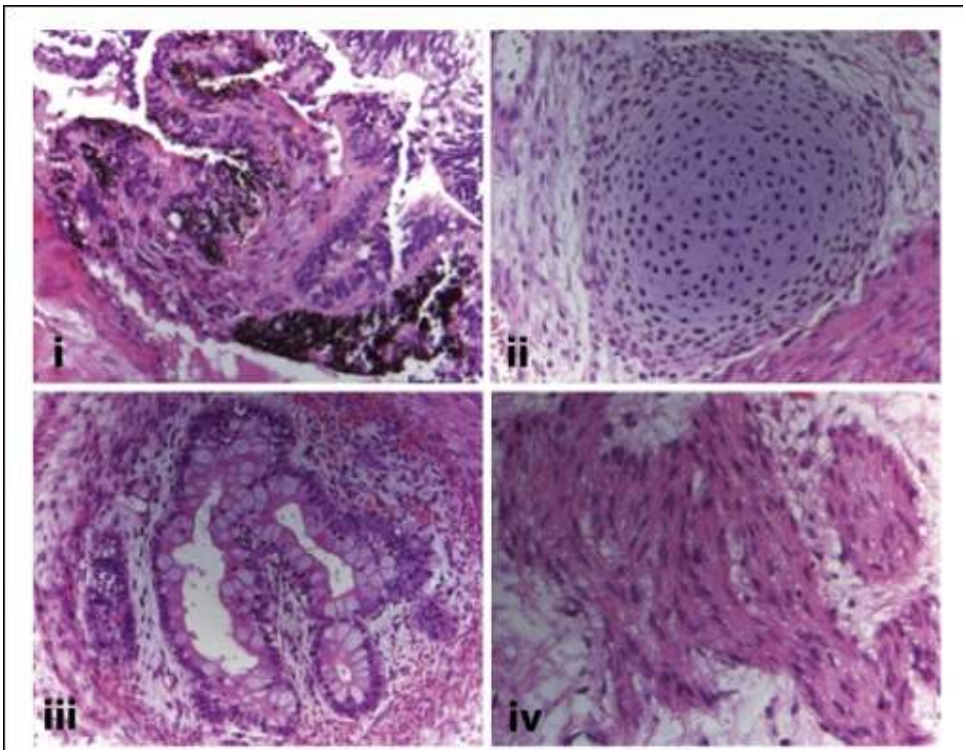


Figure 3-7 Hematoxylin and eosin stain of teratomas generated from fibroblast-derived hiPSCs. Differentiated structures from all three germ layers were present. (i) Pigmented epithelium (ectoderm), (ii) cartilage (mesoderm), (iii) gut-like epithelium (endoderm), and (iv) muscle (mesoderm).

These results indicate that hiPSCs generated with an inducible system strongly resemble hESCs and fulfill all criteria for pluripotency. Noting that keratinocyte-derived hiPSC colonies appeared faster than fibroblast-derived hiPSCs, we sought to determine the minimum amount of time required to convert keratinocytes to hiPSCs. To test this, keratinocytes were infected with rtTA and five factors (OCT4, SOX2, cMYC, KLF4, and NANOG), and Dox was withdrawn at different time points throughout the reprogramming process. The number of hESC-like colonies was counted at day 30 and plotted against the day of Dox withdrawal (Figure 4-8).

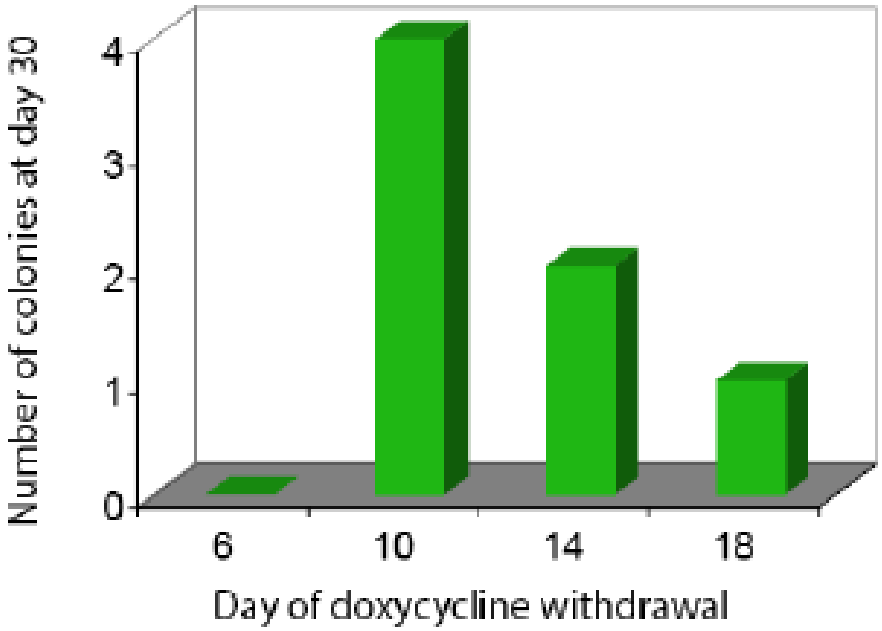


Figure 3-8 Temporal requirement of factor expression in keratinocytes.
Cells infected with five factors were seeded at 7.5x104 cells per time point and doxycycline was withdrawn every four days from days 6 through 18. The number of hES-like colonies was counted at day 30. All colonies could be expanded in the absence of doxycycline.

hESC-like colonies first appeared after 18 days when Dox had been withdrawn after 10 days. The frequency of reprogramming appeared to decline with the length of Dox exposure, which may reflect unfavorable culture conditions at later time points or adverse effects of continued transgene expression.

To establish the system of “secondary” hiPSCs, we differentiated several BJ fibroblast-derived hiPSC clones to fibroblast-like cells (MPC) in vitro according to the scheme in Figure 4-9.

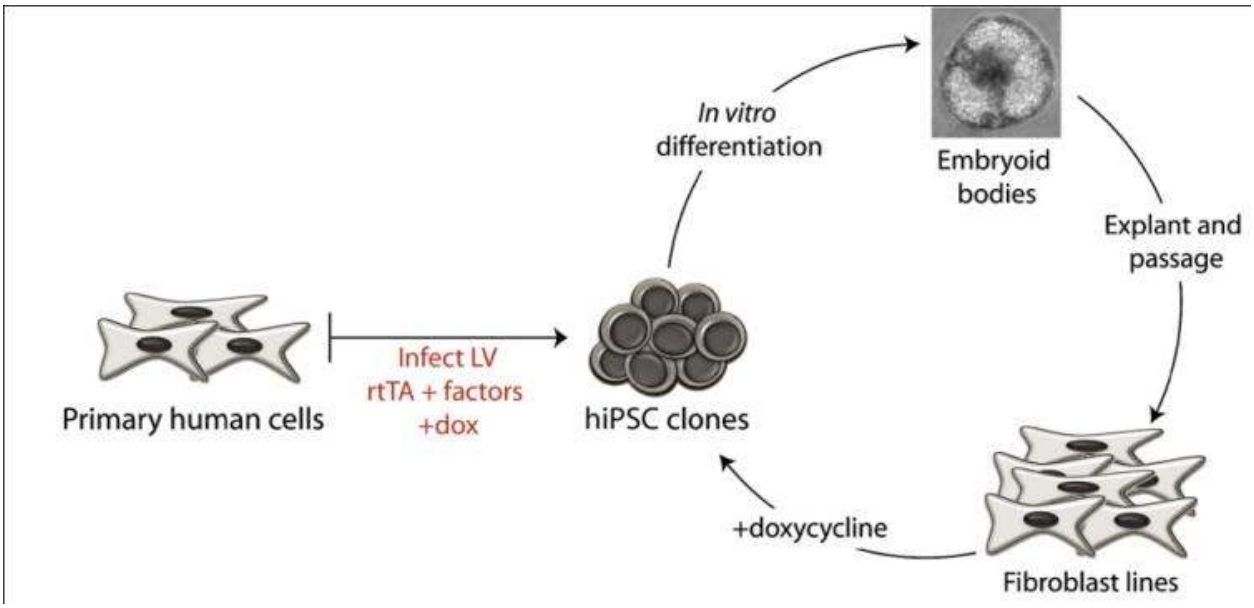


Figure 3-9 Experimental scheme depicting the generation of secondary hiPSCs. hiPSCs were differentiated in vitro as embryoid bodies for 7 days, then plated to adherent conditions. Fibroblast-like colonies were picked and expanded for at least three passages prior to undergoing reinduction by Dox.

hiPSC colonies were placed in suspension culture for 1 week, and the resulting EBs were then plated to adherent conditions. Outgrowths of fibroblast-like cells were picked and passaged a minimum of three times prior to experimental manipulation to ensure that no residual pluripotent cells were present. Quantitative RT-PCR analysis confirmed a lack of pluripotency gene expression in these populations (Figure 4-10).

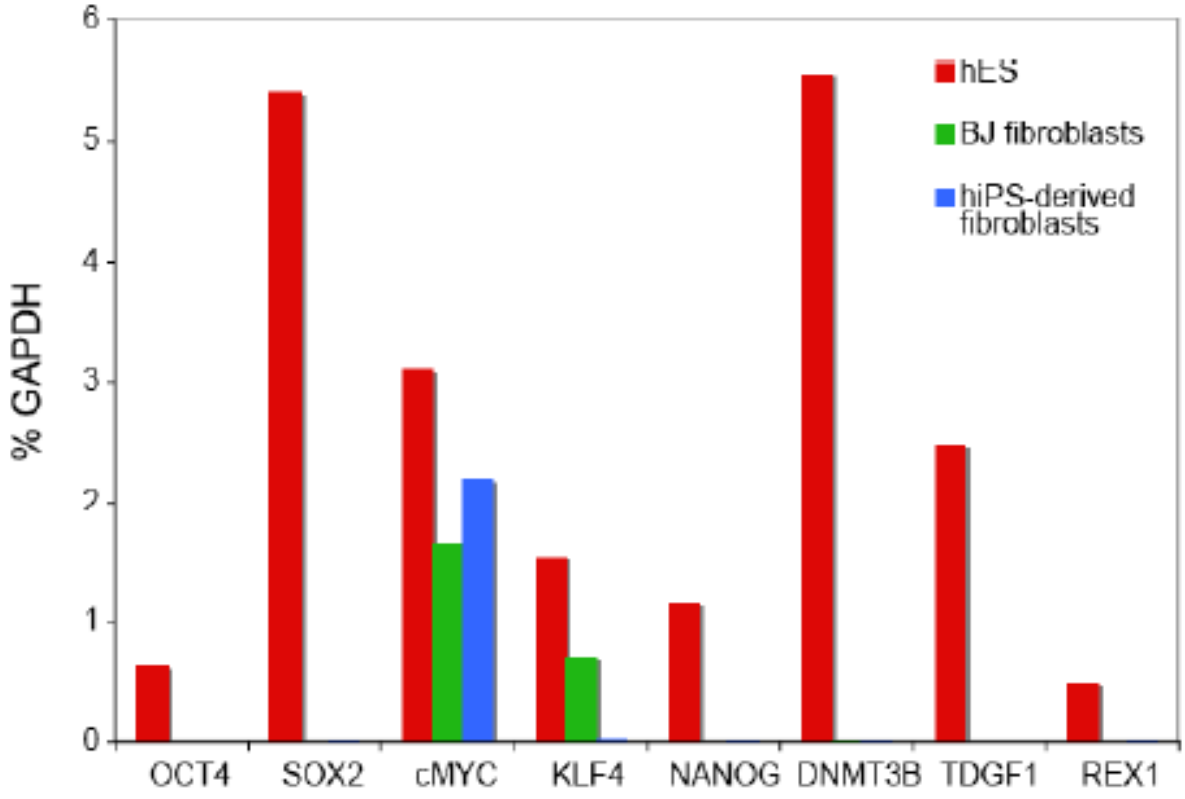


Figure 3-10 Quantitative RT-PCR analysis of reprogramming factor and pluripotency gene expression in hiPS-derived fibroblasts. WA09 hES cells, and BJ fibroblasts. Values were standardized to GAPDH.

We assessed the ability of hiPSC-derived cells to generate “secondary” hiPSCs through Dox addition and transfer to hiPSC derivation conditions. Fibroblast-like cells derived from two hiPSC clones demonstrated reprogramming in the presence, but not absence, of Dox (Figure 4-11).

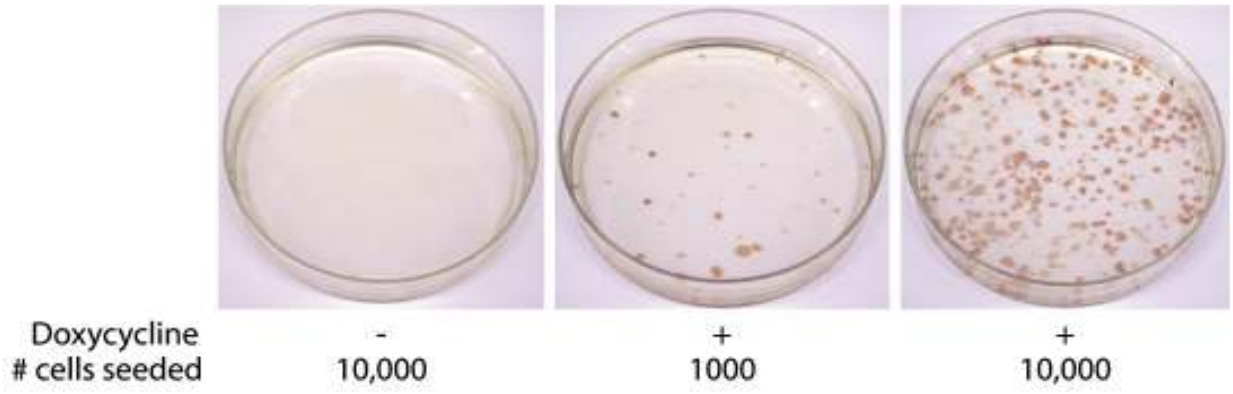


Figure 3-11 Alkaline phosphatase staining of reprogrammable cells grown in the absence or presence of Dox. Dox was withdrawn at day 21, and colonies were stained and counted at day 30.

The frequency of conversion ranged from 1% to 3%. Reinduction of viral transgenes in hiPSC-derived fibroblasts clones was also assessed by quantitative RT-PCR (Figure 4-12), demonstrating a correlation between factor reactivation and the ability of the clone to produce secondary hiPSCs.

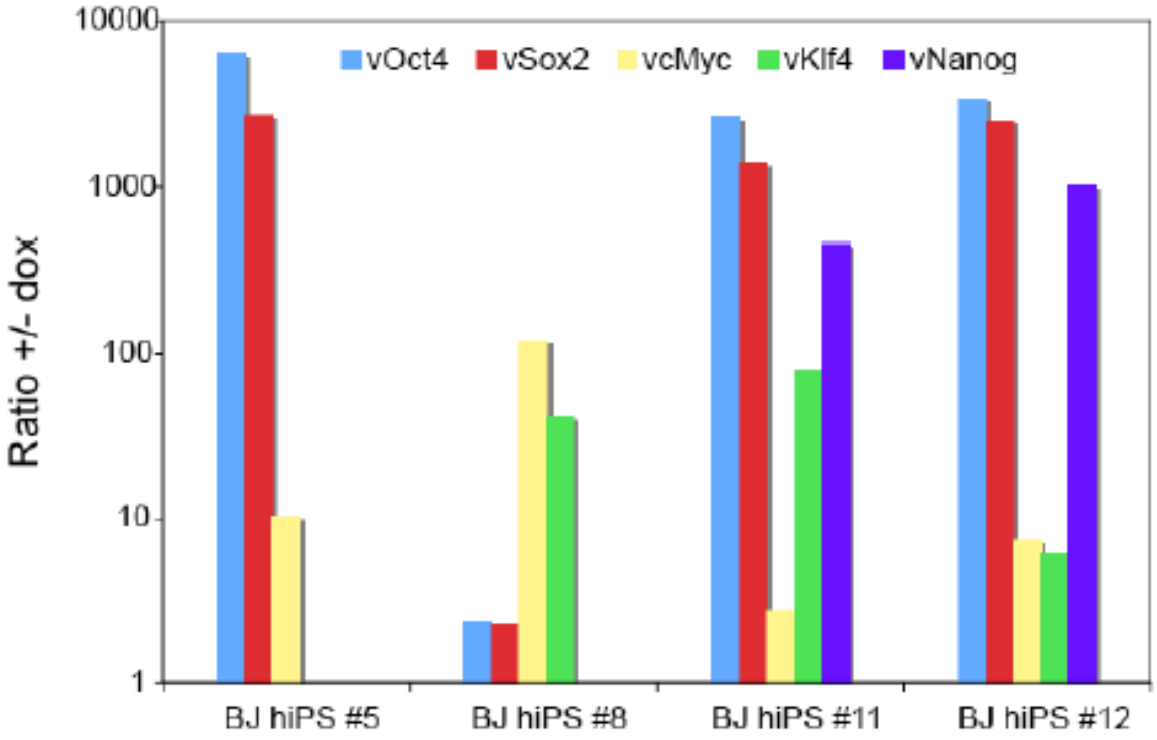


Figure 3-12 Quantitative RT-PCR analysis for viral transgene reactivation in hiPS-derived fibroblast-like cells from four BJ-hiPS clones. Cells were analyzed two days after doxycycline administration. Clones #5 and #8 were generated with four factors; clones #11 and #12 with five factors. Values were standardized to GAPDH, then calculated as the ratio of expression between +/- doxycycline.

Clones that gave rise to secondary hiPSCs showed reactivation of all factors (BJ hiPSCs #11 and #12), while those that did not lacked re-expression of a factor (BJ hiPSC #5) or re-expressed the factors at levels that are likely not permissive for the induction of pluripotency (BJ hiPSC #8).

Secondary hiPSCs were molecularly and functionally similar to primary hiPSCs and hESCs. They stained positive for OCT4 and Tra-1-81 (4-13), had a similar gene expression profile to hESCs (Figure 4-14), and demonstrated pluripotency in vitro, generating cell types from all three embryonic germ layers (Figure 4-15).

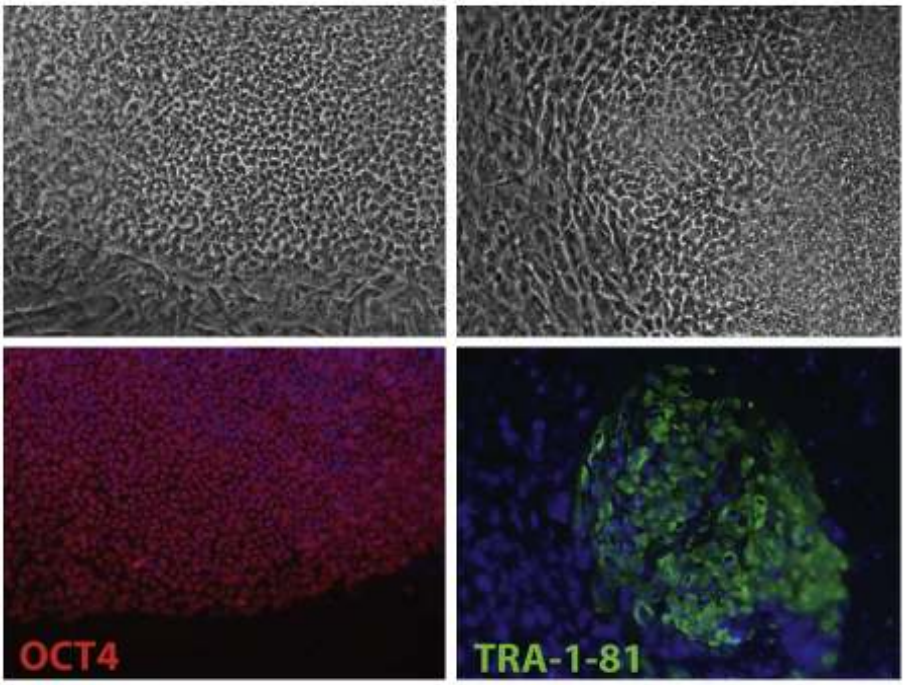


Figure 3-13 Morphology and expression of OCT4 and Tra-1-81 in Dox-independent secondary hiPSCs.

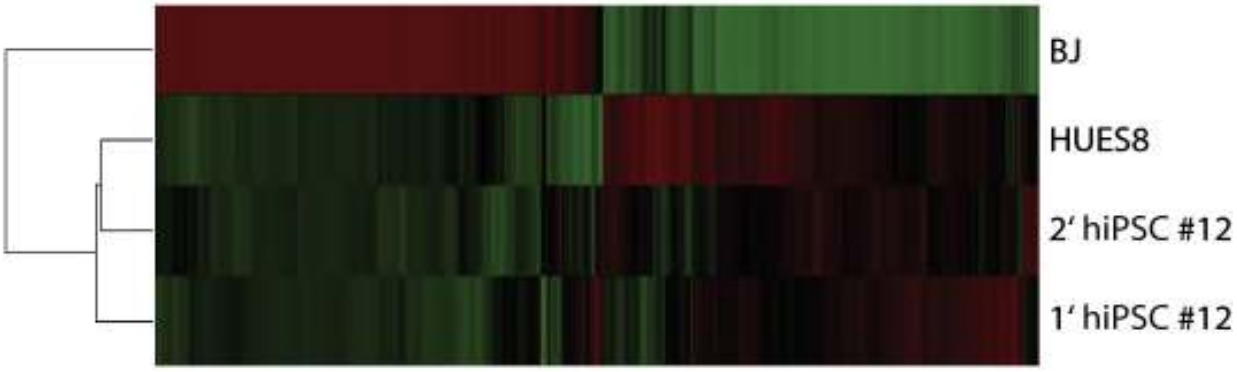


Figure 3-14 Microarray analysis of gene expression between BJ fibroblasts, HUES8 hESCs, primary fibroblast-derived hiPSCs, and a resulting secondary hiPSC clone. Shown are genes with >2-fold expression value between BJ fibroblasts and HUES8 hESCs.

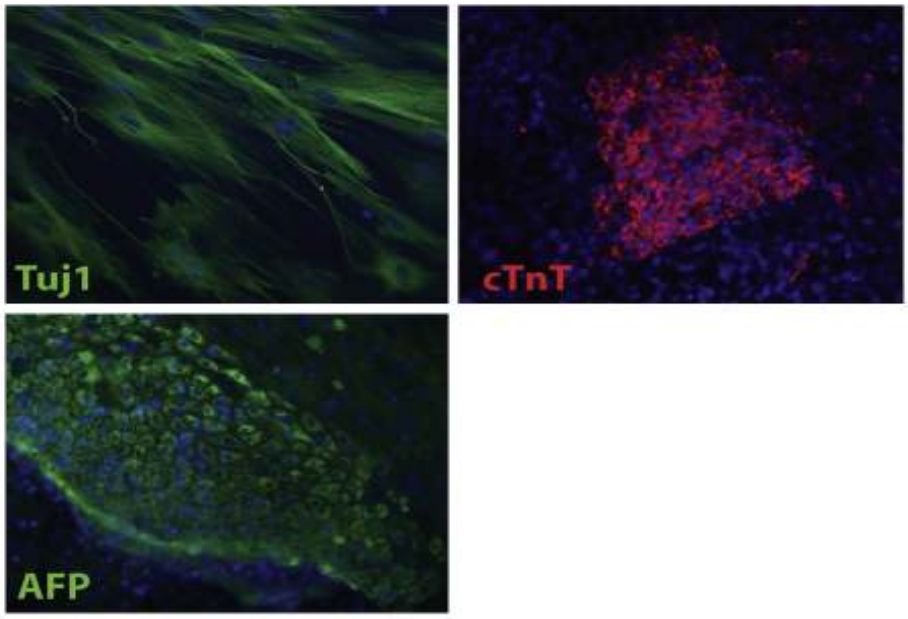


Figure 3-15 In vitro differentiation of secondary hiPSCs into lineages from all three germ layers. Immunostaining for (i) Tuj1, (ii) cardiac troponin T, and (iii) alpha-fetoprotein.

To determine the temporal requirement of transgene expression for secondary hiPSC formation, we seeded 10^4 fibroblast-like cells per time point under hiPSC derivation conditions and withdrew Dox daily from days 4-19. The final number of Tra-1-81+ hESC-like colonies was counted at day 25 (Figure 4-16).

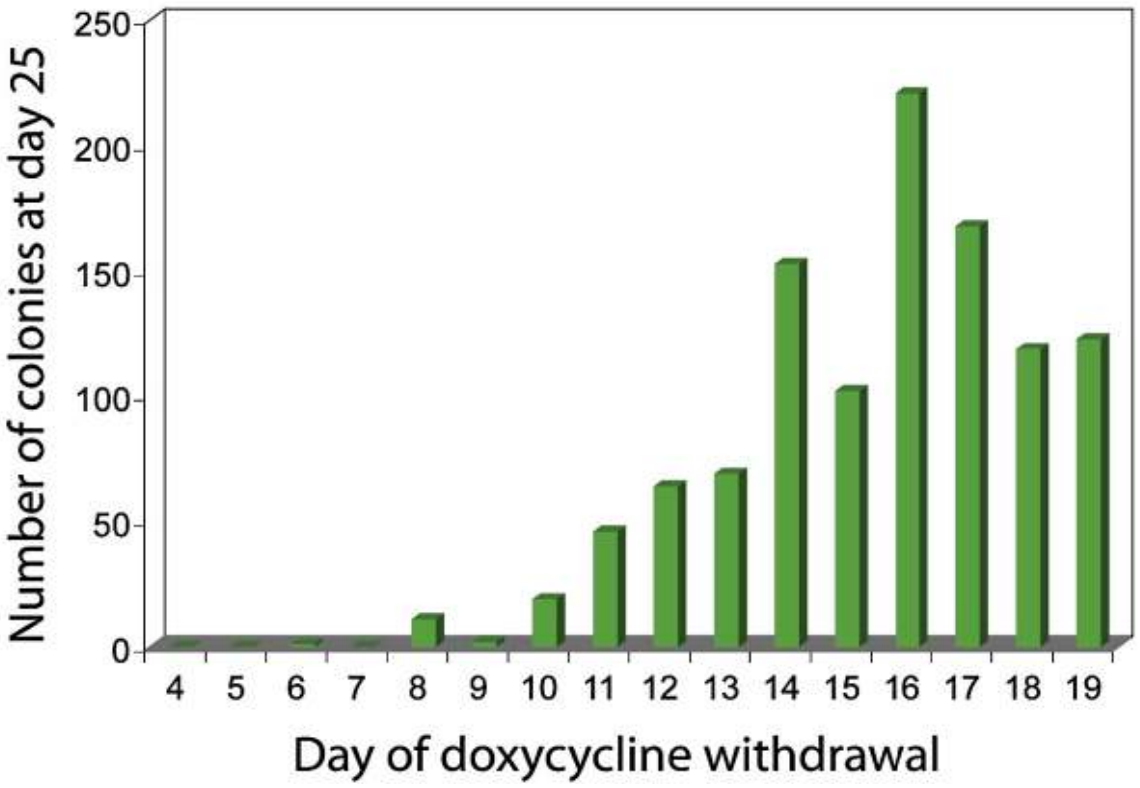


Figure 3-16 Temporal requirement of factor expression in hiPSC-derived fibroblast-like cells. 10^4 cells were plated per time point, and Dox was withdrawn daily from days 4–19. The hESC-like colonies that expressed Tra-1-81 were counted at day 25.

hiPSC colonies began to appear after withdrawal of Dox at day 6 (one colony), and the number of colonies increased with the time of Dox exposure, reaching a maximum after withdrawal at 16 days (frequency of ~2%). This increase in frequency with length of Dox exposure has also been reported in the reprogramming of mouse cells (Wernig et al., 2008).

Transformed granular colonies did not appear during secondary hiPSC induction, in contrast to the early colonies that appeared during primary induction with direct viral infection. The lack of these background colonies coupled with the high efficiency of the secondary system enabled us to monitor the progression of colonies during reprogramming. We tracked individual colonies on a daily basis with different time

points of Dox withdrawal (Figure 4-17, 4-18, 4-19) and observed that induced cells transited through non-ES-like structures prior to acquiring a hiPSC phenotype.

Not all colonies developed fully to hiPSCs; those that did not undergo successful reprogramming began to regress 2 days after Dox withdrawal and ultimately formed fibroblast-like structures. The colonies that successfully generated hiPSCs also showed some regression after withdrawal of Dox; however, hESC-like outgrowths gradually appeared which could be expanded into stable hiPSC lines.

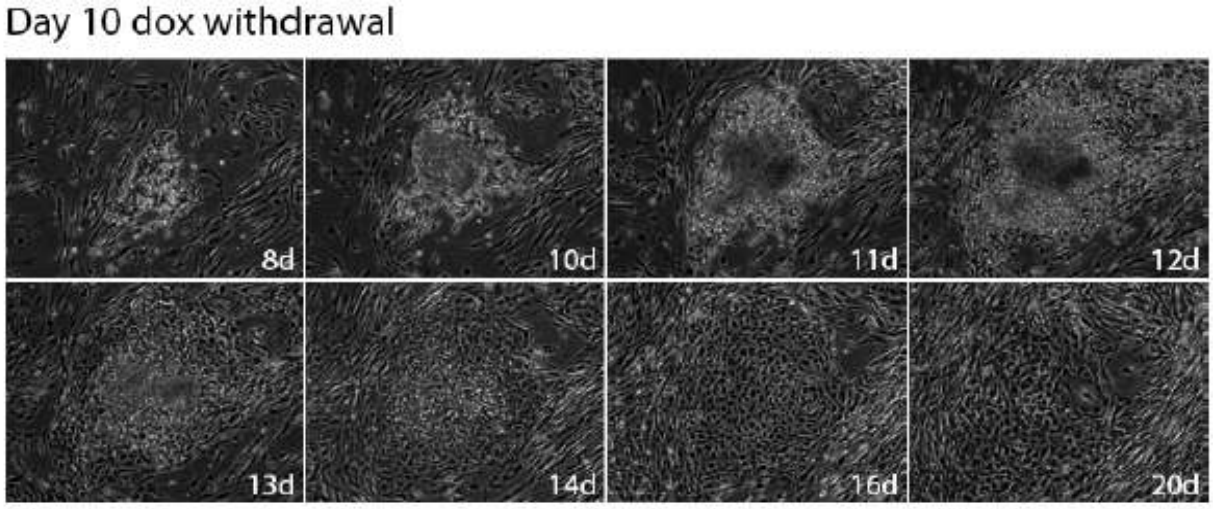


Figure 3-17 Visual tracking of hiPS colony formation. hiPS-derived fibroblast-like cells were induced to form secondary hiPS cells, and individual colonies were tracked during the reprogramming process. Doxycycline was withdrawn at various time points. Representative colony that failed to reprogram.

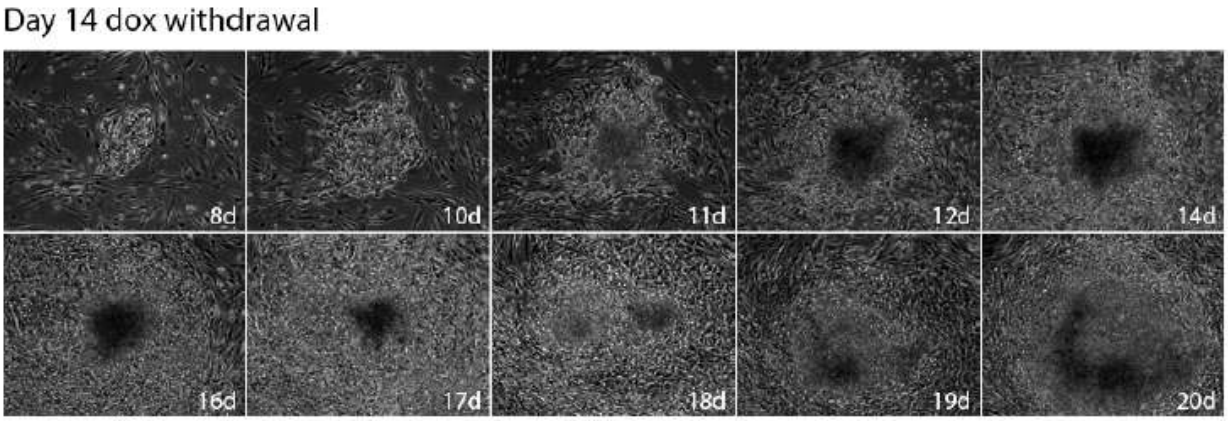


Figure 3-18 Visual tracking of hiPS colony formation: hiPS-derived fibroblast-like cells were induced to form secondary hiPS cells, and individual colonies were tracked during the reprogramming process. Doxycycline was withdrawn at various time points. Representative colony that underwent successful reprogramming.

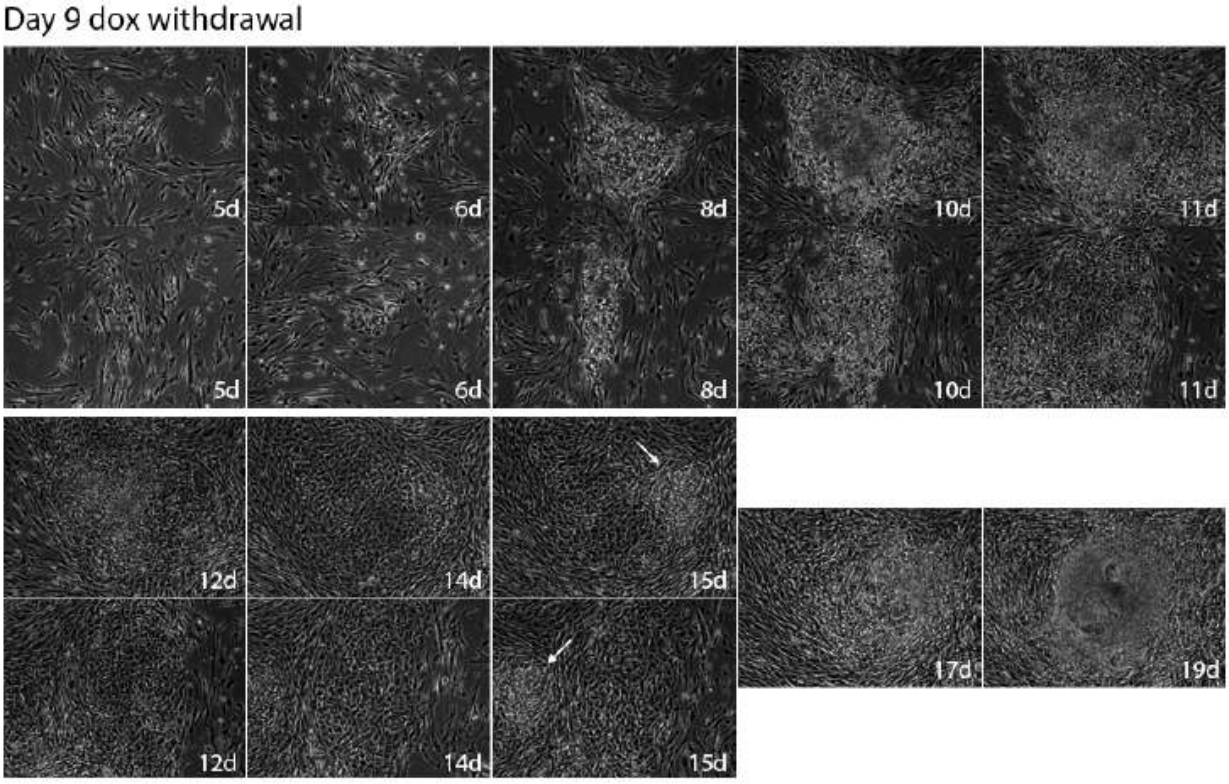


Figure 3-19 Visual tracking of hiPS colony formation. hiPS-derived fibroblast-like cells were induced to form secondary hiPS cells, and individual colonies were tracked during the reprogramming process. Doxycycline was withdrawn at various time points.

Representative colonies that gave rise to a hiPS clone; two adjacent colonies were tracked. By day 15, these colonies had regressed and an hES-like colony began to form between them (arrows), which continued to develop as hiPS cells.

3.1.4 Discussion

Here we have described the use of an inducible lentiviral system for the reprogramming of human somatic cells. Using this system, we converted neonatal foreskin fibroblasts and keratinocytes to a pluripotent state molecularly and functionally similar to hESCs. This system also enabled us to establish the temporal requirement of factor expression for cells undergoing reprogramming. While fibroblasts relied on transgene expression for several weeks, keratinocytes required only 10 days of factor expression to revert to a state that was poised to become pluripotent. It is unknown why keratinocytes are more amenable to reprogramming. Keratinocytes, like hESCs, represent an epithelial cell type and in contrast to fibroblasts may not need to undergo a mesenchymal-epithelial transition during reprogramming (Yang and Weinberg, 2008). Differences in reprogrammability between fibroblasts and keratinocytes may also be explained by differences in the cell-cycle status or viral infectivity. Moreover, keratinocytes express much higher levels of endogenous MYC and KLF4 than fibroblasts (Figure 4-3), which may accelerate their conversion to hiPSCs. The fast kinetics of reprogramming observed for keratinocytes suggests that these cells would be useful for the development and optimization of methods to reprogram cells by transient delivery of factors.

Controlled expression of the reprogramming factors provided an inherent selection strategy by eliminating cells that were dependent upon viral transgene expression and conferring a growth advantage to fully reprogrammed cells that were Dox independent. This is in contrast to the constitutive retro- and lentiviral systems that have so far been used to reprogram human cells, where the viral transgenes maintain expression in the hiPSC state. The persistence of viral gene expression could have deleterious effects in clinical applications such as in vitro disease modeling; for example, the overexpression of Oct4 or Sox2 has been shown to promote the differentiation of mouse ES cells (Kopp

et al., 2008; Niwa et al., 2000), suggesting that their continued expression during in vitro differentiation of hiPSCs may bias the resulting cell fate.

The extremely low efficiency of reprogramming led us to develop a system of "secondary" pluripotent cells in which we could reprogram hiPSC-derived differentiated cells at a high frequency. The >100-fold increase we observed was likely attributed to the ability to reactivate all factors within a given cell, thus enabling efficient reprogramming. The kinetics of this process were faster than that of primary fibroblasts but similar to keratinocytes, with the highest efficiency occurring after 16 days of factor expression. In vitro-derived fibroblasts appeared to be more amenable to reprogramming than primary fibroblasts, as previously observed (Park et al., 2008c; Yu et al., 2007a), which may reflect a lack of exposure to potent differentiation cues in vitro. However, four lines of evidence suggest that the reprogramming of hiPSC-derived differentiated cells is representative of the process that occurs in primary cells: (1) hiPSC-derived fibroblast-like cells lacked detectable expression of key pluripotency genes, (2) no colonies formed in the absence of Dox, (3) clones that did not reactivate all factors could not successfully form secondary hiPSCs, and (4) visual tracking of colonies demonstrated that cells transit through a non-hESC-like state prior to becoming bona fide hiPSC colonies. These data collectively support the use of a secondary system as a platform for mechanistic dissection of the reprogramming process.

Despite the fact that viral transgenes were reactivated in most of the hiPSC-derived cells, the frequency of reprogramming remained quite low, ranging from 1% to 3%. The basis for the low efficiency is poorly understood but may reflect a multitude of factors such as the starting cell type, cell-cycle status and the ability to undergo replication, variability in factor reactivation, and other stochastic events. Epigenetic events have been implicated in the reprogramming process; for example, it has recently been shown that valproic acid, which acts primarily as a histone deacetylase inhibitor, can enhance the efficiency of reprogramming (Huangfu et al., 2008). Also, DNA methylation and the activation of differentiation pathways have been shown to impede the reprogramming of mouse fibroblasts (Mikkelsen et al., 2008). However, these events account only for a

small proportion of cells, and it will be interesting to further define the limiting factors that constrain the efficiency of reprogramming.

The secondary system of hiPSCs presents an attractive model for which to study the molecular events that underlie the reversion of human somatic cells to a pluripotent state. By providing a homogeneous population of cells that harbor all the viral transgenes, the cultures are not subject to the background of cells that do not receive all factors, allowing proper analysis of the reprogramming process. The secondary system will enable chemical and genetic screening efforts to identify key molecular constituents of reprogramming, as well as important obstacles in this process, and will ultimately lend itself as a powerful tool in the development and optimization of methods to produce hiPSCs.

3.1.5 Experimental Procedures

Virus Production

Vectors were constructed as previously described (Stadtfeld et al., 2008a). To generate virus, 293T cells were transfected at 30% confluence using FuGENE 6 reagent (Roche). For a 10 cm plate, 560 µL DMEM, 27 µL FuGENE, and 12 µg DNA (4:3:2 vector:Δ8.9:vsv-g) were used. Virus was harvested over 3 days and concentrated 300-fold. For a standard infection in a 35 mm dish (~10^5 cells), 10 µL rtTA + 5 µL factors (OCT4, SOX2, KLF4, and NANOG) + 2 µL cMYC was used in an overnight infection supplemented with 6 µg/mL polybrene.

Cell Culture and hiPSC Generation

Fibroblasts were grown in DMEM with 10% FBS, nonessential amino acids, glutamine, and β-mercaptoethanol; keratinocytes were cultured on collagen IV in keratinocyte serum-free medium and growth supplement (Invitrogen). Human ES and iPS cells were cultured as previously described (Cowan et al., 2004). hiPSCs were generated as follows: day 0, to 1 µg/mL Dox in hESC media + 2% defined FBS (GIBCO) for fibroblasts, 1% FBS for keratinocytes; day 2, hESC + 1 µg/mL Dox + 1% FBS

(fibroblasts) or hESC + Dox (keratinocytes); day 4, hESC + 1 μg/mL Dox; day 10, hESC + 0.5 μg/mL Dox (continued until colonies appeared).

Differentiation

For in vitro differentiation, hiPSC colonies were mechanically picked and placed in suspension culture with fibroblast media. After 1 week, embryoid bodies were plated to adherent conditions with gelatin. For teratoma formation, ~10^7 hiPSCs were pelleted and injected into SCID mice, either subcutaneously above the dorsal flank or underneath the kidney capsule. Tumors were harvested after 10–12 weeks and processed for histological analysis.

Bisulfite Sequencing

Genomic DNA was converted using the Epitect bisulfite kit (QIAGEN). OCT4 and NANOG promoters were PCR amplified using primers listed in Table 4-1 . PCR products were cloned into TOPO vectors and sequenced.

Table 3-1 Primers used in this study

Gene name	Forward primer sequence	Reverse primer sequence
Total OCT4	GAG GAG TCC CAG GAC ATC AA	TGG CTG AAT ACC TTC CCA AA
Total SOX2	AGC TAC AGC ATG ATG CAG GA	GGT CAT GGA GTT GTA CTG CA
Total cMYC	ACT CTG AGG AGG AAC AAG AA	TGG AGA CGT GGC ACC TCT T
Total KLF4	CCC AAT TAC CCA TCC TTC CT	ACG ATC GTC TTC CCC TCT TT
Total NANOG	TAC CTC AGC CTC CAG CAG AT	CCT TCT GCG TCA CAC CAT T
Lentiviral OCT4	CCC CTG TCT CTG TCA CCA CT	CCA CAT AGC GTA AAA GGA GCA
Lentiviral SOX2	ACA CTG CCC CTC TCA CAC AT	CAT AGC GTA AAA GGA GCA ACA
Lentiviral cMYC	AAG AGG ACT TGT TGC GGA AA	TTG TAA TCC AGA GGT TGA TTA TCG
Lentiviral KLF4	GAC CAC CTC GCC TTA CAC AT	CAT AGC GTA AAA GGA GCA ACA
Lentiviral NANOG	ACA TGC AAC CTG AAG ACG TG	CAC ATA GCG TAA AAG GAG CAA
Endogenous OCT4	TGT ACT CCT CGG TCC CTT TC	TCC AGG TTT TCT TTC CCT AGC
Endogenous SOX2	GCT AGT CTC CAA GCG ACG AA	GCA AGA AGC CTC TCC TTG AA
Endogenous cMYC	CGG AAC TCT TGT GCG TAA GG	CTC AGC CAA GGT TGT GAG GT
Endogenous KLF4	TAT GAC CCA CAC TGC CAG AA	TGG GAA CTT GAC CAT GAT TG
Endogenous NANOG	CAG TCT GGA CAC TGG CTG AA	CTC GCT GAT TAG GCT CCA AC
OCT4 promoter	AAG TTT TTG TGG GGG ATT TGT AT	CCA CCC ACT AAC CTT AAC CTC TA
NANOG promoter	TTA ATT TAT TGG GAT TAT AGG GGT G	AAA CCT AAA AAC AAA CCC AAC AAC
DNMT3B	CCA ATC CTG GAG GCT ATC CG	ACT GGG GTG TCA GAG CCA T
TDGF1 (CRIPTO)	AAG ATG GCC CGC TTC TCT TAC	AGA TGG ACG AGC AAA TTC CTG
ZFP42 (REX1)	AAC GGG CAA AGA CAA GAC AC	GCT GAC AGG TTC TAT TTC CGC
GAPDH	TGT TGC CAT CAA TGA CCC CTT	CTC CAC GAC GTA CTC AGC G

Immunostaining

Immunostaining was performed using the following antibodies: α-Oct4 (sc-8628, Santa Cruz Biotech), α-Tra-1-81 (MAB4381, Millipore), α-β-III tubulin (T2200, Sigma), α-cardiac troponin T (clone 13-11, Neomarkers), α-myosin heavy chain (clone MF20, Developmental Studies Hybridoma Bank), and α-AFP (sc-15375, Santa Cruz Biotech).

qPCR

RNA was extracted by using a QIAGEN RNeasy kit, then converted to cDNA with the Superscript III First-Strand synthesis system (Invitrogen) using oligo-dT primers. qRT-PCRs were carried out using Brilliant II SYBR Green mix (Stratagene) and run on a Stratagene MXPro400. Reactions were carried out in duplicate with −RT controls, and data were analyzed using the delta-delta Ct method. Primer sequences are listed in Table 4-1

Whole-Genome Expression Analysis

Total RNA was isolated using an RNeasy kit (QIAGEN). Samples were processed as independent triplicates. RNA probes for microarray hybridization were prepared and hybridized to Affymetrix HGU133 plus two oligonucleotide microarrays. Data were extracted and analyzed using the Rosetta Resolver system. During importation, the data were subjected to background correction, intrachip normalization, and the Rosetta Resolver Affymetrix GeneChip error model (Weng et al., 2006). For the generation of intensity plots, genes that showed greater than a 2-fold difference in expression value ($p < 0.01$) in HUES8 hESCs and BJ fibroblasts were noted (19,663 probes) and their expression analyzed. A hierarchical clustering was performed.

3.2 To generate integration free human induced pluripotent stem cells using modified RNA

This chapter includes the publication (Warren et al., 2010) as well as preliminary data that helped developing this project. The underlying idea is simple as depicted in the experimental scheme (Figure 4-20).

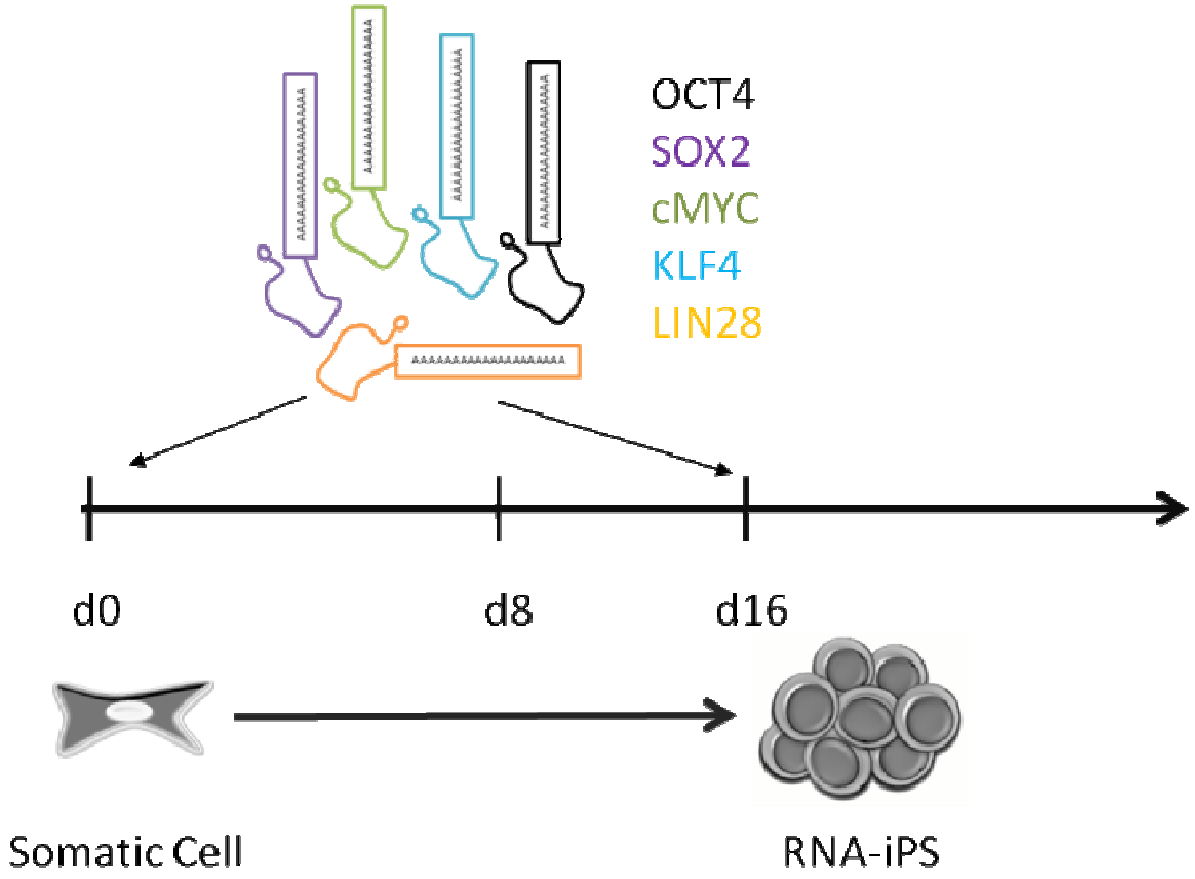


Figure 3-20 Experimental scheme for the derivation of hIPS using modified RNA of the reprogramming factors OCT4, SOX2, cMYC, KLF and Lin 28

Highly efficient reprogramming to pluripotency and directed differentiation of human cells using synthetic modified mRNA.

3.2.1 Summary

Clinical application of induced pluripotent stem (iPS) cells is limited by low efficiency of iPS derivation, and protocols that permanently modify the genome to effect cellular reprogramming. Moreover, safe and effective means of directing the fate of patient-specific iPS cells towards clinically useful cell types are lacking. Here we describe a simple, non-integrating strategy for reprogramming cell fate based on administration of synthetic mRNA modified to overcome innate anti-viral responses. We show that this

approach can reprogram multiple human cell types to pluripotency with efficiencies that greatly surpass established protocols. We further show that the same technology can be used to efficiently direct the differentiation of RNA-induced pluripotent stem (RiPS) cells into terminally differentiated myogenic cells. This technology represents a safe, efficient strategy for somatic cell reprogramming and directing cell fate that has broad applicability for basic research, disease modeling and regenerative medicine.

3.2.2 Introduction

The reprogramming of differentiated cells to pluripotency holds great promise as a tool for studying normal development, while offering hope that patient-specific induced pluripotent stem (iPS) cells could be used to model disease, or to generate clinically useful cell types for autologous therapies aimed at repairing deficits arising from injury, illness, and aging. Induction of pluripotency was originally achieved by Yamanaka and colleagues by enforced expression of four transcription factors, KLF4, c-MYC, OCT4, and SOX2 (KMOS) using retroviral vectors (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Viral integration into the genome initially presented a formidable obstacle to therapeutic use of iPS cells. The search for ways to induce pluripotency without incurring genetic change has thus become the focus of intense research effort. Towards this end, iPS cells have been derived using excisable lentiviral and transposon vectors, or through repeated application of transient plasmid, episomal, and adenovirus vectors (Chang et al., 2009; Kaji et al., 2009; Okita et al., 2008; Stadtfeld et al., 2008c; Woltjen et al., 2009; Yu et al., 2009b). Human iPS cells have also been derived using two DNA-free methods: serial protein transduction with recombinant proteins incorporating cell-penetrating peptide moieties (Kim et al., 2009a; Zhou et al., 2009), and transgene delivery using the Sendai virus, which has a completely RNA-based reproductive cycle (Fusaki et al., 2009).

Despite such progress, considerable limitations accompany the non-integrative iPS derivation strategies devised thus far. For example, while DNA transfection-based methodologies are ostensibly safe, they nonetheless entail some risk of genomic recombination or insertional mutagenesis. In protein-based strategies, the recombinant proteins used are challenging to generate and purify in the quantities required (Zhou et

al., 2009). Use of Sendai virus requires stringent steps to purge reprogrammed cells of replicating virus, and the sensitivity of the viral RNA replicase to transgene sequence content may limit the generality of this reprogramming vehicle (Fusaki et al., 2009). Importantly, methods that rely on repeat administration of transient vectors, whether DNA or protein-based, have so far shown very low iPS derivation efficiencies (Jia et al., 2010; Kim et al., 2009a; Okita et al., 2008; Stadtfeld et al., 2008c; Yu et al., 2009b; Zhou et al., 2009), presumably due to weak or inconstant expression of reprogramming factors.

Here we demonstrate that repeated administration of synthetic messenger RNAs incorporating modifications designed to bypass innate anti-viral responses can reprogram differentiated human cells to pluripotency with conversion efficiencies and kinetics substantially superior to established viral protocols. Furthermore, this simple, non-mutagenic, and highly controllable technology is applicable to a range of tissue engineering tasks, exemplified here by RNA-mediated directed differentiation of RNA-iPS (RiPS) cells to terminally differentiated myogenic cells.

3.2.3 Results

Development of modified-RNAs for directing cell fate

We manufactured mRNA using in vitro transcription (IVT) reactions templated by PCR amplicons (Figure 4-21).

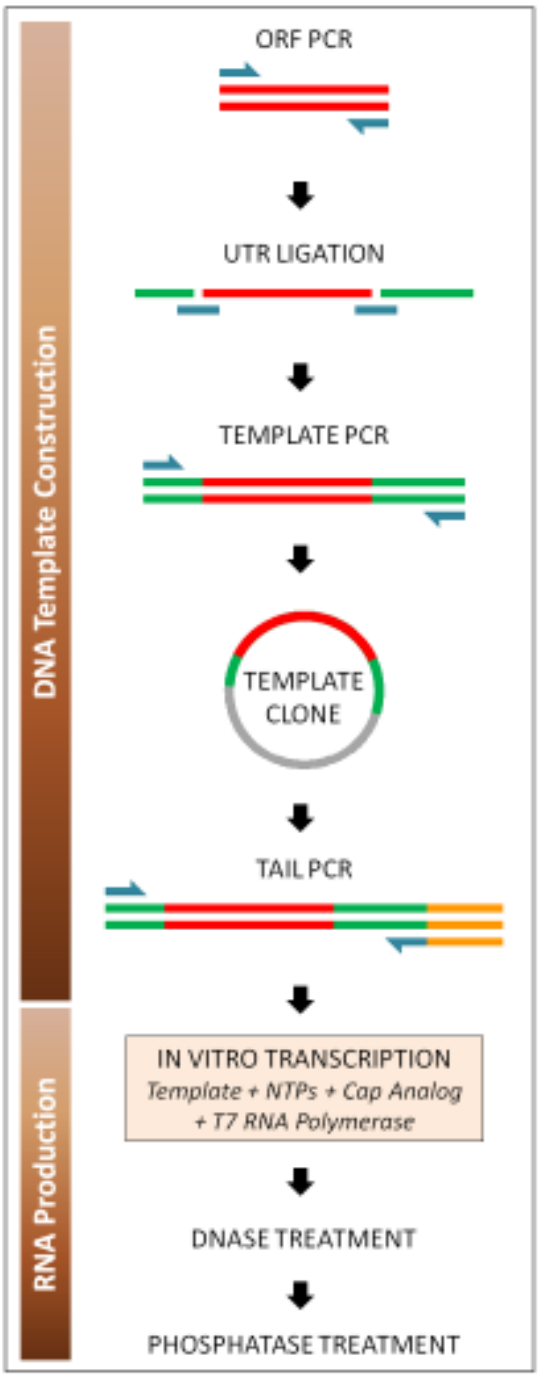


Figure 3-21 Schematic of how the modified synthetic mRNA is produced

To promote efficient translation and boost RNA half-life in the cytoplasm, a 5' guanine cap was incorporated by inclusion of a synthetic cap analog in the IVT reactions (Yisraeli et al., 1989). Within our IVT templates, the open reading frame (ORF) of the gene of interest is flanked by a 5' untranslated region (UTR) containing a strong Kozak

translational initiation signal, and an alpha-globin 3' UTR terminating with an oligo(dT) sequence for templated addition of a polyA tail.

Cytosolic delivery of mRNA into mammalian cells can be achieved using electroporation or by complexing the RNA with a cationic vehicle to facilitate uptake by endocytosis (Audouy and Hoekstra, 2001; Elango et al., 2005; Holtkamp et al., 2006; Van den Bosch et al., 2006; Van Tendeloo et al., 2001). We focused on the latter approach, reasoning that this would allow for repeated transfection to sustain ectopic protein expression over the days to weeks required for cellular reprogramming. In preliminary experiments in which synthetic RNA encoding GFP was transfected into murine embryonic fibroblasts and human epidermal keratinocytes, a high, dose-dependent cytotoxicity was observed that was not attributable to the cationic vehicle, which was exacerbated on repeated transfection. These experiments revealed a serious impediment to achieving sustained protein expression by mRNA transfection, and highlighted a need to develop a technology that would permit sustained protein expression with mRNA with reduced cellular toxicity.

It is known that exogenous single-stranded RNA (ssRNA) activates antiviral defenses in mammalian cells through interferon and NF-□B dependent pathways (Diebold et al., 2004; Hornung et al., 2006; Kawai and Akira, 2007; Pichlmair et al., 2006; Uematsu and Akira, 2007). We sought approaches to reduce the immunogenic profile of synthetic RNA in order to increase the sustainability of RNA-mediated protein expression. Co-transcriptional capping yields a significant fraction of uncapped IVT products bearing 5' triphosphates, which can be detected by the ssRNA sensor RIG-I (Hornung et al., 2006; Pichlmair et al., 2006), and have also been reported to activate PKR, a global repressor of protein translation (Nallagatla and Bevilacqua, 2008). We therefore treated synthesized RNA with a phosphatase, which resulted in modest reductions in cytotoxicity upon transfection (data not shown).

Eukaryotic mRNA is extensively modified *in vivo*, and the presence of modified nucleobases has been shown to reduce signaling by RIG-I and PKR, as well as by the less widely expressed but inducible endosomal ssRNA sensors TLR7 and TLR8 (Kariko et al., 2005; Kariko et al., 2008; Kariko and Weissman, 2007; Nallagatla and

Bevilacqua, 2008; Nallagatla et al., 2008; Uzri and Gehrke, 2009). In an attempt to further reduce innate immune responses to transfected RNA, we synthesized mRNAs incorporating modified ribonucleoside bases. Complete substitution of either 5-methylcytidine (5mC) for cytidine, or pseudouridine (psi) for uridine in GFP-encoding transcripts markedly improved viability and increased ectopic protein expression, while the most significant improvement was seen when both modifications were used together (Figure 4-22 A-C).

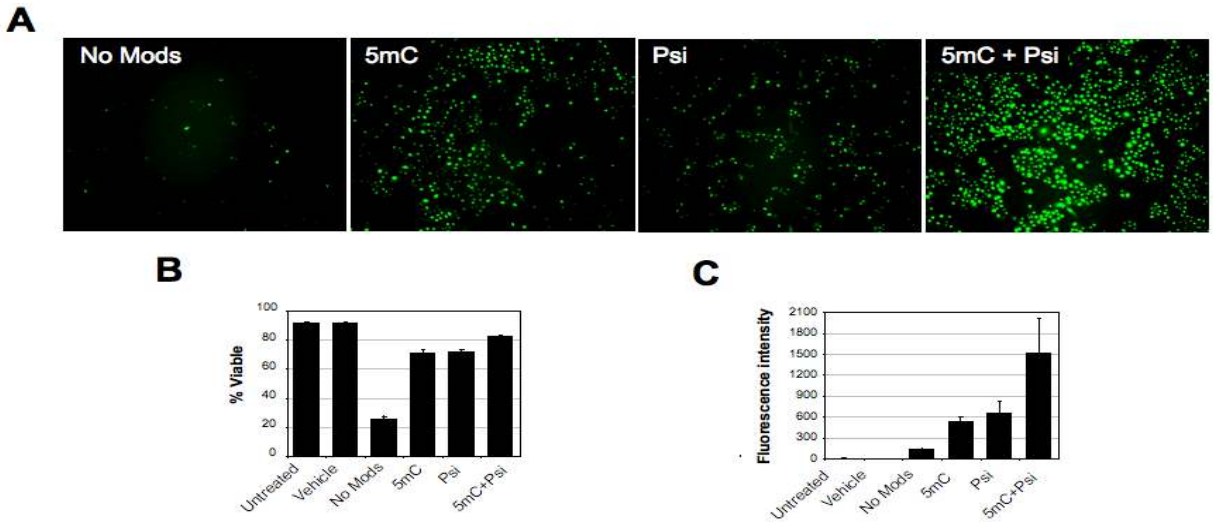


Figure 3-22 Modified-RNA overcomes anti-viral responses and can be used to direct cell fate. (A) Microscopy images showing keratinocytes transfected 24 hours earlier with 400 ng/well of synthetic unmodified (No Mods), 5-methyl-cytosine modified (5mC), pseudouridine modified (Psi), or 5mC + Psi modified-RNA encoding GFP. (B) Percent viability and (C) mean fluorescence intensity of the cells shown in (A) as measured by flow cytometry.

These modifications dramatically attenuated interferon signaling as revealed by qRT-PCR for a panel of interferon response genes, although residual upregulation of some interferon targets was still detected (Figure 4-23).

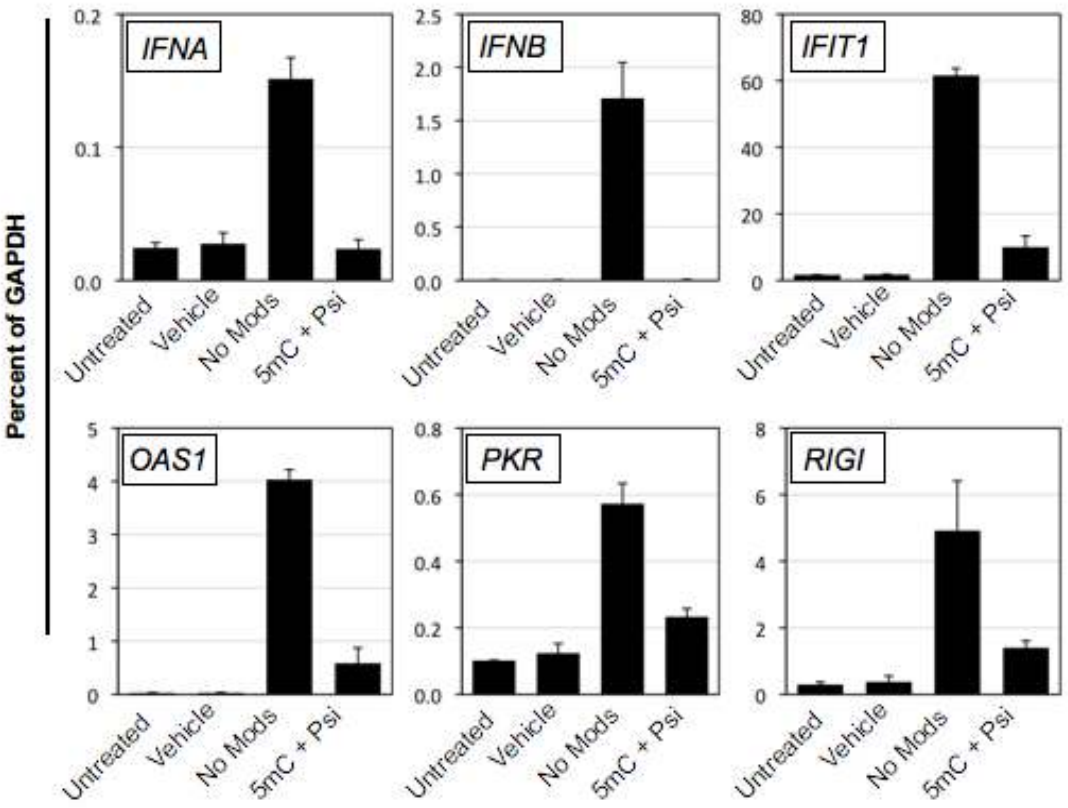


Figure 3-23 Quantitative RT-PCR data showing expression of six interferon-regulated genes in BJ fibroblasts 24 hours after transfection with unmodified (No Mods), or modified (5mC + Psi) RNA encoding GFP (1200 ng/well), and vehicle and untransfected controls.

It is known that cellular anti-viral defenses can self-prime through a positive-feedback loop involving autocrine and paracrine signaling by Type I interferons (Randall and Goodbourn, 2008). Media supplementation with a recombinant version of B18R protein, a Vaccinia virus decoy receptor for Type I interferons (Symons et al., 1995), further increased cell viability of RNA transfection in some cell types (data not shown). Synthesis of RNA with modified ribonucleotides and phosphatase treatment (henceforth, modified-RNAs), used in conjunction with media supplementation with the interferon inhibitor B18R allowed for high, dose-dependent levels of protein expression with high cell viability (Figure 4-24).

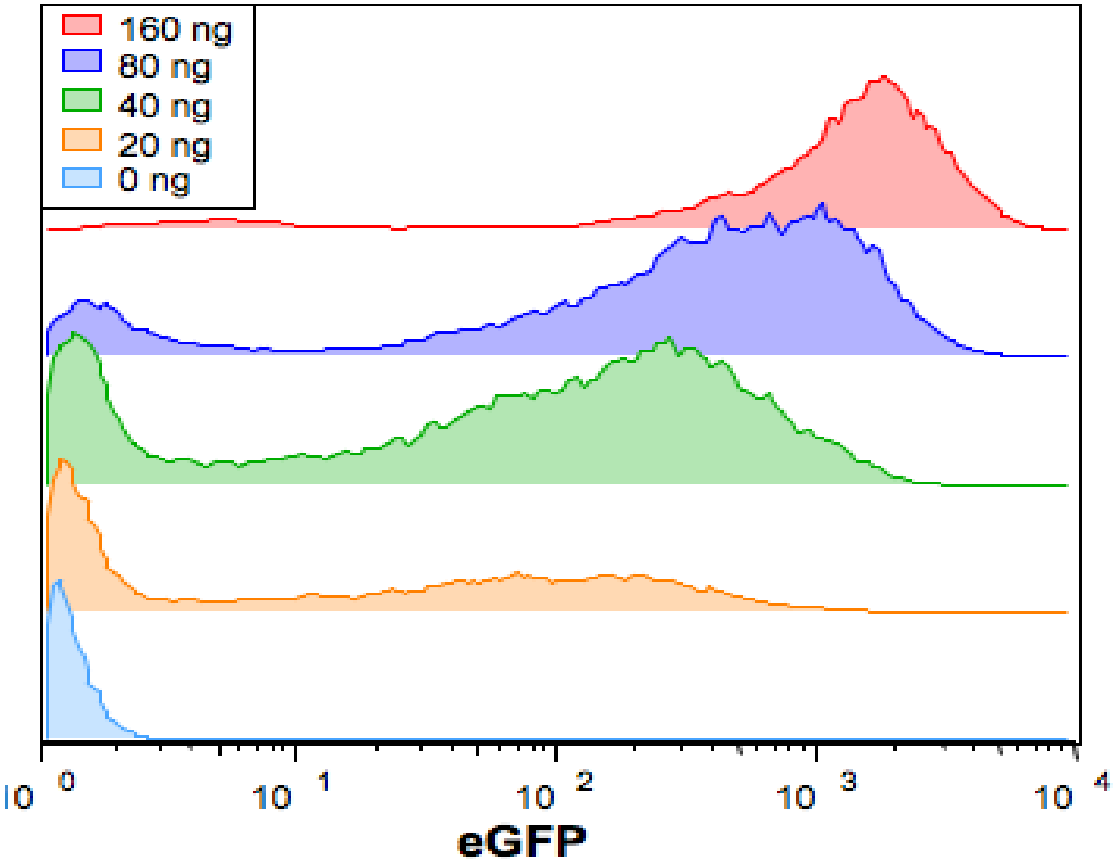
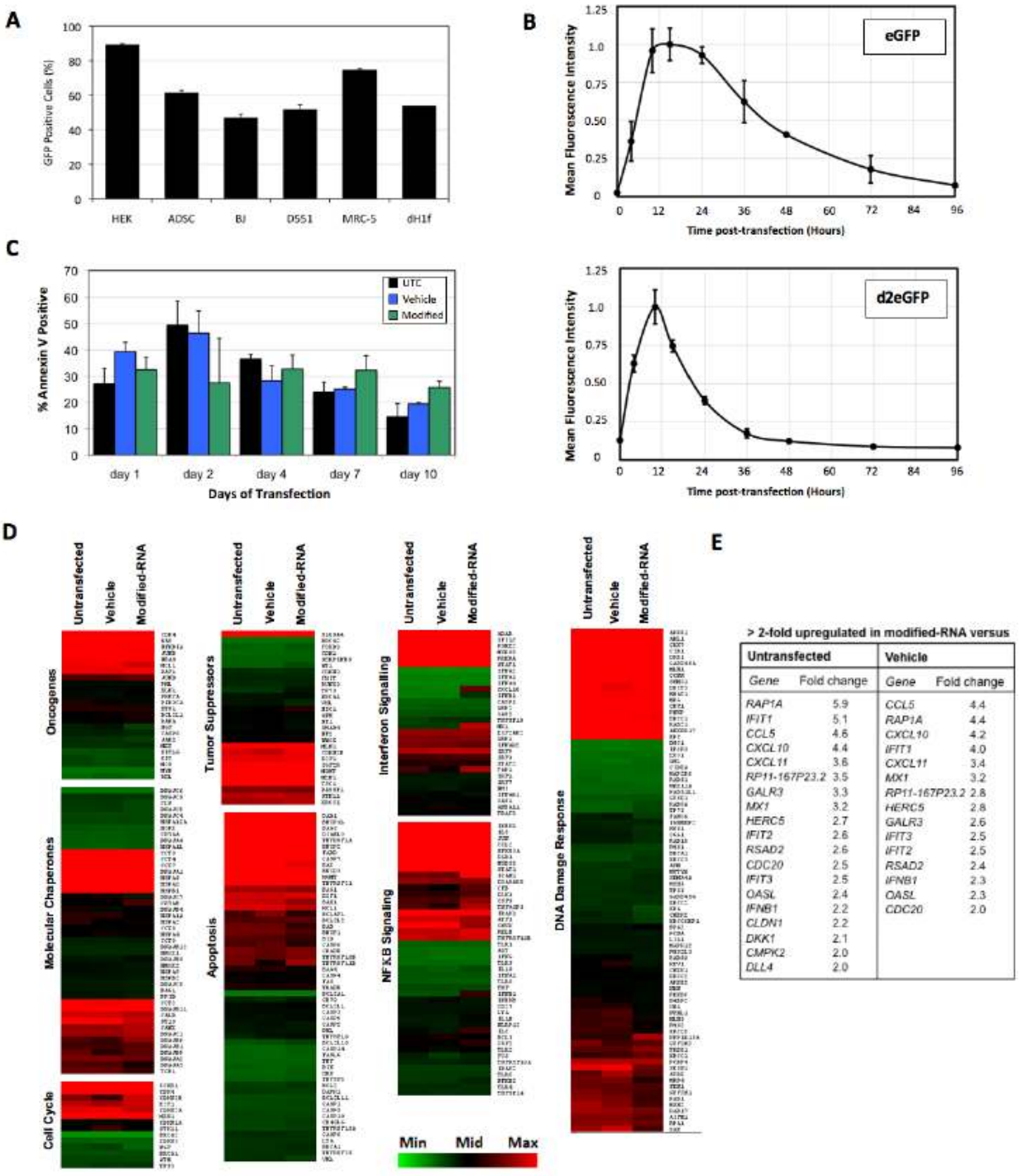


Figure 3-24 (Flow cytometry histograms showing GFP expression in keratinocytes transfected with 0-160 ng of modified-RNA, 24 hours post transfection.

Transfection of modified-RNA encoding GFP into six human cell types showed highly penetrant expression (50-90% positive cells), demonstrating the applicability of this technology to diverse cell types (Figure 4-25 A).



Untransfected		Vehicle	
Gene	Fold change	Gene	Fold change
RAP1A	5.9	*CCL5*	4.4
IFIT1	5.1	*RAP1A*	4.4
CCL5	4.6	*CXCL10*	4.2
CXCL10	4.4	*IFIT1*	4.0
CXCL11	3.6	*CXCL11*	3.4
RP11-167P23.2	3.5	*MX1*	3.2
GALR3	3.3	*RP11-167P23.2*	2.8
MX1	3.2	*HERC5*	2.8
HERC5	2.7	*GALR3*	2.6
IFIT2	2.6	*IFIT3*	2.5
RSAD2	2.6	*IFIT2*	2.5
CDC20	2.5	*RSAD2*	2.4
IFIT3	2.5	*IFNB1*	2.3
OASL	2.4	*OASL*	2.3
IFNB1	2.2	*CDC20*	2.0
CLDN1	2.2		
DKK1	2.1		
CMPK2	2.0		
DLL4	2.0		

Figure 3-25 Modified RNA can be used to express proteins in multiple human cell types (A), the timecourse expression of high and low stability GFPs (B), apoptosis assays over a time course of repeated transfection (C), heatmaps showing microarray data of

stress pathways in cells repeat transfected with modified-RNA (D), and genes upregulated after repeat transfection (E).

Simultaneous delivery of modified-RNAs encoding mCherry, and GFP containing a nuclear localization signal confirmed that generalized co-expression of multiple proteins could be achieved in mammalian cells, and that expressed proteins could be correctly localized to different cellular compartments (Figure 4-26).

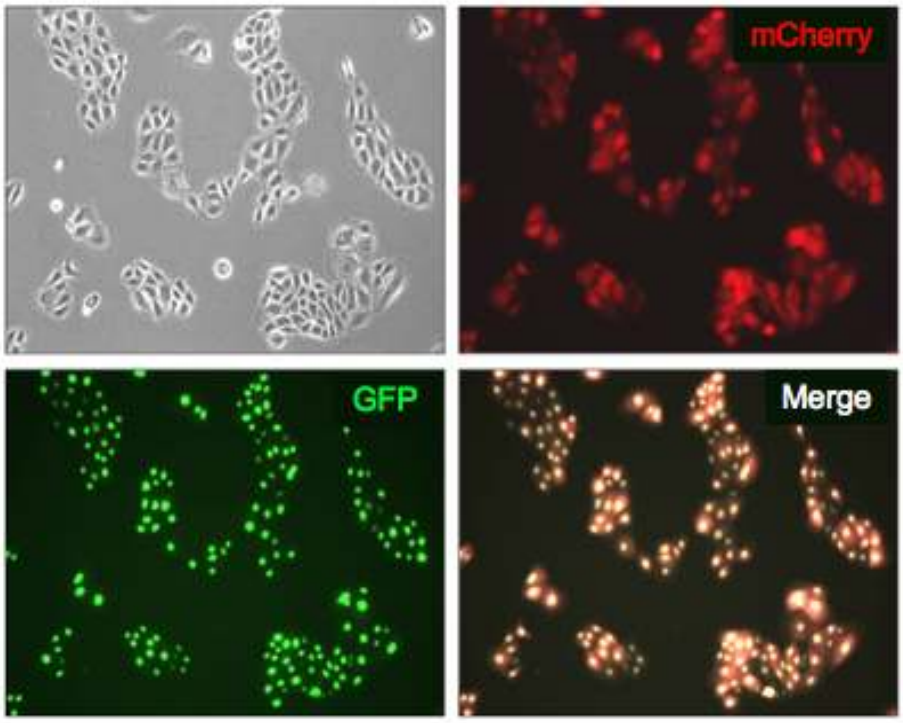


Figure 3-26 (F) Microscopy images of keratinocytes co-transfected with modified-RNAs encoding GFP with a nuclear localization signal, and mCherry (cytosolic) proteins.

Ectopic protein expression after RNA transfection is transient owing to RNA and protein degradation and the diluting effect of cell division. To establish the kinetics and persistence of protein expression, modified-RNA encoding GFP variants designed for high and low protein stability (Li et al., 1998) were synthesized and transfected into keratinocytes. Time course analysis by flow cytometry showed that protein expression persisted for several days for the high-stability variant, but peaked within 12 hours and decayed rapidly thereafter for the destabilized GFP (Figure 4-25 B).

These results indicated that a repetitive transfection regimen would be required to sustain high levels of ectopic expression for short-lived proteins over an extended time course. To address this and further examine the impact of repeated RNA transfection on cell growth and viability we transfected BJ fibroblasts for 10 consecutive days with either unmodified, or modified-RNAs encoding a destabilized nuclear GFP and appropriate controls (Figure 4-27 A).

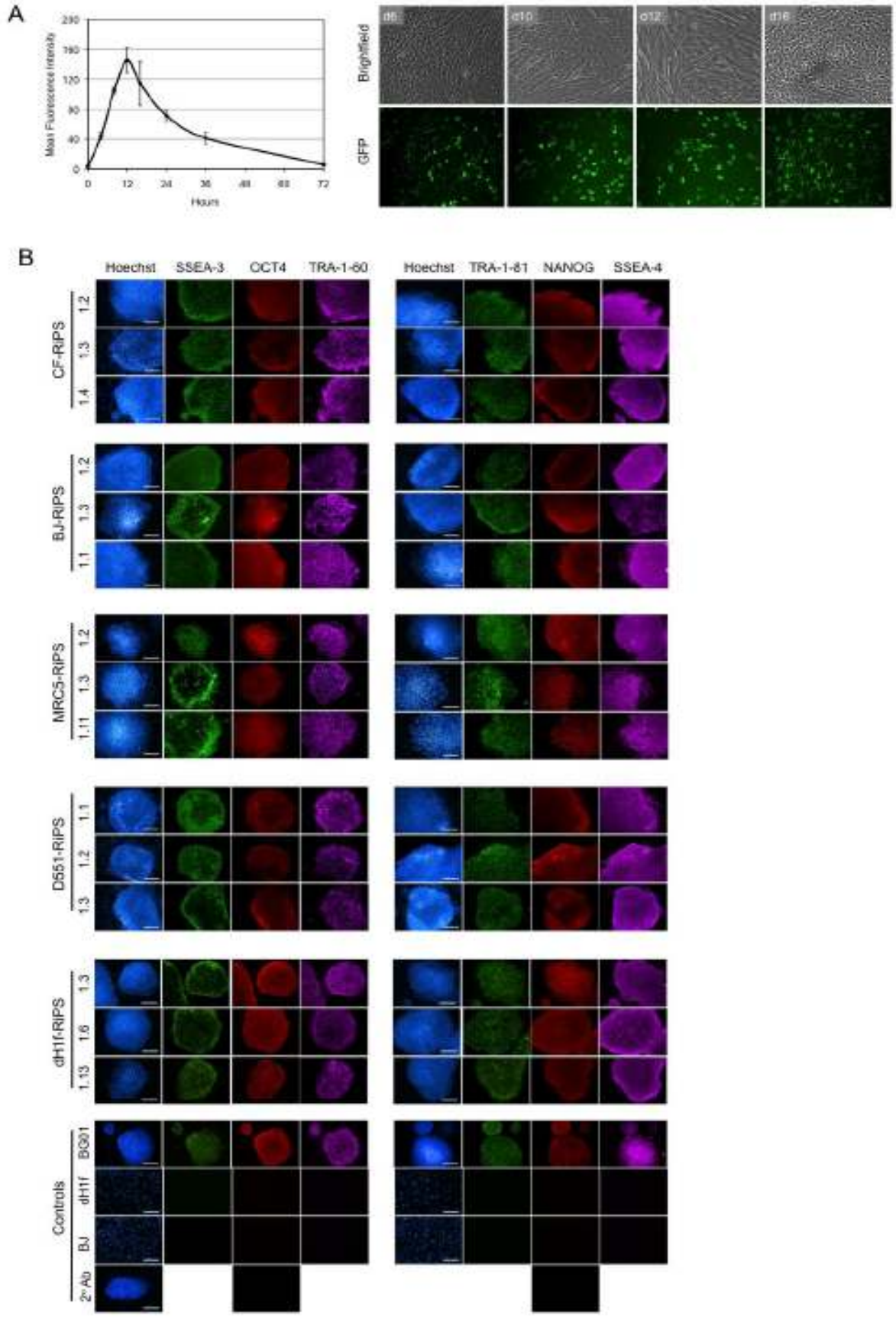

A
Mean Fluorescence Intensity
Hours
Brightfield
GFP
d8
d10
d12
d16
B
Hoechst
SSEA-3
OCT4
TRA-1-60
Hoechst
TRA-1-81
NANOG
SSEA-4
CF-RiPS
1.2
1.3
1.4
BJ-RiPS
1.2
1.3
1.1
MRC5-RiPS
1.2
1.3
1.11
D551-RiPS
1.1
1.2
1.3
dH1f-RiPS
1.3
1.6
1.13
Controls
BG01
dH1f
BJ
2° Ab

Figure 3-27 *iPS-derivation from five human cell types*. (A) Expression time course of low-stability nuclear GFP after a single transfection into keratinocytes, assessed by flow cytometry (right panel). Bright-field and GFP images (right panel) taken at four different time points during a reprogramming experiment. RNA-encoding the low-stability GFP analyzed in the left panel was spiked into the reprogramming cocktail (KMOSL) to visualize sustained protein expression from transfected modified-RNAs during iPS reprogramming (right panel). (B) Antibody stains of independent RiPS clones derived from cells taken from an adult cystic fibrosis patient (CF cells), BJ postnatal fibroblasts, MRC-5 and Detroit 551 fetal fibroblasts, and human ES-derived dH1f fibroblasts. Panels show cell-surface staining for SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, and intracellular staining for OCT4 and NANOG. Control stains of BG01 hES cells, dH1f and BJ fibroblasts are shown. Additional control stains show the specificity of the secondary antibody used for the OCT4 and NANOG intracellular stains.

Daily transfection with modified-RNA permitted sustained protein expression without substantially compromising the viability of the culture beyond a modest reduction in growth kinetics that was largely attributable to the transfection reagent (Figure 4-28 A-B, Figure 4-25 C).

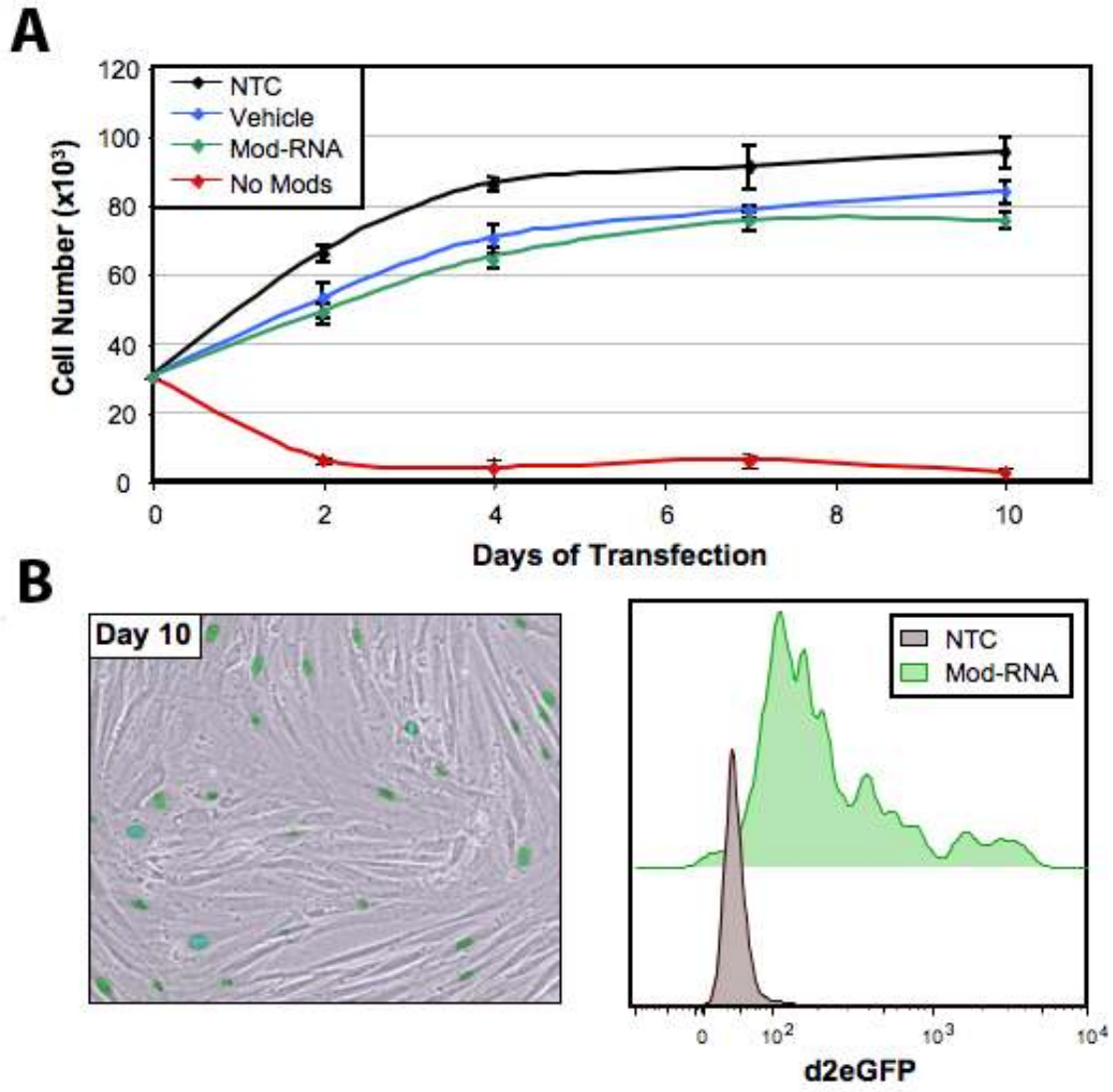


Figure 3-28 (G) Growth kinetics of BJ fibroblasts transfected daily with unmodified, or modified-RNAs encoding a destabilized nuclear-localized GFP, and vehicle and untransfected controls for 10 days. (H) Sustained GFP expression of modified-RNA transfected cells described in (G) at day 10 of transfection shown by fluorescence imaging with bright field overlay (left panel), and flow cytometry (right panel).

Microarray analysis of the cultures following the tenth and final transfection revealed that prolonged daily transfection with modified-RNA did not significantly alter the molecular profile of the transfected cells (Figure 4-25 D), although upregulation of a number of interferon response genes was noted consistent with our previous

observation that the RNA modifications did not completely abrogate interferon signaling (Figure 4-23, Figure 4-25 E). By contrast, repeated transfection with unmodified RNA severely compromised the growth and viability of the culture through elicitation of a massive interferon response (Figure 4-23), indicating that the use of unmodified RNA was not a viable strategy for sustaining polypeptide expression in cells (Figure 4-28 A).

To determine if modified-RNAs could be used to directly alter cell fate, we synthesized modified-RNA encoding the myogenic transcription factor *MYOD* (Davis et al., 1987) and transfected it into murine C3H10T1/2 cells over the course of 3 days, followed by continued culturing in a low serum media for an additional 3 days. The emergence of large, multi-nucleated myotubes that stained positive for the myogenic markers myogenin and myosin heavy chain (MyHC) provided proof of principle that transfection with modified-RNAs could be utilized to efficiently direct cell fate (Figure 4-29).

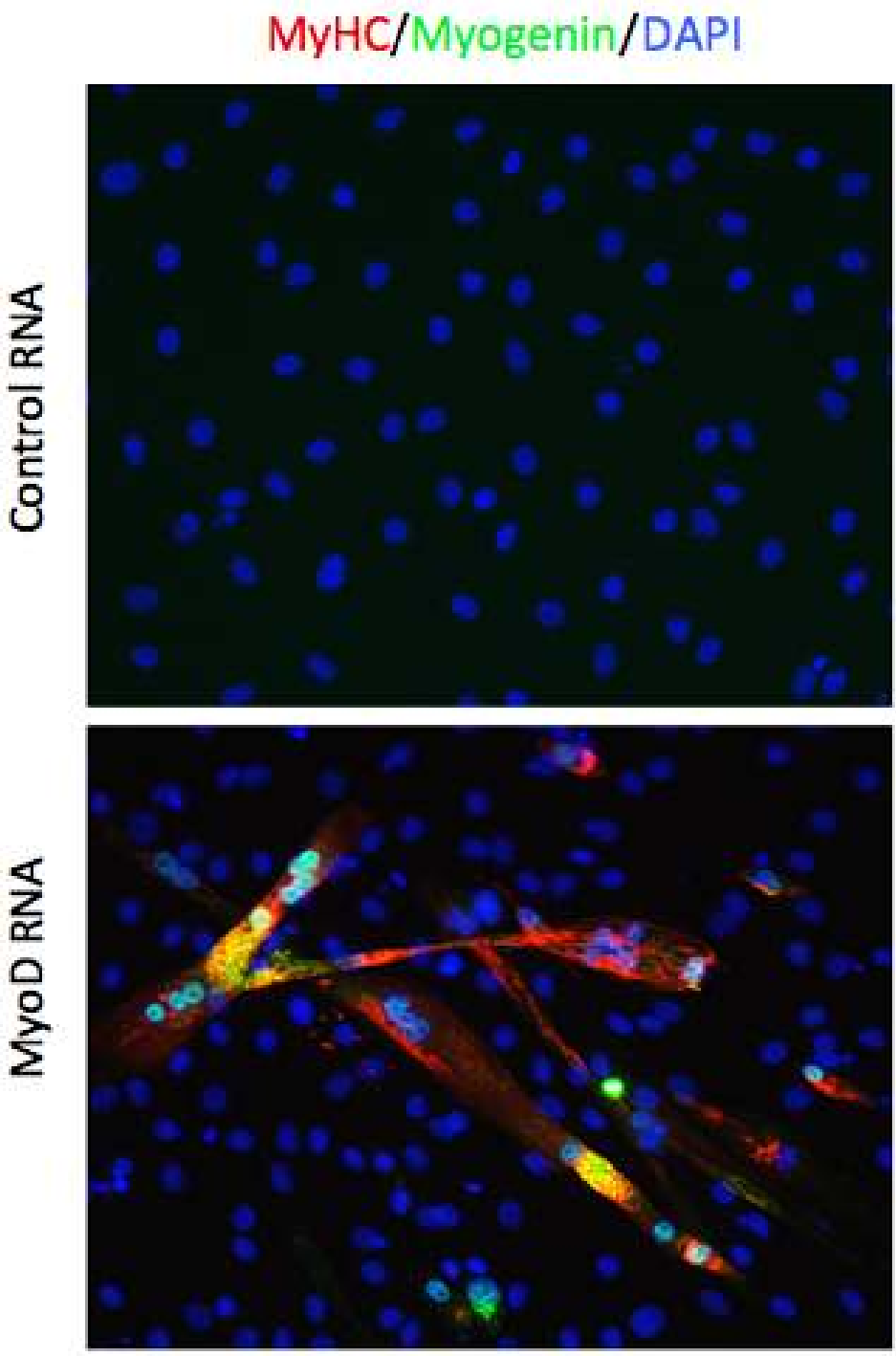


Figure 3-29 Immunostaining for the muscle-specific proteins myogenin and myosin heavy chain (MyHC) in murine C3H/10T1/2 cell cultures 3 days after 3 consecutive daily transfections with a modified-RNA encoding *MYOD*. Error bars indicate s.d., n=3 for all panels.

Generation of induced pluripotent stem cells using modified-RNAs

We next sought to determine if induced pluripotent stem cells (iPS) could be derived using modified-RNAs. To this end, modified-RNAs encoding the four canonical Yamanaka factors, *KLF4* (K), *c-MYC* (M), *OCT4* (O), and *SOX2* (S), were synthesized, and transfected into cells. Immuno-staining with antibodies directed against OCT4, KLF4 and SOX2 demonstrated that each of the factors was robustly expressed and correctly localized to the nucleus (Figure 4-30).

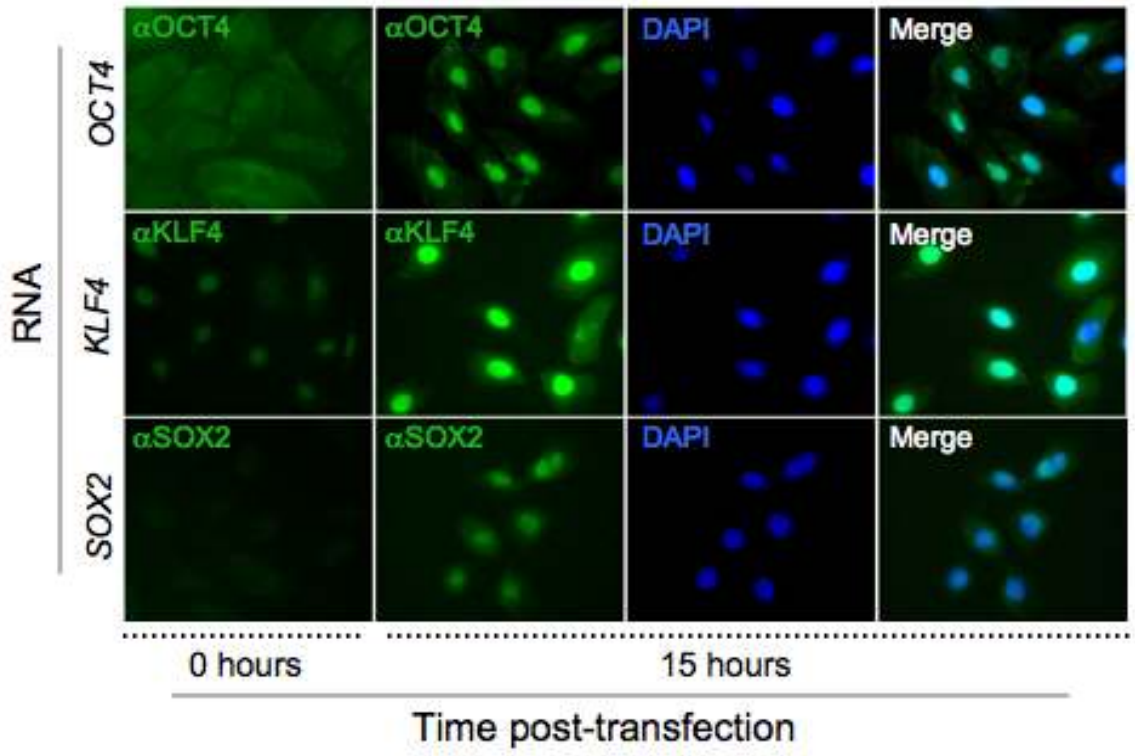


Figure 3-30 *Generation of RNA-induced pluripotent stem cells (RiPS).* (A) Immunostaining for human KLF4, OCT4, and SOX2 proteins in keratinocytes 15 hours post-transfection with modified-RNA encoding KLF4, OCT4, or SOX2.

Expression kinetics was monitored by flow cytometry, which showed maximal protein expression 12 to 18 post-transfection, followed by rapid turnover of these transcription factors (Figure 4-31).

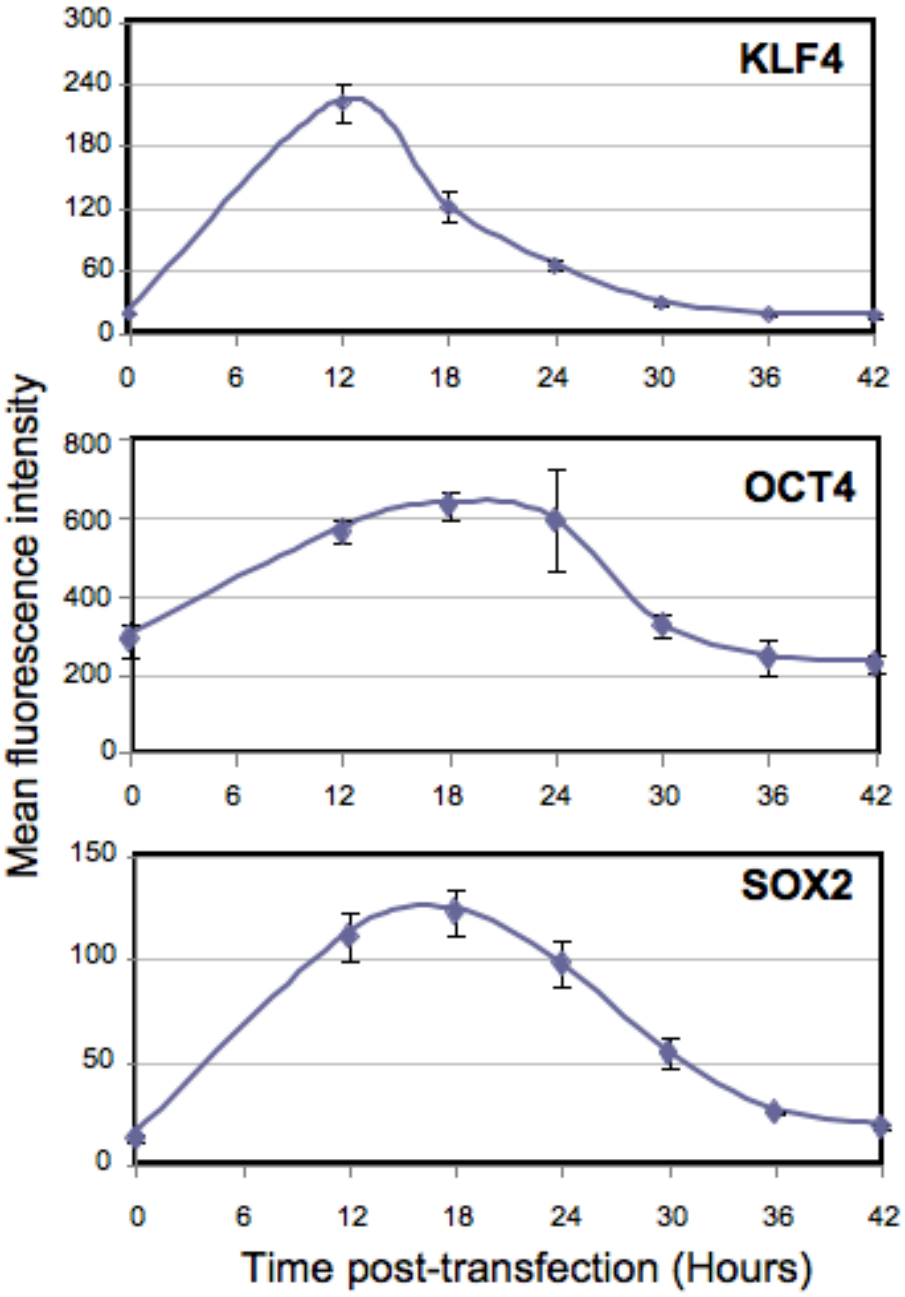


Figure 3-31 Time course showing kinetics and stability of KLF4, OCT4, and SOX2 proteins after modified-RNA transfection, assayed by flow cytometry following intracellular staining of reach protein.

From this we concluded that daily transfections would be required to maintain high levels of expression of the Yamanaka factors during long-term, multi-factor reprogramming regimens.

We next sought to establish a protocol to ensure sustained, high-level protein expression through daily transfection of modified-RNAs by exploring a matrix of conditions encompassing different transfection reagents, culture media, feeder cell types, and RNA doses (data not shown). Once optimized, we initiated long-term reprogramming experiments with human ES-derived dH1f fibroblasts, which display relatively efficient viral-mediated iPS cell conversion (Chan et al., 2009; Park et al., 2008a). Low-oxygen (5% O2) culture conditions and a KMOS stoichiometry of 1:1:3:1 were employed, as these have been reported to promote efficient iPS conversion (Kawamura et al., 2009; Papapetrou et al., 2009; Utikal et al., 2009; Yoshida et al., 2009). Modified-RNA encoding a destabilized nuclear GFP was spiked into the KMOS RNA cocktail to allow visualization of continued protein expression throughout the experimental time course (Figure 4-27A). Widespread transformation of fibroblast morphology to a compact, epithelioid morphology was observed within the first week of modified-RNA transfection, followed by emergence of canonical hES-like colonies with tight morphology, well-defined borders, and prominent nucleoli towards the end of the second week of transfection (Figure 4-32).

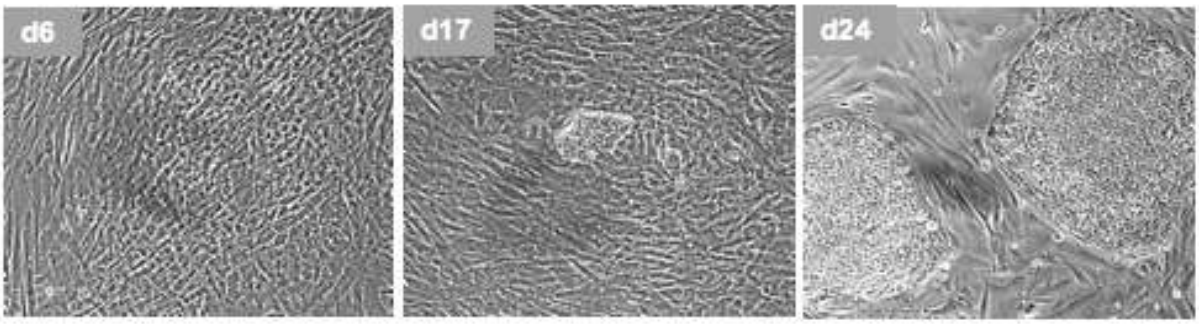


Figure 3-32 Brightfield images taken during the derivation of RNA-iPS cells (RiPS) from dH1f fibroblasts showing early epitheliod morphology (day 6), small hES-like colonies (day 17), and appearance of mature iPS clones after mechanical picking and expansion (day 24).

RNA transfection was terminated on day 17, and colonies were mechanically picked three days later which were then expanded under standard ES culture conditions to establish 14 prospective iPS lines, designated dH1f-RiPS (RNA-derived iPS) 1-14.

We next attempted to reprogram somatically-derived cells to pluripotency using a similar reprogramming regimen. Anticipating that these cells might be more challenging to reprogram, we employed a five-factor cocktail including a modified-RNA encoding LIN28 (KMOSL), which has been shown to facilitate reprogramming (Yu et al., 2007b), and supplemented the media with valproic acid (VPA), a histone deacetylase inhibitor, which has been reported to increase reprogramming efficiency (Huangfu et al., 2008). Four human cell types were tested: Detroit 551 (D551) and MRC-5 fetal fibroblasts, BJ post-natal fibroblasts, and fibroblast-like cells cultured from a primary skin biopsy taken from an adult cystic fibrosis patient (CF cells). Daily transfection with the modified-RNA KMOSL cocktail gave rise to numerous hES-like colonies in the D551, BJ, CF and MRC5 cultures that were mechanically picked at day 18, 20, 21 and 25, respectively. More than 10 RiPS clones were expanded for each of the somatic lines, with notably very few clones failing to establish. Immunostaining confirmed the expression of OCT4, NANOG, TRA-1-60, TRA-1-81, SSEA3, and SSEA4 in all the RiPS lines examined (Figure 4-33, Figure 4-27 B).

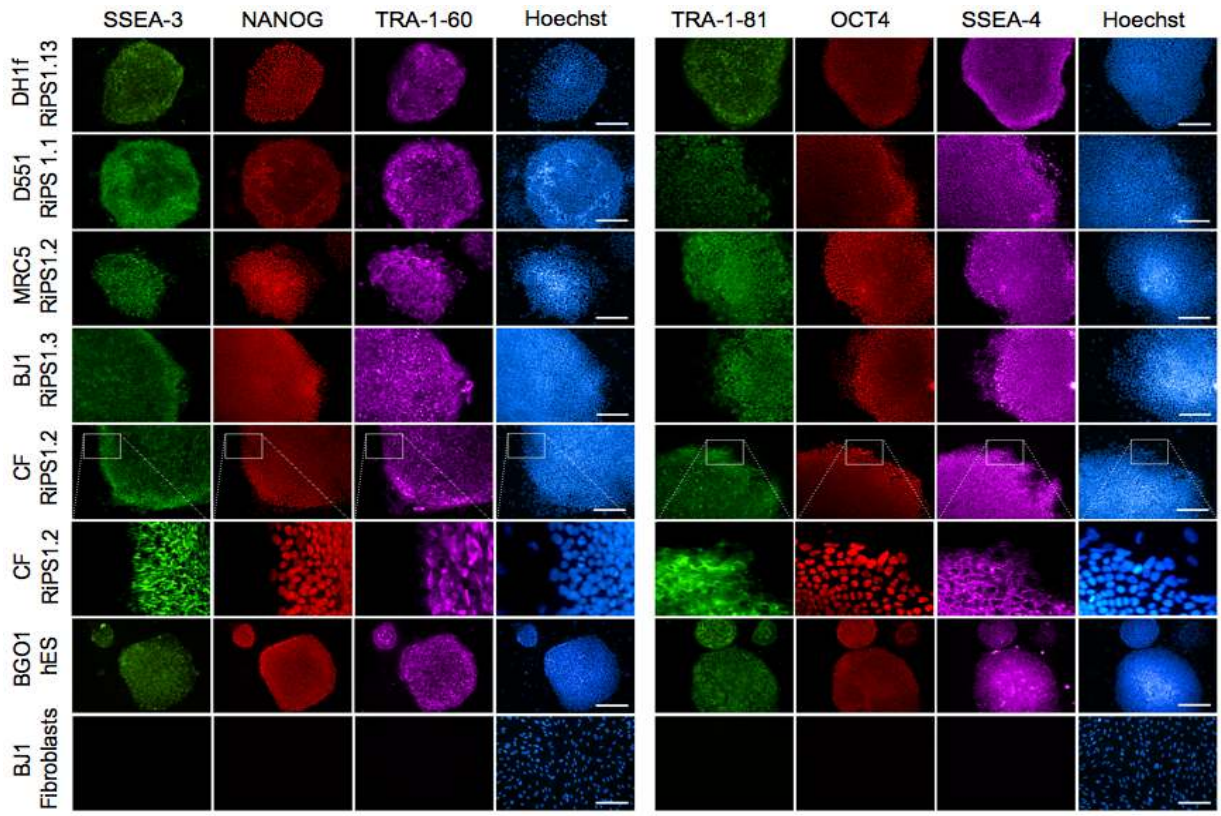


Figure 3-33 Immunohistochemistry showing expression of a panel of pluripotency markers in expanded RiPS clones derived from dH1f fibroblasts, Detroit 551 (D551) and MRC-5 fetal fibroblasts, BJ post-natal fibroblasts, and cells derived from a skin biopsy taken from an adult cystic fibrosis patient (CF), shown also in high magnification. BG01 hES cells and BJ1 fibroblasts are included as positive and negative controls, respectively.

DNA fingerprinting confirmed parental origin of three RiPS clones from each somatic cell derivations, and all clones presented normal karyotypes (data not shown). Of note, additional experiments conducted in the presence or absence of VPA showed little difference in reprogramming efficiency (data not shown), and VPA was therefore not used in subsequent experiments.

Molecular characterization and functional potential of RiPS cells

A number of molecular and functional assays were performed to further assess whether the RiPS cells had been reprogrammed to pluripotency (Table 4-34).

Immunostaining[#]	qRT-PCR	Bisulfite Sequencing[Ω]	Microarray	Developmental Potential	
				In vitro	Teratoma
dH1F-RiPS-1.3 dH1F-RiPS-1.6 dH1F-RiPS-1.13 BJ-RiPS-1.1 BJ-RiPS-1.2 BJ-RiPS-1.3 MCR5-RiPS-1.2 MCR5-RiPS-1.3 MCR5-RiPS-1.11 CF-RiPS-1.2 CF-RiPS-1.3 CF-RiPS-1.4 D551-RiPS-1.1 D551-RiPS-1.2 D551-RiPS-1.3	dH1F-RiPS-1.2 dH1F-RiPS-1.3 dH1F-RiPS-1.6 dH1F-RiPS-1.7 BJ-RiPS-1.1 BJ-RiPS-1.2 BJ-RiPS-1.3 MCR5-RiPS-1.8 MCR5-RiPS-1.9 MCR5-RiPS-1.11 CF-RiPS-1.2 CF-RiPS-1.3 CF-RiPS-1.4 D551-RiPS-1.1 D551-RiPS-1.2 D551-RiPS-1.3	dH1F-RiPS-1.2 dH1F-RiPS-1.3 dH1F-RiPS-1.6 BJ-RiPS-1.2 BJ-RiPS-1.3 MCR5-RiPS-1.8 MCR5-RiPS-1.9 MCR5-RiPS-1.11 CF-RiPS-1.2 CF-RiPS-1.3 CF-RiPS-1.4	dH1F-RiPS-1.2 dH1F-RiPS-1.3 dH1F-RiPS-1.6 dH1F-RiPS-1.7 BJ-RiPS-1.1 BJ-RiPS-1.2 BJ-RiPS-1.3 MCR5-RiPS-1.8 MCR5-RiPS-1.9 MCR5-RiPS-1.11 CF-RiPS-1.2 CF-RiPS-1.3 CF-RiPS-1.4	dH1F-RiPS-1.2^†ø* dH1F-RiPS-1.6^ø dH1F-RiPS-1.13^ø dH1F-RiPS-1.14^ø MCR5-RiPS-1.8^†* MCR5-RiPS-1.9^†* MCR5-RiPS-1.11^* BJ-RiPS-1.1^†ø* BJ-RiPS-1.2^†ø* BJ-RiPS-1.3^†* CF-RiPS-1.2^†* CF-RiPS-1.3^†* CF-RiPS-1.4^ø* D551-RiPS-1.1^†* D551-RiPS-1.2^* D551-RiPS-1.3^*	dH1F-RiPS-1.3 dH1F-RiPS-1.5 dH1F-RiPS-1.6 dH1F-RiPS-1.7 dH1F-RiPS-1.11 BJ-RiPS-1.1 BJ-RiPS-1.2 CF-RiPS-1.4

Figure 3-34 *Pluripotency validation assays performed in this study.* The table shows the RiPS clones that were validate in each assay. # Validated for immuno-staining for all of TRA-1-60, TRA-1-80, SSEA3, SSEA4, OCT4, NANOG. **Ω** Demethylation of the *OCT4* promoter. *In vitro* differentiation including ^embryoid body formation, [ø]trilineage by directed differentiation, [†] beating cardiomyocytes, and * blood formation by CFC assays in methylcellulose.

Multiple RiPS lines derived from each of the five starting cell types were evaluated by quantitative RT-PCR (qRT-PCR), and all demonstrated robust expression of the pluripotency-associated transcripts *OCT4*, *SOX2*, *NANOG,* and *hTERT* (Figure 4-35).

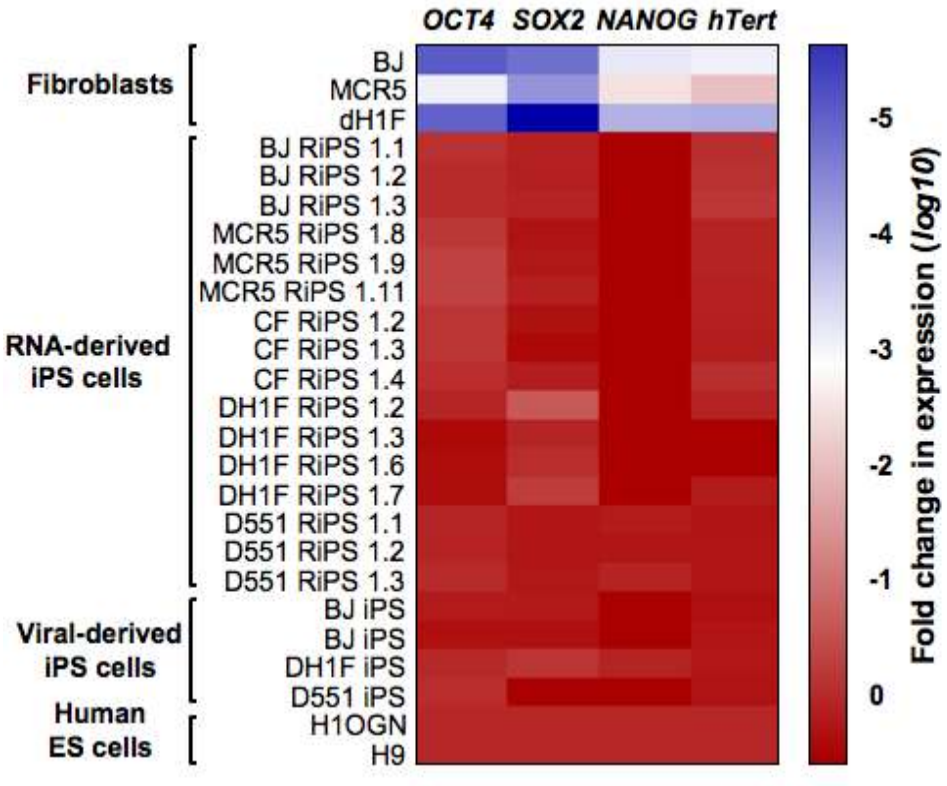


Figure 3-35 Heatmap showing results of qRT-PCR analysis measuring the expression of pluripotency-associated genes in RiPS cell lines, parental fibroblasts and viral-derived iPS cells relative to hES cell controls.

Bisulfite sequencing of the Oct4 locus revealed extensive demethylation relative to the parental fibroblasts, an epigenetic state equivalent to human ES cells (Figure 4-36).

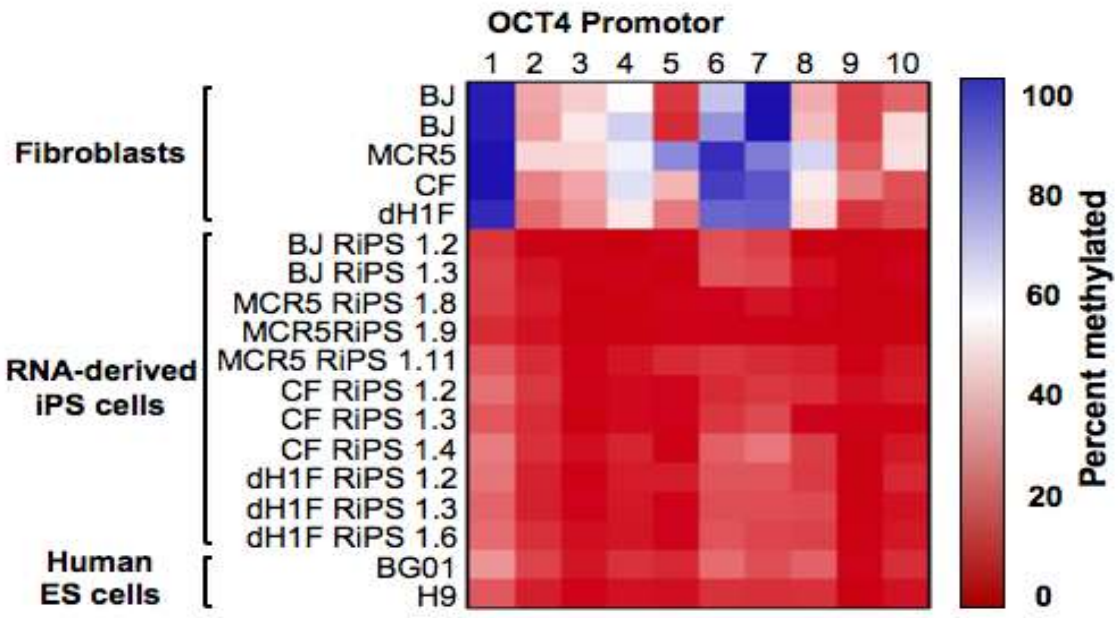


Figure 3-36 Heatmap showing results of OCT4 promoter methylation analysis of RiPS cell lines, parental fibroblasts, and hES cell controls.

To gain more global insight into the molecular properties of RiPS cells, gene expression profiles of RiPS clones from multiple independent derivations were generated and compared to fibroblasts, human ES cells, and virally-derived iPS cell lines. These analyses revealed that all modified-RNA-derived iPS clones examined had a molecular signature that very closely recapitulated that of human ES cells while being highly divergent from the profile of the parental fibroblasts (Figure 4-37).

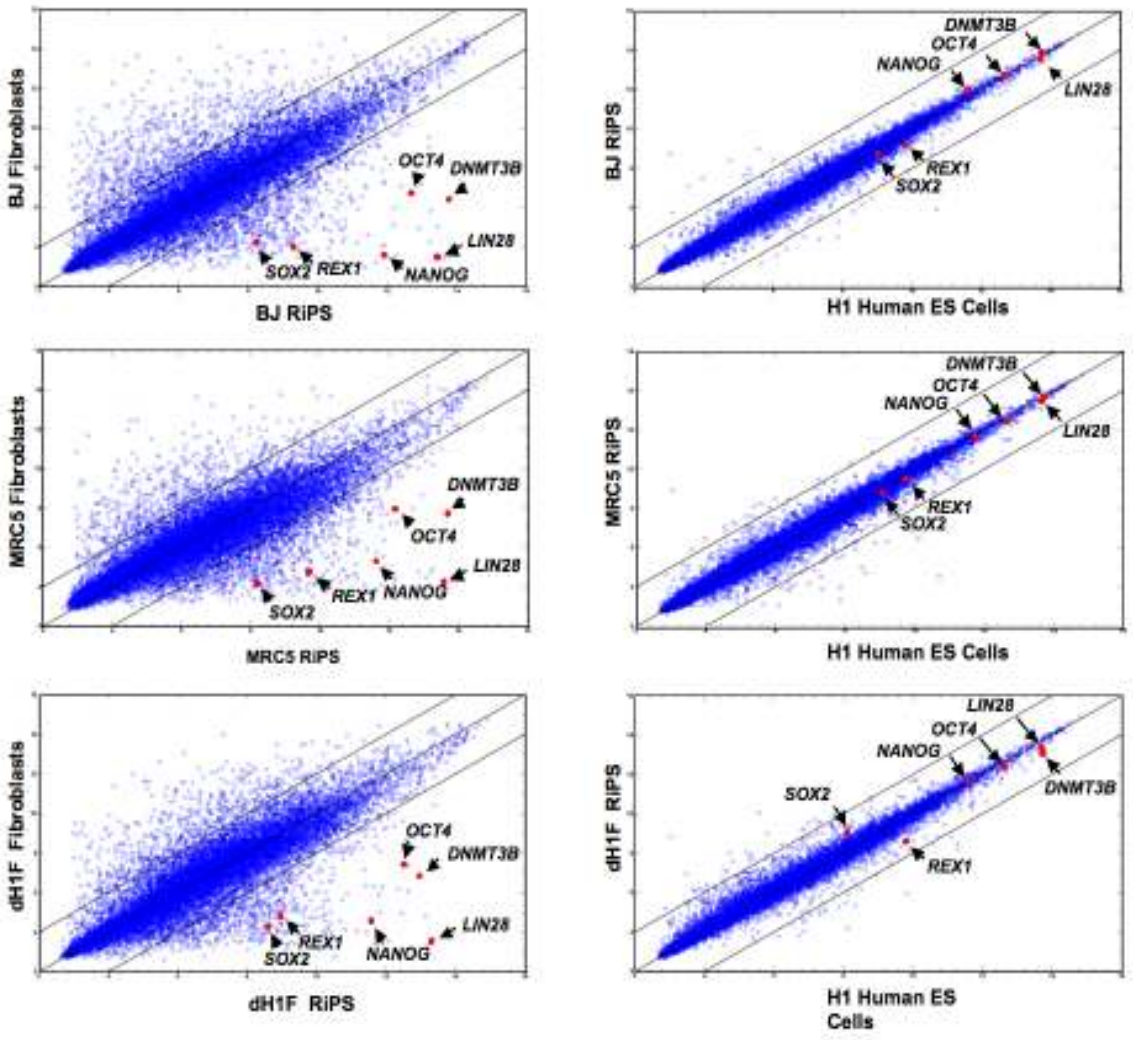


Figure 3-37 Global gene expression profiles of BJ-, MRC5- and dH1F-derived RiPS cells shown in scatter plots against parental fibroblasts and hES cells with pluripotency-associated transcripts indicated.

Importantly, pluripotency-associated transcripts including *SOX2, REX1, NANOG, OCT4, LIN28* and *DNMT3B* were substantially upregulated in the RiPS cells compared to the parental fibroblast lines to levels comparable to hES cells (Figure 4-37). Furthermore, when the transcriptional profiles were subjected to unsupervised hierarchical clustering analysis, all RiPS clones analyzed clustered more closely to hES than did virally-derived iPS cells suggesting that modified-RNA-derived iPS cells more fully recapitulated the molecular signature of human ES cells (Figure 4-38).

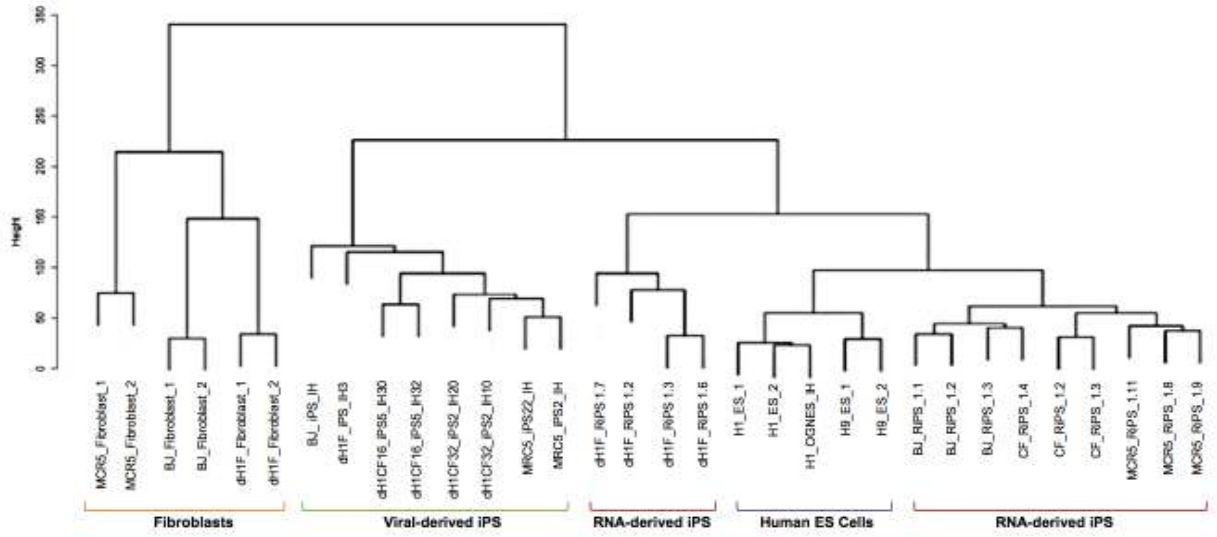


Figure 3-38 Dendrogram showing unsupervised hierarchical clustering of the global expression profiles for RiPS cells, parental fibroblasts, hES cells, and virus-derived iPS cells.

To test the developmental potential of RiPS cells, embryoid bodies (EBs) were generated from multiple clones from five independent RiPS derivations, and beating cardiomyocytes were observed for the vast majority of the EBs. Mesodermal potential was further evaluated in methylcellulose blood forming assays which showed that all lines tested were robustly able to differentiate into hematopoietic precursors capable of giving rise to colony numbers and a spectrum of blood colony types comparable to human ES cells (Figure 4-39, Table 4-34).

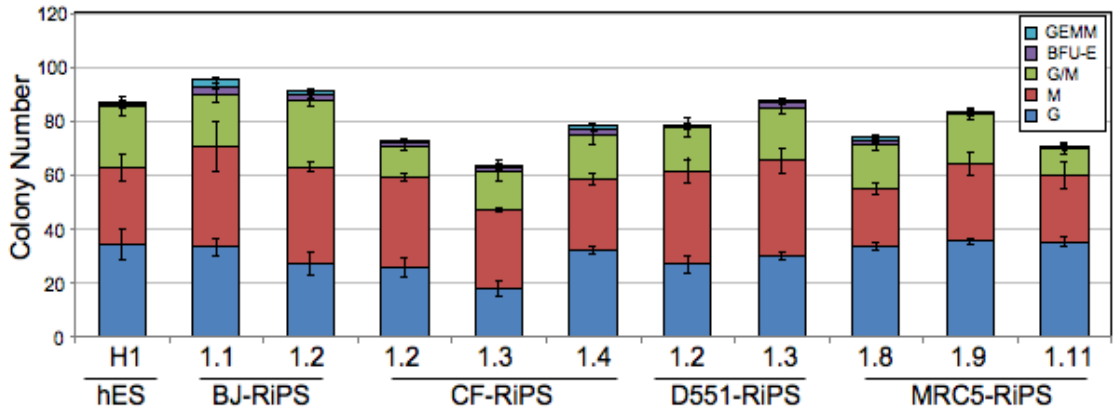


Figure 3-39 *Trilineage differentiation of RiPS cells.* Yield and typology of blood-lineage colonies produced by directed differentiation of embryoid bodies in methylcellulose assays with RiPS clones derived from BJ, CF, D551 and MCR5 fibroblasts, and a human ES (H1) control.

A subset of clones was further plated onto matrigel and differentiated into Tuj1-positive neurons (ectoderm), and alpha-fetoprotein-positive endodermal cells (Figure 4-40, Table 4-34).

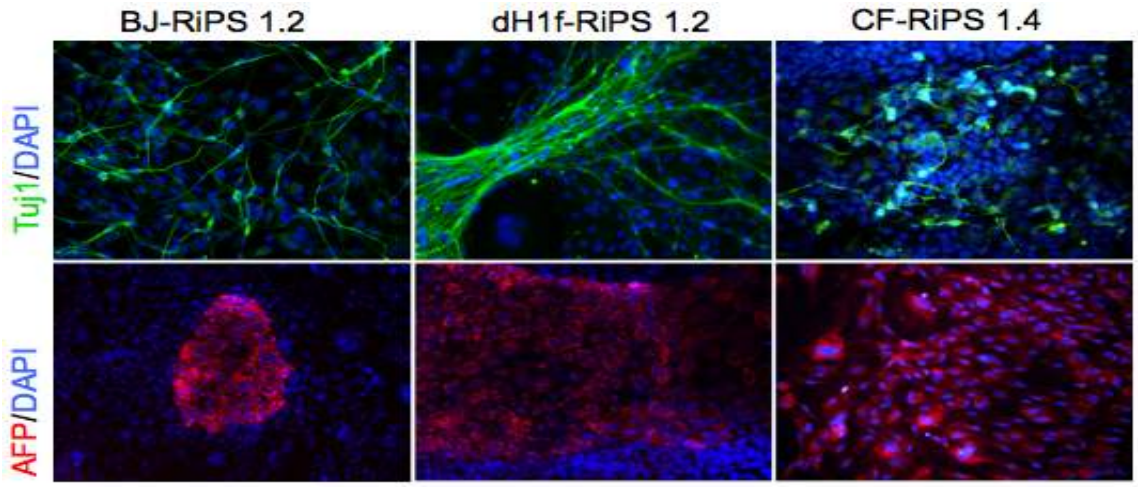


Figure 3-40 *Trilineage differentiation of RiPS cells.* Immunostaining showing expression of the lineage markers Tuj1 (neuronal, ectodermal), and alpha-fetoprotein (epithelial, endodermal) in RiPS clones from 3 independent RiPS derivations subjected to directed differentiation.

Finally, tri-lineage differentiation potential was confirmed *in vivo* by the formation of teratomas from dH1F-, CF- and BJ-RiPS cells, that histologically revealed cell types of the three germ layers (Figure 4-41, Table 4-34).

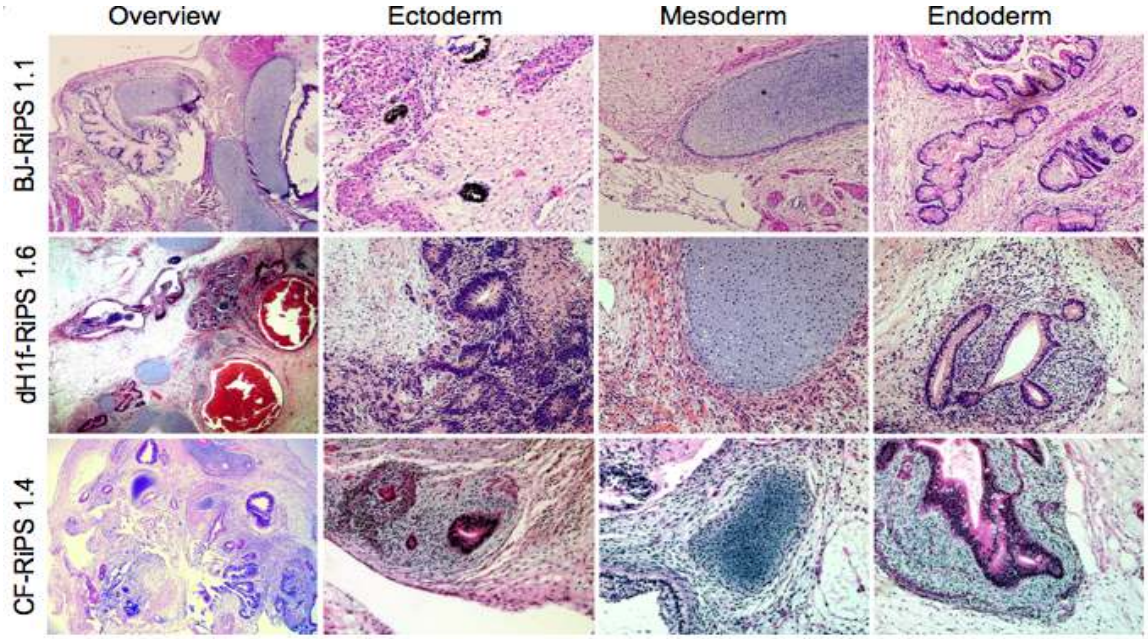


Figure 3-41 *Differentiation of RiPS cells.* Hematoxylin and eosin staining of BJ-, CF- and dH1F-RiPS-derived teratomas showing histological overview, ectoderm (pigmented epithelia (BJ and CF), neural rosettes (dH1F)), mesoderm (cartilage, all), and endoderm (gut-like endothelium, all).

Taken together, these data demonstrate by the most stringent criteria available to human pluripotent cells (Chan et al., 2009; Smith et al., 2009), that modified-RNA-derived iPS clones from multiple independent derivations were reprogrammed to pluripotency, and closely recapitulated the functional and molecular properties of human ES cells.

Modified RNAs generate iPS cells at very high efficiency

During the course of our experiments, we noted surprisingly high reprogramming efficiencies and rapid kinetics with which RiPS cells were generated. To quantify this more thoroughly, a number of reprogramming experiments were undertaken in which quantitative readout of efficiency was based on colony morphology and expression of

the stringent pluripotency markers TRA-1-60 and TRA-1-81, (Chan et al., 2009; Lowry et al., 2008). In one set of experiments, BJ fibroblasts transfected with a five-factor modified-RNA cocktail (KMOSL), demonstrated an iPS conversion efficiency of over 2%, regardless of whether the cells were passaged in the presence or absence of Rho-associated kinase (ROCK), Y-27632 (Figure 4-42 A-B, Table 4-34).

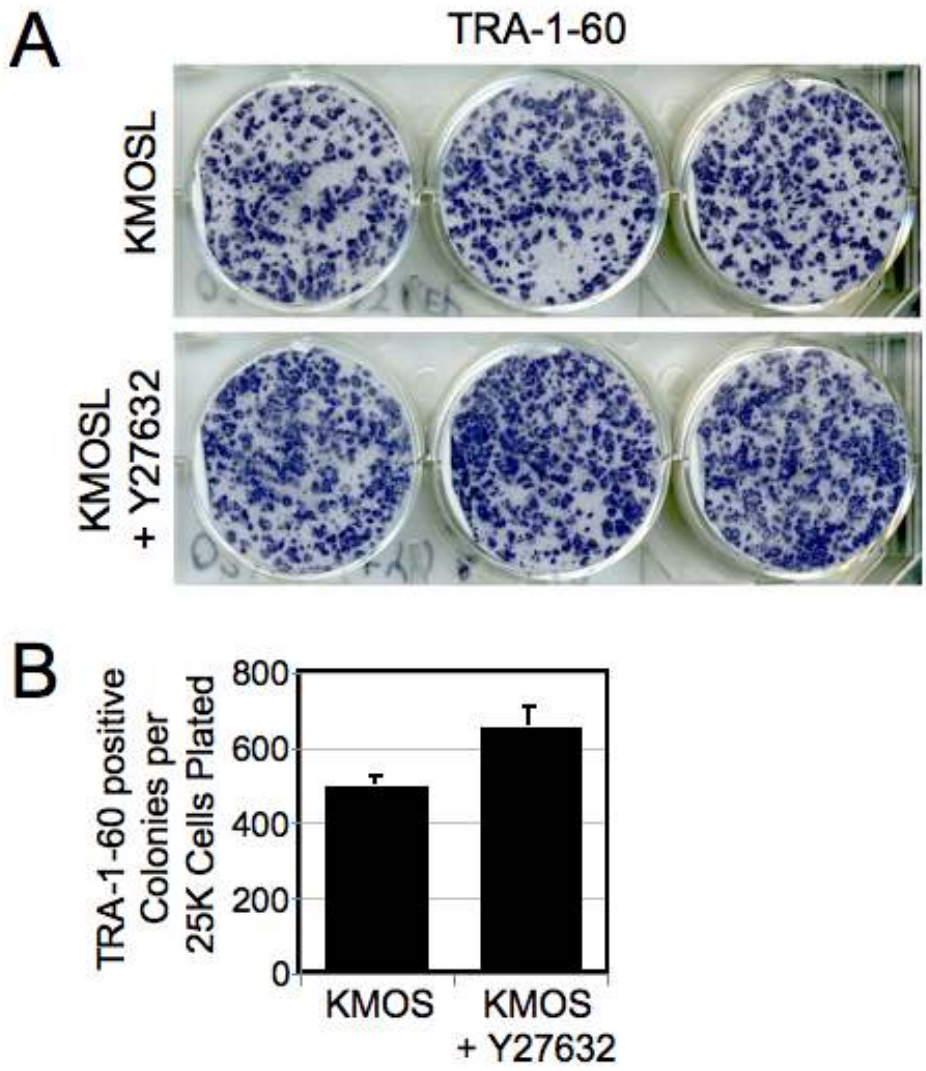


Figure 3-42 *Pluripotency induction by modified-RNAs is highly efficient.* (A) TRA-1-60 horseradish peroxidase (HRP) staining conducted at day 18 of a BJ-RiPS derivation with modified-RNAs encoding KMOSL and (B) frequency of TRA-1-60-positive colonies produced in the experiment relative to number of cells initially seeded. Error bars show s.d., n=6 for each condition.

This efficiency was two orders of magnitude higher than those typically reported for virus-based derivations. Moreover, in contrast to virus-mediated BJ-iPS derivations, in which iPS colonies typically take around 4 weeks to emerge, by day 17 of RNA transfection the plates had already become overgrown with ES-like colonies (Figure 4-42 A).

We next evaluated the contributions of low-oxygen culture and LIN28 to the efficiency of RiPS derivation. The yield of TRA-1-60/TRA-1-81-positive colonies in the ambient (20%) oxygen condition was four-fold lower than in the cultures maintained at 5% O2 when using KMOS RNA, but this deficit was negated when LIN28 was added to the cocktail (Figure 4-43 A-B, Table 4-34).

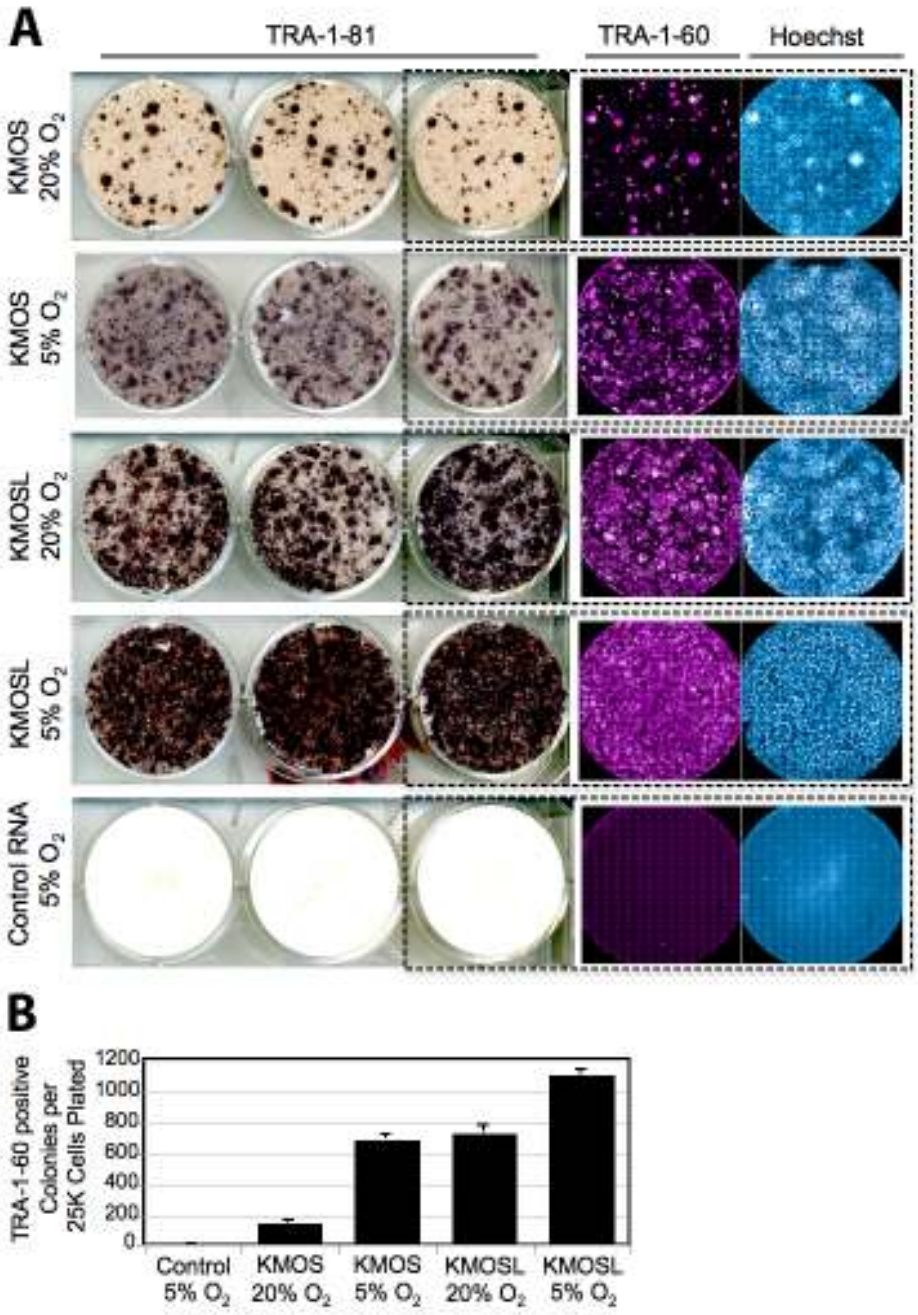


Figure 3-43 (*Pluripotency induction by modified-RNAs is highly efficient.* (A) TRA-181 HRP, TRA-160 immunofluorescence and Hoechst staining, and (B) colony frequencies for dH1f-RiPS experiments done using 4-factor (KMOS) and 5-factor (KMOSL) modified-RNA cocktails under 5% O_2 or ambient oxygen culture conditions quantified at day 18. Control wells were transfected with equal doses of modified-RNA encoding GFP.

The highest conversion efficiency (4.4%) was observed when low-oxygen culture and the five-factor KMOSL cocktail were combined.

To directly compare the kinetics and efficiency of our RiPS derivation protocol against an established viral protocol, we conducted an experiment in which dH1f fibroblasts were transfected with either KMOS modified-RNAs, or transduced with KMOS retroviruses in parallel. As had been observed in previous experiments, ES-like colonies began to emerge towards the end of the second week on the RNA-transfected cultures, and the plates became overgrown with ES-like colonies by the 16th and final By contrast, no ES-like colonies had appeared in the retrovirally transduced cultures by this timepoint, and colonies only began to emerge on the 24th day post-transduction, a time point consistent with previous reports describing iPS derivation by retroviruses (Lowry et al., 2008; Takahashi et al., 2007). The retroviral cultures were fixed for analysis on day 32. Both arms of the experiment were then immunostained for TRA-1-60 and colonies were counted. iPS derivation efficiencies were 1.4% and 0.04% for modified-RNA and retrovirus, respectively, corresponding to 36-fold higher conversion efficiency with the modified-RNA protocol (Figure 4-44 A-B, Table 4-34).

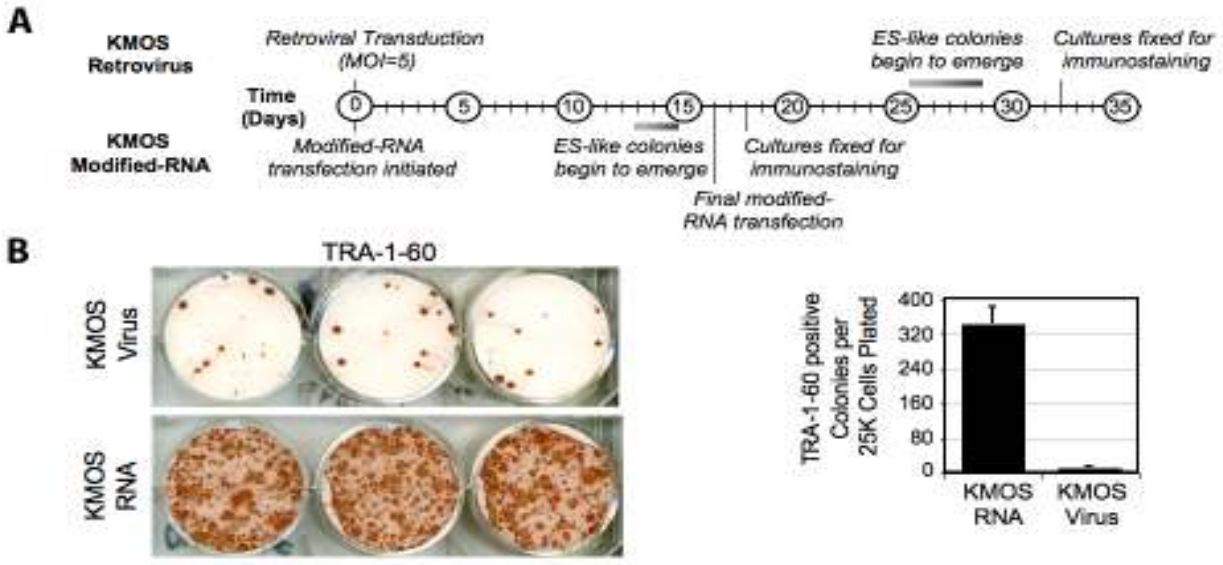


Figure 3-44 (*Pluripotency induction by modified-RNAs is highly efficient.* (A) Kinetics and efficiency of retroviral and modified-RNA reprogramming. (B) Timeline of colony formation (top panel), TRA-1-60 HRP immuno-staining (lower left panel), and TRA-1-60

positive colony counts (lower right panel) of dH1f cells reprogrammed using KMOS retroviruses (MOI=5 of each) or modified-RNA KMOS cocktails (n=3 for each condition).

These experiments also revealed that the kinetics of modified-RNA iPS derivation were almost twice as fast as retroviral iPS derivation. Thus by the combined criteria of colony numbers and kinetics of reprogramming, the efficiency of modified-RNA iPS derivation greatly exceeds that of conventional retroviral approaches.

It should be noted that in the experiments described above, transfected fibroblast cultures were passaged once at an early time point (day 6 or 7) in order to promote fibroblast proliferation, which has been shown to facilitate reprogramming (Hanna et al., 2009). However, in preliminary experiments, RiPS cells were also efficiently derived from BJ and Detroit 551 fibroblasts in the absence of cell passaging indicating that splitting the culture during the reprogramming process was not required for modified-RNA iPS-derivation (Figure 4-45, and data not shown).

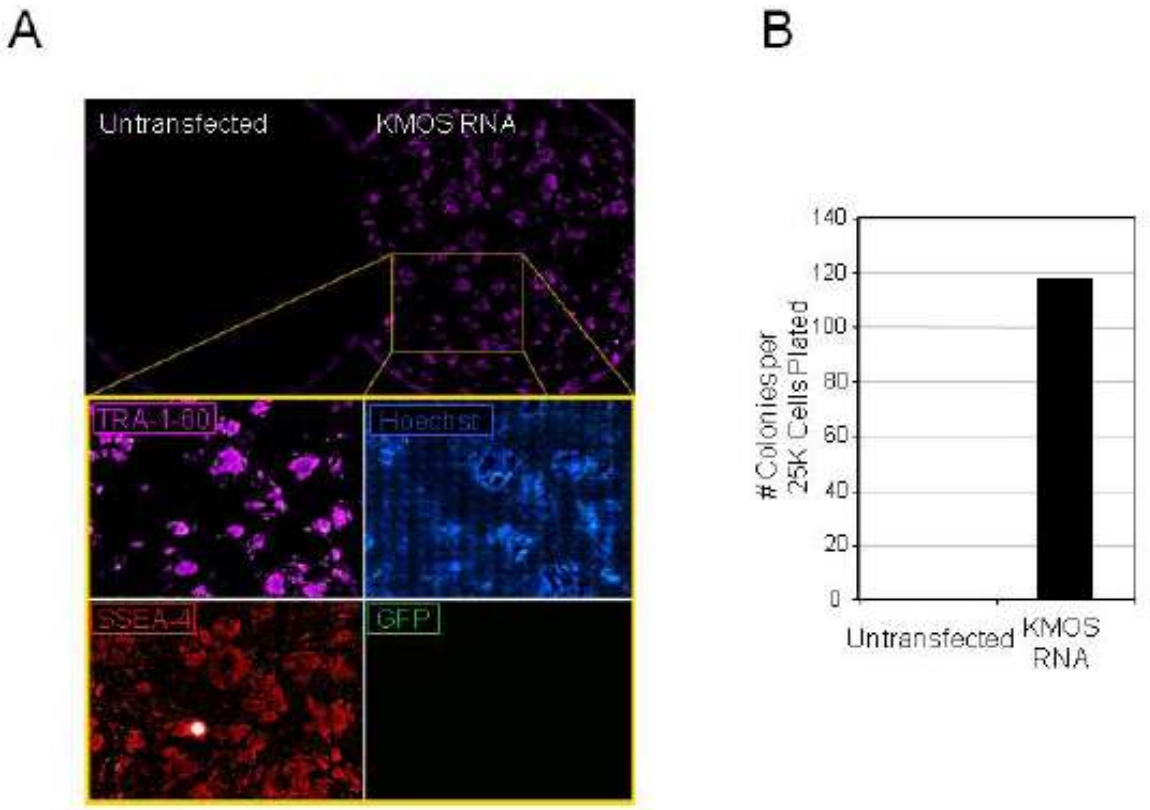
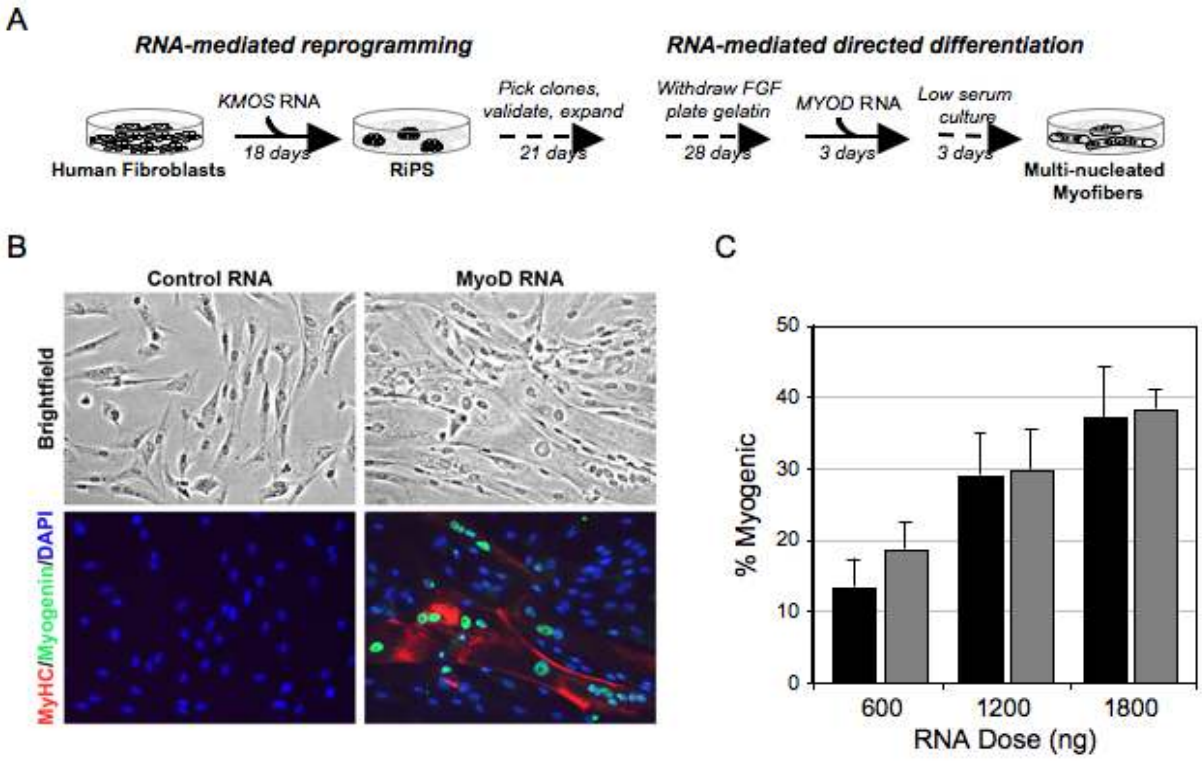


Figure 3-45 *Efficient RiPS derivation from BJ fibroblasts without passaging*. (A) Immunohistochemistry showing expression of pluripotency markers SSEA-4 and TRA-1-60 in a BJ fibroblast reprogramming experiment transfected for 16 days with 600ng per day of a KMOSL modified RNA cocktail containing a destabilized GFP spike-in. Cultures were fixed for staining at day 18. 50,000 BJ cells were originally seeded onto feeder cells and went unpassaged throughout the course of the experiment. (B) Quantification of TRA-1-60 colony count relative to the number of cells seeded.

Utilization of modified-RNA to direct differentiation of pluripotent RiPS cells to a terminally-differentiated cell fate.

To realize the promise of iPS cell technology for regenerative medicine or disease modeling it is imperative that the multi-lineage differentiation potential of pluripotent cells be harnessed. Although progress has been made in directing the differentiation of pluripotent ES cells to various lineages by modulating the extracellular cytokine milieu, such protocols remain relatively inefficient. Given the high efficiency of iPS derivation by

modified-RNAs, we reasoned that this technology might also be utilized to redirect pluripotent cells towards differentiated cell fates. To test this hypothesis, we subjected one of our validated RiPS lines to a simple *in vitro* differentiation protocol in which FGF was withdrawn, serum added, and the cells plated onto gelatin (Figure 4-46).



3-46 *Efficient directed differentiation of RiPS cells to terminally differentiated myogenic fate using modified-RNA.* (A) Schematic of experimental design. (B) Bright-field and immunostained images showing large, multi-nucleated, myosin heavy chain (MyHC) and myogenin positive myotubes in cells fixed three days after cessation of *MYOD* modified-RNA transfection. Modified-RNA encoding GFP was administered to the controls. (C) Penetrance of myogenic conversion relative to daily RNA dose. Black bars refer to an experiment in which cultures were plated at 10^4 cells/cm^2, grey bars to cultures plated at $5x10^3$ cells/cm^2. Error bars show s.d. for triplicate wells.

Cells obtained under these conditions were then subjected to three consecutive days of transfection with a MYOD-encoding modified-RNA followed by an additional 3 days of culture in low serum conditions. The cultures were then fixed and immunostained for the

myogenic markers myogenin and MyHC, which revealed a high percentage of large multi-nucleated myogenin and MyHC double positive myotubes (Figure 4-46).

Taken together, these experiments provide proof of principle that modified-RNAs can be used to both efficiently reprogram cells to a pluripotent state, and direct the fate of such cells to a terminally differentiated somatic cell type.

3.2.4 Discussion

Using a combination of RNA modifications and a soluble interferon inhibitor to overcome innate anti-viral responses, we have developed a novel technology that enables highly efficient reprogramming of somatic cells to pluripotency, and can likewise be harnessed to direct the differentiation of pluripotent cells towards a desired lineage. The methodology described here offers several key advantages over established reprogramming techniques. By obviating the need to perform experiments under stringent biological containment, modified-RNA technology should make reprogramming accessible to a wider community of researchers. More fundamentally, our approach allows protein stoichiometry to be exquisitely regulated within cultures while avoiding the stochastic variation of expression typical of integrating vectors, as well as the uncontrollable effects of viral silencing. Given the stepwise character of the phenotypic changes observed during pluripotency induction (Smith et al., 2010), it seems likely that individual transcription factors play distinct, stage-specific roles during reprogramming. The unprecedented potential for temporal control over individual factor expression afforded by our technology should help researchers unravel these nuances, yielding insights that can be applied to further enhance the efficiency and kinetics of reprogramming.

The risk of mutagenesis is not the only safety concern holding back clinical exploitation of induced pluripotency, and it has become increasingly apparent that all iPS cells are not created equal with respect to epigenetic landscape and developmental plasticity (Hu et al., 2010; Miura et al., 2009). In this regard, we have applied the most stringent molecular and functional criteria for reprogramming human cells to pluripotency (Chan et al., 2009; Smith et al., 2009). Our results demonstrate that modified-RNA derived iPS

clones from multiple independent derivations were fully reprogrammed to pluripotency, and that the resulting cells very closely recapitulated the functional and molecular properties of human ES cells. Our observation that modified-RNA derived iPS cells more faithfully recapitulated the global transcriptional signature of human ES cells than retrovirally-derived iPS cells is important as it suggests that RNA reprogramming may produce higher quality iPS cells, possibly owing to the fact that they are transgene-free.

The transient and non-mutagenic character of RNA-based protein expression could also deliver important clinical benefits outside the domain of lineage reprogramming. Indeed, the use of RNA transfection to express cancer or pathogen antigens for immunotherapy is already an active research area (Rabinovich et al., 2008; Rabinovich et al., 2006; Van den Bosch et al., 2006; Weissman et al., 2000), which may benefit from the non-immunogenic properties of modified-RNAs. One can readily envisage employing modified-RNA to transiently express surface proteins such as homing receptors to target cellular therapies toward specific organs, tissues, or diseased cells.

For tissue engineering to progress further into the clinic there is a pressing need for safe and efficient means to redirect cell fate. This is doubly apparent when one considers that iPS cells are only a starting point for patient-specific therapies, and specification of clinically useful cell types is still required to produce autologous tissues for transplantation or for disease modeling. Importantly, we have demonstrated that our modified-RNA-based technology enables highly efficient reprogramming, and that it can equally be applied to efficiently redirect pluripotent cell fate to terminally differentiated fates without compromising genomic integrity. In light of these considerations, we believe that our approach has the potential to become a major and perhaps even central enabling technology for cell-based therapies and regenerative medicine.

3.2.5 Experimental Procedures

Construction of IVT templates

The pipeline for production of IVT template constructs and subsequent RNA synthesis is schematized in Figure S1. The oligonucleotide sequences used in the construction of IVT templates are shown in Table.

	ORF Forward Primer	ORF Reverse Primer
eGFP	GTGAGCAAGGGCGAGGAGCTGTT	TTACTTGTACAGCTCGTCCATGCCGAGA
d2eGFP	GTGAGCAAGGGCGAGGAGCTGTT	CTACACATTGATCCTAGCAGAAGCACAGGCT
KLF4	GCTGTCAGCGACGCGCTGCTC	TTAAAAATGCCTCTTCATGTGTAAGGCGAGGT
c-MYC	CCCCTCAACGTTAGCTTCACCAACAGG	TTACGCACAAGAGTTCCGTAGCTGTTCA
OCT4	GCGGGACACCTGGCTTCGGATTTC	TCAGTTTGAATGCATGGGAGAGCCCAGA
SOX2	TACAACATGATGGAGACGGAGCTGAAGC	TCACATGTGTGAGAGGGGCAGTGTG
LIN28	GGCTCCGTGTCCAACCAG	TCAATTCTGTGCCTCCGG
MYOD	GAGCTTCTATCGCCGCCACTCC	TCAAAGCACCTGATAAATCGCATTGG
	5' Splint Oligo	3' Splint Oligo
eGFP	TCCTCGCCCTTGCTCACCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTTACTTGTACAGCTCGTCCATGC
d2eGFP	TCCTCGCCCTTGCTCACCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCCTACACATTGATCCTAGCAGA
KLF4	GCGCGTCGCTGACAGCCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTTAAAAATGCCTCTTCATGTGTAA
c-MYC	GTGAAGCTAACGTTGAGGGGCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTTACGCACAAGAGTTCCGTAG
OCT4	AAGCCAGGTGTCCCGCCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTCAGTTTGAATGCATGGGAG
SOX2	CTCCGTCTCCATCATGTTGTACATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTCACATGTGTGAGAGGGGC
LIN28	CTGGTTGGACACGGAGCCCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTCAATTCTGTGCCTCCGG
MYOD	TGGCGGCGATAGAAGCTCCATGGTGGCTCTTATATTTCTTCTT	CCCGCAGAAGGCAGCTCAAAGCACCTGATAAATCGCATTGG
	UTR Oligos	
5' UTR	TTGGACCCTCGTACAGAAGCTAATACGACTCACTATAGGGAAATAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGAGCCACCATG	
3' UTR	GCTGCCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTTCTTCTCTCCCTTGCACCTGTACCTCTTGGTCTTTGAATAAAGCCTGAGTAGGAAGTGAGGGTCTAGAACTAGTGTCGACGC	
	Forward Primer	Reverse Primer
Template PCR	TTGGACCCTCGTACAGAAGCTAATACG	GCGTCGACACTAGTTCTAGACCCTCA
Tail PCR	TTGGACCCTCGTACAGAAGCTAATACG	T_{120}CTTCCTACTCAGGCTTTATTCAAAGACCA

Figure 3-47 *Oligonucleotides for IVT template construction.* 5' and 3' UTR oligos are ligated to the top strand of gene-specific ORF amplicons to produce a basic template construct for cloning. Underlined bases in the 5' UTR oligo sequence indicate the upstream T7 promoter, and in the 3' UTR oligo sequence show downstream restriction sites,introduced to facilitate linearization of template plasmids. Template PCR primers are used to amplify ligation products for sub-cloning. Tail PCR primers are used to append an oligo(dT) sequence immediately after the 3' UTR to drive template addition of a poly(A) tail during IVT reactions. Gene-specific ORF primers are used to capture the coding region (minus the start codon) from cDNA templates. Splint oligos mediate ligation of UTR oligos to the top strand of ORF amplicons.

All oligos were synthesized by Integrated DNA Technologies (Coralville, IA). ORF PCRs were templated from plasmids bearing human KLF4, c-MYC, OCT4, SOX2, human ES cDNA (LIN28), Clontech pIRES-eGFP (eGFP), pRVGP (d2eGFP) and CMV-MyoD from Addgene. The ORF of the low-stability nuclear GFP was constructed by combining the d2eGFP ORF with a 3' nuclear localization sequence. PCR reactions were performed using HiFi Hotstart (KAPA Biosystems, Woburn, MA) per the manufacturer's instructions. Splint-mediated ligations were carried out using Ampligase Thermostable

DNA Ligase (Epicenter Biotechnologies, Madison, WI). UTR ligations were conducted in the presence of 200 nM UTR oligos and 100 nM splint oligos, using 5 cycles of the following annealing profile: 95°C for 10 seconds; 45°C for 1 minute; 50°C for 1 minute; 55°C for 1 minute; 60°C for 1 minute. A phosphorylated forward primer was employed in the ORF PCRs to facilitate ligation of the top strand to the 5' UTR fragment. The 3' UTR fragment was also 5'-phosphorylated using polynucleotide kinase (New England Biolabs, Ipswich, MA). All intermediate PCR and ligation products were purified using QIAquick spin columns (Qiagen, Valencia, CA) before further processing. Template PCR amplicons were sub-cloned using the pcDNA 3.3-TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid inserts were excised by restriction digest and recovered with SizeSelect gels (Invitrogen) before being used to template tail PCRs.

Synthesis of modified-RNA

RNA was synthesized with the MEGAscript T7 kit (Ambion, Austin, TX), using 1.6 ug of purified tail PCR product to template each 40 uL reaction. A custom ribonucleoside blend was used comprising 3´-0-Me-m^{7}G(5')ppp(5')G ARCA cap analog (New England Biolabs), adenosine triphosphate and guanosine triphosphate (USB, Cleveland, OH), 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies, San Diego, CA). Final nucleotide reaction concentrations were 33.3 mM for the cap analog, 3.8 mM for guanosine triphosphate, and 18.8 mM for the other nucleotides. Reactions were incubated 3-6 hours at 37°C and DNAse-treated as directed by the manufacturer. RNA was purified using Ambion MEGAclear spin columns, then treated with Antarctic Phosphatase (New England Biolabs) for 30 minutes at 37°C to remove residual 5'-triphosphates. Treated RNA was re-purified, quantitated by Nanodrop (Thermo Scientific, Waltham, MA), and adjusted to 100 ng/uL working concentration by addition of Tris-EDTA (pH 7.0). RNA reprogramming cocktails were prepared by pooling individual 100 ng/uL RNA stocks to produce a 100 ng/uL (total) blend. The KMOS[L]+GFP cocktails were formulated to give equal molarity for each component except for OCT4, which was included at 3x molar concentration. Volumetric ratios used for pooling were as follows: 170:160:420:130:120[:90] (KLF4:c-MYC:OCT4:SOX2:GFP[:LIN28]).

Cells

Primary human neonatal epidermal keratinocytes, BJ human neonatal foreskin fibroblasts, MRC-5 human fetal lung fibroblasts, and Detroit 551 human fetal skin fibroblasts were obtained from ATCC (Manassas, VA). CF cells were obtained with informed consent from a skin biopsy taken from an adult cystic fibrosis patient. dH1f fibroblasts were sub-cloned from fibroblasts produced by directed differentiation of the H1-OGN human ES cell line as previously described (Park et al., 2008a). BGO1 hES cells were obtained from BresaGen (Athens, GA). H1 and H9 hES cells were obtained from WiCell (Madison, Wi).

RNA transfection

RNA transfections were carried out using RNAiMAX (Invitrogen) or TransIT-mRNA (Mirus Bio, Madison, WI) cationic lipid delivery vehicles. RNAiMAX was used for RiPS derivations, the RiPS-to-myogenic conversion, and for the multiple cell-type transfection experiments. All other transfections were performed with TransIT-mRNA. For RNAiMAX transfections, RNA and reagent were first diluted in Opti-MEM basal media (Invitrogen). 100 ng/uL RNA was diluted 5x and 5 uL of RNAiMAX per microgram of RNA was diluted 10x, then these components were pooled and incubated 15 minutes at room temperature (RT) before being dispensed to culture media. For TransIT-mRNA transfections, 100 ng/uL RNA was diluted 10x in Opti-MEM and BOOST reagent was added (2 uL per microgram of RNA), then TransIT-mRNA was added (2 uL per microgram of RNA), and the RNA-lipid complexes were delivered to culture media after a 2-minute incubation at RT. RNA transfections were performed in Nutristem xeno-free hES media (Stemgent, Cambridge, MA) for RiPS derivations, Dermal Cell Basal Medium plus Keratinocyte Growth Kit (ATCC) for keratinocyte experiments, and Opti-MEM plus 2% FBS for all other experiments described. The B18R interferon inhibitor (eBioscience, San Diego, CA) was used as a media supplement at 200 ng/mL.

qRT-PCR

Transfected cells were lysed using 400 uL CellsDirect reagents (Invitrogen), and 20 uL of each lysate was taken forward to a 50 uL reverse transcription reaction using the VILO cDNA synthesis kit (Invitrogen). Reactions were purified on QIAquick columns (Qiagen). qRT-PCR reactions were performed using SYBR FAST qPCR supermix (KAPA Biosystems).

Transcript	Forward Primer	Reverse Primer
GAPDH	GAAGGCTGGGGCTCATTT	CAGGAGGCATTGCTGATGAT
IFNA	ACCCACAGCCTGGATAACAG	ACTGGTTGCCATCAAACTCC
IFNB	CATTACCTGAAGGCCAAGGA	CAGCATCTGCTGGTTGAAGA
IFIT1	AAAAGCCCACATTTGAGGTG	GAAATTCCTGAAACCGACCA
OAS1	CGATCCCAGGAGGTATCAGA	TCCAGTCCTCTTCTGCCTGT
PKR	TCGCTGGTATCACTCGTCTG	GATTCTGAAGACCGCCAGAG
RIG-I	GTTGTCCCCATGCTGTTCTT	GCAAGTCTTACATGGCAGCA

Figure 3-48 *Primers for qRT-PCR analysis of interferon-regulated genes*.

Reprogramming to pluripotency

Gamma-irradiated human neonatal fibroblast feeders (GlobalStem, Rockville, MD) were seeded at 33,000 cells/cm^2. Nutristem media was replaced daily, four hours after transfection, and supplemented with 100 ng/mL bFGF and 200 ng/mL B18R (eBioscience, San Diego, CA). Where applied, VPA was added to media at 1 mM final concentration on days 8-15 of reprogramming. Low-oxygen experiments were carried out in a NAPCO 8000 WJ incubator (Thermo Scientific). Media were equilibrated at 5% O_2 for approximately 4 hours before use. Cultures were passaged using TrypLE Select recombinant protease (Invitrogen). Y27632 ROCK inhibitor (Watanabe et al., 2007) was used at 10 uM in recipient plates until the next media change. The daily RNA dose applied in the RiPS derivations was 1200 ng per well (6-well plate format) or 8 ug to a 10-cm dish.

For RNA vs. retrovirus experiments, starting cultures were seeded with 100,000 cells in individual wells of a 6-well plate using fibroblast media (DMEM+10% FBS). The

following day (day 1) KMOS RNA transfections were initiated in the RNA plate, and the viral plate was transduced with a KMOS retroviral cocktail (MOI=5 for each virus). All wells were passaged on day 6, using split ratios of 1:6 for the RNA wells and 1:3 for the virus wells. The conditions applied in the RNA arm of the trial were as in the initial RiPS derivation, including the use of Nutristem supplemented with 100 ng/mL bFGF, 5% O_2 culture, and human fibroblast feeders. Ambient oxygen tension and other conventional iPS derivation conditions were used in the viral arm, the cells being grown in fibroblast media without feeders until the day 6 split, then being replated onto CF1 MEF feeders (GlobalStem) with a switch to hES media based on Knockout Serum Replacement (Invitrogen) supplemented with 10 ng/mL bFGF.

RiPS cell culturing

RiPS colonies were mechanically picked and transferred to MEF-coated 24-well plates with standard hES medium containing 5 uM Y27632 (BioMol, Plymouth Meeting, PA). The hES media comprised DMEM/F12 supplemented with 20% Knockout Serum Replacement (Invitrogen), 10 ng/mL of bFGF (Gembio, West Sacramento, CA), 1x non-essential amino acids (Invitrogen), 0.1mM β-ME (Sigma), 1mM L-glutamine (Invitrogen), plus antibiotics. Clones were mechanically passaged once more to MEF-coated 6-well plates, and then expanded using enzymatic passaging with collagenase IV (Invitrogen). For RNA and DNA preparation, cells were plated onto hES-qualified Matrigel (BD Biosciences) in mTeSR (Stem Cell Technologies, Vancouver, BC), and further expanded by enzymatic passaging using dispase (Stem Cell Technologies).

Immunostaining

Cells were fixed in 4% paraformaldehyde for 20 minutes. Washed cells were treated with 0.2% Triton X (Sigma) in PBS for 30 minutes. Cells were blocked with 3% BSA (Invitrogen) and 5% donkey serum (Sigma) for 2 hours at RT. Cells were stained in blocking buffer with primary antibodies at 4°C overnight. Cells were washed and stained with secondary antibodies and 1 ug/mL Hoechst 33342 (Invitrogen) in blocking buffer for 3 hours at 4°C or for 1 hour at RT, protected from light. Antibodies were used, at 1:100

dilution: TRA-1-60-Alexa Fluor 647, TRA-1-81-Alexa Fluor 488, SSEA-4-Alexa Fluor 647, and SSEA-3-Alexa 488 (BD Biosciences). Primary OCT4 and NANOG antibodies (Abcam, Cambridge, MA) were used at 0.5 ug/mL, and an anti-rabbit IgG Alexa Fluor 555 (Invitrogen) was used as the secondary. Images were acquired with a Pathway 435 bioimager (BD Biosciences). Live imaging was performed as described previously (Chan et al., 2009). For pluripotency factor time course experiments, transfected human keratinocytes were trypsinized, washed with PBS, and fixed in 4% paraformaldehyde for 10 minutes. Fixed cells were washed with 0.1M glycine, then blocked and permeabilized in PBS/0.5% saponin/1% goat serum (Rockland Immunochemicals, Gilbertsville, PA) for 20 minutes. Cells were incubated for 1 hour at RT with 1:100 diluted primary antibodies for KLF4, OCT4, SOX2 (Stemgent), washed, then for 45 minutes at RT with 1:200-diluted DyLight 488-labeled secondary antibodies (goat anti-mouse IgG+IgM and goat anti-rabbit IgG). Cells were suspended in PBS and analyzed by flow cytometry.

Gene expression analysis

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was primed with oligo(dT) primers and qPCR was performed with primer sets as described previously (Park et al., 2008a), using Brilliant SYBR Green master mix (Stratagene, La Jolla, CA). For the microarray analysis, RNA probes were prepared and hybridized to Human Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA) per the manufacturer's instructions. Arrays were processed by the Coriell Institute Genotyping and Microarray Center (Camden, NJ). Microarray data will be uploaded to the GEO database at the time of publication. Gene expression levels were normalized with the Robust Multichip Average (RMA) algorithm. Hierarchical clustering was performed using the Euclidean distance with average linkage method. The similarity metric for comparison between different cell lines is indicated on the height of cluster dendrogram.

Bisulfite sequencing

DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. Bisulfite treatment of genomic DNA was carried out using EZ DNA Methylation™ Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. For pyrosequencing analysis, the bisulfite treated DNA was first amplified by HotStar Taq Polymerase (Qiagen) for 45 cycles of (95ºC 30 s; 53ºC 30 s; 72ºC 30 s). The analysis was performed by EpigenDx using the PSQ™96HS system according to standard procedures using primers that were developed by EpigenDx for the CpG sites at positions (-50) to (+96) from the start codon of the *OCT4* gene.

Tri-lineage differentiation

Embryoid body (EB) hematopoietic differentiation was performed as previously described (Chadwick et al., 2003). Briefly, RiPS cells and hES cell controls were passaged with collagenase IV and transferred (3:1) in differentiation medium to 6-well low-attachment plates and placed on a shaker in a 37°C incubator overnight. Starting the next day, media was supplemented with the following hematopoietic cytokines: 10 ng/mL of interleukin-3 (R&D Systems, Minneapolis, MN) and interleukin-6 (R&D), 50 ng/mL of G-CSF (Amgen, Thousand Oaks, CA) and BMP-4 (R&D), and 300 ng/mL of SCF (Amgen) and Flt-3 (R&D). Media was changed every 3 days. On day 14 of differentiation, EBs were dissociated with collagenase B (Roche, Indianapolis, IN). $2x10^4$ differentiated cells were plated into methylcellulose H4434 (Stem Cell Technologies) and transferred using a blunt needle onto 35mm dishes (Stem Cell Technologies) in triplicate and incubated at 37°C and 5°CO_2 for 14 days. Colony Forming Units (CFUs) were scored based on morphological characteristics.

For neuronal differentiation, cells were differentiated at 70% confluency as a monolayer in neuronal differentiation medium (DMEM/F12, Glutamax 1%, B27-Supplement 1%, N2-Supplement 2%, P/S 1% and noggin 20ng/ml). After 7 days neuronal structures were visible. For endoderm differentiation (AFP stain), cells were differentiated as a monolayer in endoderm differentiation medium (DMEM, B27(-RA) and 100 ng/ml

activin-a) for 7 days, then switched to growth medium (DMEM, 10% FBS, 1% P/S) and continued differentiation for 7 days. Antibodies used were as follows: Anti-β-Tubulin III (Tuj1) rabbit anti-human (Sigma, St. Louis, MO), 1:500; AFP (h-140) rabbit polyclonal IgG, (Santa Cruz Biotechnology, Santa Cruz, CA), 1:100 dilution. Secondary antibodies were conjugated to Alexa Fluor 488, or Alexa Fluor 594.

For cardiomyocyte differentiation, colonies were digested at 70% confluency using dispase and placed in suspension culture for embryoid body (EB) formation in differentiation medium (DMEM, 15% FBS, 100 uM ascorbic acid). After 11 days, EBs were plated to adherent conditions using gelatin and the same medium. Beating cardiomyocytes were observed 3 days after replating.

For teratomas, $2.5x10^6$ cells were spun down, and all excess media was removed. In 20-week old female SCID mice, the capsule of the right kidney was gently elevated, and one droplet of concentrated cells was inserted under the capsule. Tumors harvested at 6-12 weeks were fixed in 4% PFA, run through an ethanol gradient, and stored in 70% ethanol. Specimens were sectioned and stained with H&E.

Myogenic differentiation of RiPS cells

Validated RiPS cells were plated into wells coated with 0.1% gelatin (Millipore, Billerica, MA), and cultured in DMEM+10% FBS for 4 weeks with passaging every 4-6 days using trypsin. The culture media was switched to Opti-MEM+2% FBS, and the cells were transfected with modified-RNA encoding either murine MYOD or GFP the following day, and for the following two days. Media was supplemented with B18R, and replaced 4 hours after each transfection. After the third and final transfection, the media was switched to DMEM+3% horse serum, and cultures were incubated for a further 3 days. Cells were then fixed in 4% PFA and immuno-stained as previously described (Shea et al., 2010). The percentage of myogenin-positive nuclei/total nuclei and nuclei/MyHC-positive myotubes was quantified, with a minimum of 500 nuclei counted per condition.

3.3 To differentiate human pluripotent stem cells into adipocytes

Programming human embryonic stem cells and human induced pluripotent stem cells into adipocytes (Submission to Cell Stem Cells)

Tim Ahfeldt, Youn-Kyoung Lee, Adam Kaplan, Dave Lum, Robert Schinzel, Frank Lau, Rahul Deo, Jennifer Shay, Greg Mowrer, Eugene Rhee, Robert Gerszten and Chad A. Cowan

3.3.1 Summary

The utility of human pluripotent stem cells as tools for understanding disease and as a source of cells for transplantation therapies are limited by efficient differentiation protocols that guide the fate of these cells to adult cell types of interest. Here, we report the reliable and efficient differentiation of human pluripotent stem cells to adipocytes. We find that the inducible overexpression of the master regulator PPARγ in pluripotent stem cell-derived mesenchymal progenitor cells efficiently programs their development towards an adipocyte cell fate. Utilizing this approach, we differentiated multiple human pluripotent cell lines into adipocytes with efficiencies of 80 to 85%. These pluripotent stem cell-derived adipocytes were found to be transgene independent, could be maintained in culture for several weeks, expressed mature markers, and exhibited mature functional properties such as lipid catabolism in response to a beta adrenergic stimulus. Lipid content analysis by mass spectroscopy further confirmed the identity and maturity of these pluripotent stem cell-derived adipocytes. The differentiation of human pluripotent stem cells into adipocytes provides a renewable source of adult cells for understanding diseases such as diabetes and obesity.

3.3.2 Introduction:

The derivation of human embryonic stem cells (hESC) (Thomson et al., 1998) and subsequent discovery of methods for generating human induced pluripotent stem cells (hiPSC) (Park et al., 2008c; Takahashi et al., 2007; Yu et al., 2007a)have made possible the creation of cell-based models of disease. As these human pluripotent stem cells (hPSC) self-renew indefinitely and have the ability to differentiate into any adult cell

type they represent an inexhaustible supply of cells for studying disease progression in the particular cell type affected by disease. When combined with the ability to either genetically manipulate hESCs or generate hiPSCs from any given patient it is now feasible to create a human pluripotent cell-based model for almost any disease. To this end, several groups have now established a number of cell-based surrogates for diseases such as amyotrophic lateral sclerosis, spinal muscular atrophy, type-1 diabetes, Duchenne muscular dystrophy, and familial dysautonomia, to name just a few (Dimos et al., 2008; Lee et al., 2009; Park et al., 2008b). The hope is that these cell based models have the potential to provide insight into the pathophysiology of disease progression, identify new prognostic biomarkers, and ensure a continuous supply of cells for drug screening and discovery. And while the number of cellular models for disease continues to increase rapidly, the principal obstacle for understanding disease mechanisms remains the ability to differentiate hPSCs to a particular adult cell fate.

Obesity is a physical state in which the natural energy reserves, stored in the adipose tissue of humans and other mammals, is increased to a point where it is associated with adverse health effects and mortality. Obesity is influenced by environmental, genetic, and behavioral components. It is the second leading cause of preventable death in the U.S. (Ogden et al., 2002). Adipose tissue from patients is one of the more readily obtained primary cell types, but is very difficult to maintain in culture and impossible to expand. As a consequence, *in vitro* systems for understanding mature primary adipocyte function as it relates to obesity and metabolic disease do not exist. With the establishment of the murine 3T3 cell culture system, adipocytes have become a popular model for the study of cell differentiation and transcriptional regulation (Green and Kehinde, 1975). When exposed to an adipogenic cocktail, the immortalized mouse adipogenic 3T3-L1 and 3T3-F442A cell lines change their fibroblastic appearance to that of adipocytes. The transcriptional control during adipogenesis has been intensively studied in the mouse model and has been extensively reviewed (Rosen and Spiegelman, 2006). Of note, using these cellular models the transcription factor PPARγ2 was identified as a tissue specific regulator (Tontonoz et al., 1994) of adipogenesis. PPARγ2 is now widely accepted as a master regulator of adipogenesis as it is both sufficient and necessary for adipocyte formation. The derivation of

multipotent mesenchymal stem cells (MSCs) from bone marrow (Pittenger et al., 1999), and other tissues such as adipose tissue (Zuk et al., 2001) allowed for the in vitro differentiation of clinical relevant cells into adipocytes. These cells are mainly characterized by surface marker expression such as CD105 and $CD73^+$ and their potential to differentiate into bone, cartilage and adipocytes. Major limitations for the clinical translation are the limited proliferative potential and the decreasing differentiation potential over time (Barberi and Studer, 2006), as well as difficulties to genetically modify cells by homologous recombination.

To overcome these obstacles many groups seek to use hPSCs to generate human adipocytes. Even though published data on the differentiation of adipocytes - using hPSCs exist, these reports merely represent a formal proof of the ability of pluripotent cells to form adipocytes but have so far been inefficient in generating large numbers of adipocytes and have failed to thoroughly characterize the pluripotent cell-derived adipocytes (Dani, 1999; van Harmelen et al., 2007; Wolvetang and Hannan, 2008; Xiong et al., 2005). In addition, a few groups have explored the differentiation of pluripotent stem cells into multipotent mesenchymal progenitor cells (MPC) (Barberi et al., 2005; Olivier et al., 2006) and then using standard adipogenic differentiation medium containing insulin, isobutylxanthine and dexamethasone (Pittenger et al., 1999) have shown that these cells can be further differentiated into adipocytes. Unfortunately, the efficiency of differentiation into adipocytes is extremely variable between different MPC lines and the adipocytes have not been extensively characterized or shown to be functional. In order to fulfill the promise of hPSCs as cellular models of obesity and metabolic disease it is necessary to develop reliable and efficient protocols for the differentiation of hPSCs to adipocytes.

Here, we report the reliable and efficient differentiation of hPSCs to adipocytes. We find that the inducible overexpression of the master regulator PPARγ in hPSC-derived MPCs efficiently programs their development towards an adipocyte cell fate. Utilizing this approach, we differentiated multiple hPSCs into adipocytes with efficiencies of up to 88%. These pluripotent stem cell-derived adipocytes were found to be transgene independent, could be maintained in culture for several weeks, expressed mature

markers, and exhibited mature functional properties such as lipid catabolism in response to a beta adrenergic stimulus. Global proteomic lipid content analysis further confirmed the identity and maturity of these pluripotent stem cell-derived adipocytes. The differentiation of human pluripotent stem cells into adipocytes provides a renewable source of adult cells for understanding diseases such as diabetes and obesity.

3.3.3 Results:

Generating pluripotent cell-derived adipocytes:

Differentiation of pluripotent cells into mesenchymal progenitor cells

We reasoned that the efficient differentiation of hPSCs into adipocytes might be achieved by first establishing an intermediate mesenchymal progenitor cell population that could then be examined with any number of adipogenic-promoting stimuli. Several protocols concerning the generation multipotent stem cells or so called mesenchymal progenitor cells (MPCs) from human embryonic stem cells have been previously published. For example, Barberi and colleagues established a co-culture system with OP9 cells that when combined with a FACS sorting strategy for the antigen CD73 generated hESC-derived MPCs (Barberi et al., 2005). Notably, they demonstrated these MPCs could form adipocytes. Although successful, this approach was labor intensive, inefficient and provided limited characterization of the resulting adipocytes. In an effort to streamline this approach, we sought to first simplify the differentiation of hPSC-derived MPCs. We differentiated hESCs and hiPSCs into embryoid bodies (EBs) that after 2 days displayed a characteristic rounded shape with defined and smoothened edges. These EBs were replated to adherent cell culture dishes after 10 days of suspension culture and cells with a fibroblast-like morphology were observed growing over the next few days. These adherent fibroblast-like cells were subsequently passaged at least 3 times and appeared to become more homogenous over time. We analyzed the resulting cells using flow cytometry after staining for the surface marker CD73. Pluripotent cells lack the CD73 surface antigen but the fibroblast-like cells as well as adipose derived stromal vascular (ADSV) cells expressed the antigen in almost the entire cell population (Figure and Table 4-49) . As mesenchymal progenitor cells have

previously been shown to also uniformly express CD73, we termed our resulting pluripotent stem cell-derived fibroblast-like cells mesenchymal progenitor cells or MPCs.

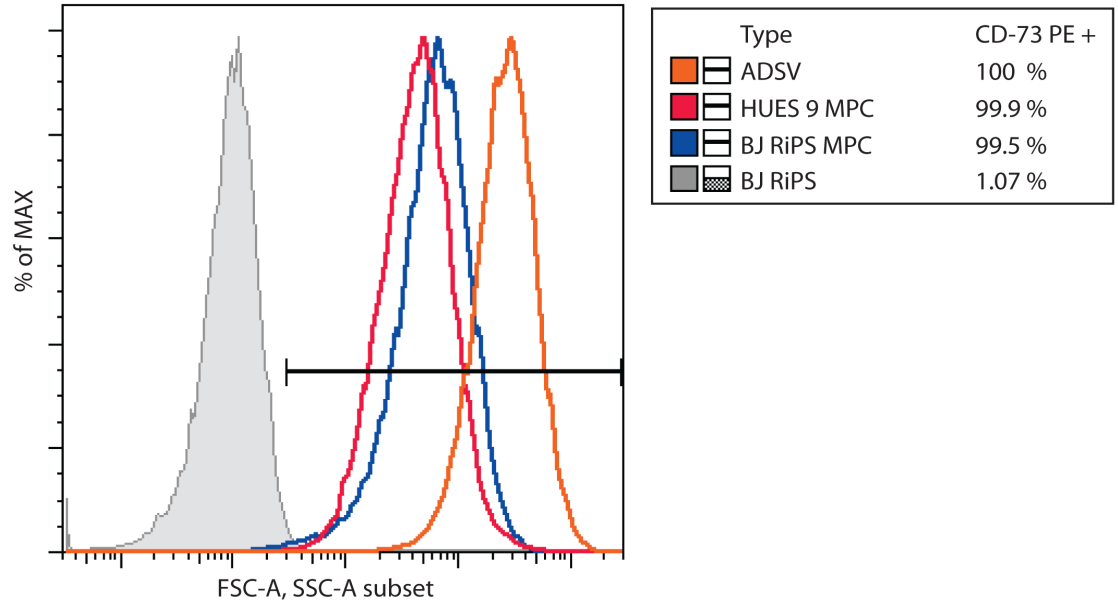


Figure 3-49 Flow Cytometry for CD73 surface marker; Histogram showing populations gated to scatter; (Orange) ADSV, (Red) HUES 9 MPC, (Blue) BJ RiPS MPC, (Grey) BJ RiPS pluripotent

MPCs could generally be expanded and used in experiments until passage 12 at which point we observed that the cell population became non-replicative and was presumably senescent.

Differentiation of mesenchymal progenitor cells and ADSV cells into adipocytes.

To test whether our MPCs responded as those previously reported by Barberi and colleagues we examined their response to medium that contained known inducers of adipocytes including Insulin, Rosiglitazone and Dexamethasone. MPCs and ADSV cells were exposed to this medium for 21 days. After 8 days, small lipid droplets could be observed and after 21 days many cells exhibited multiloccular lipid droplets often found

in immature adipocytes. ADSV lines from distinct donors showed variances in their efficiency to differentiate into adipocytes (Figure 4-50).

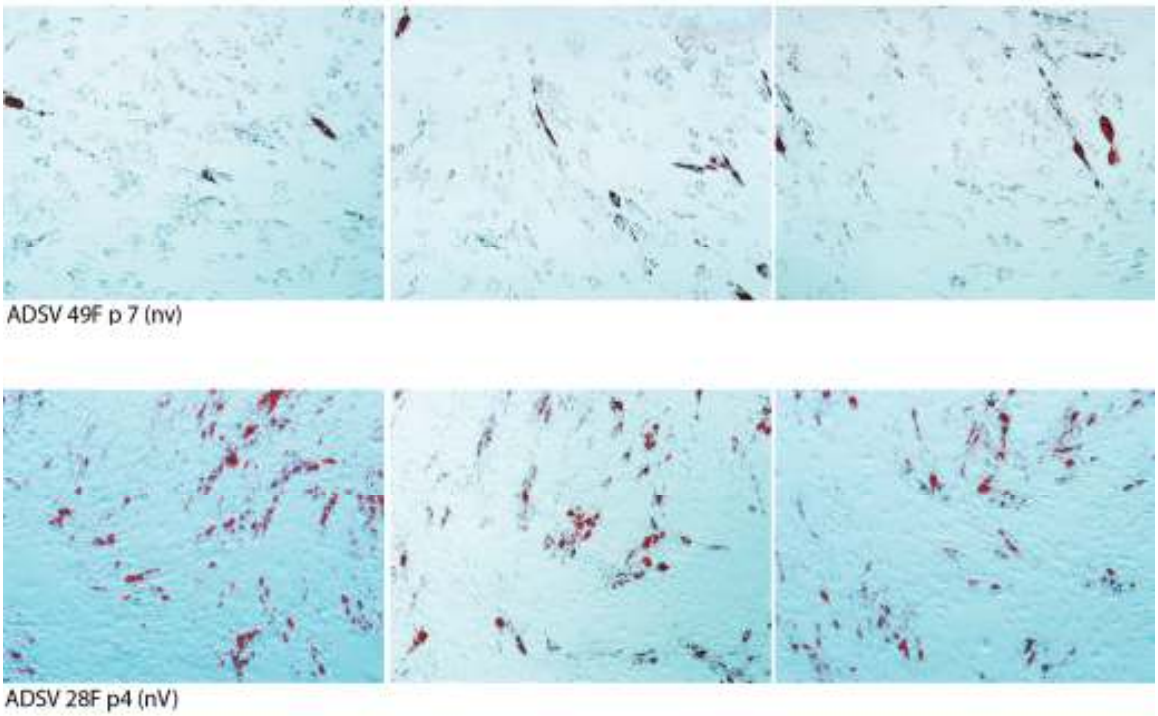


Figure 3-50 Morphology of ADSV derived adipocytes shown in Oil-Red-O (upper panel) ADSV (2) p7, (bottom panel) ADSV (1) p4

The differentiation potential of the MPC cells varied as well. In general we observed a lower efficiency of differentiation in MPC cells compared to ADSV lines. We also observed that the adipogenic differentiation potential of ADSV as well as MPCs decreased with increasing passage numbers (data not shown).

Doxycycline-inducible overexpression of PPARγ

To further increase the efficiency of adipogenic differentiation we reasoned that brief overexpression of genes known to control adipocyte differentiation would likely result in a higher yield of adipocytes. PPARγ is a key regulator of the genetic program controlling adipogenesis, and is both necessary and sufficient for normal adipocyte differentiation (Rosen et al., 2002) , Moreover, overexpression of PPARγ has resulted in adipogenic conversion of some cell lines that would not normally give rise to fat cells (Rosen et al.,

2002) . In order to test whether PPARγ overexpression in our system would enhance adipogenesis, we generated lentiviruses where a PPARγ-encoding cDNA was under the control of a doxycycline-inducible promoter (Lenti-tet-PPARγ). When co-infected with a virus constitutively expressing the reverse tetracycline transactivator (rtTA) under the human ubiquitin C-promoter (Lenti-rtTA) (Maherali et al., 2008), expression of PPARγ could be induced by the addition of doxycycline to the cell culture media. MPCs or ADSVs cells were transduced with both viruses, and semi-quantitative PCR analysis revealed tight control and robust inducibile transgene expression (Figure 4-51).

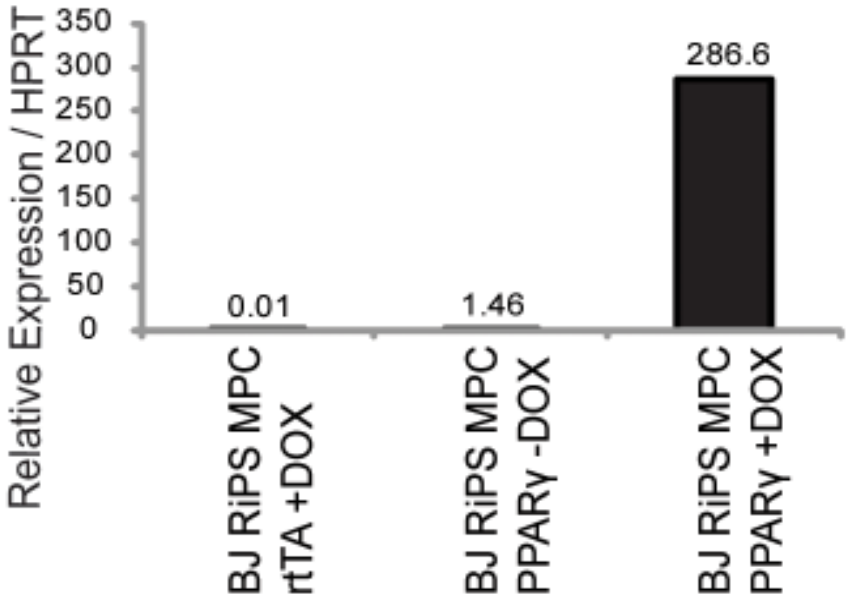


Figure 3-51 qRT-PCR for the viral PPARγ expression normalized to HPRT; from left to right: BJ RiPS MPC (control), BJ RiPS MPC transduced (-DOX Control), BJ RiPS MPC transduced (+DOX). 48 hours of culture in the presence or absence of 1ug/mL doxycycline. Semi-Quantitative PCR with viral specific primers for PPARγ was performed and in the induction group PPARγ was expressed 200 fold higher than in the control group. In the no transduction control no transgene expression was observed

To further examine the efficiency of this viral system for inducible transgene expression we generated a virus carrying an inducible EGFP cassette. MPCs were infected with Lenti-tet-EGFP and Lenti-rtTA. In the presence of doxycycline we found strong and

robust expression of the transgene but only a very faint expression in the -dox control. Overall transduction efficiency of the cells was approximately 95% as measured by the number of GFP positive cells (Figure 4-52).

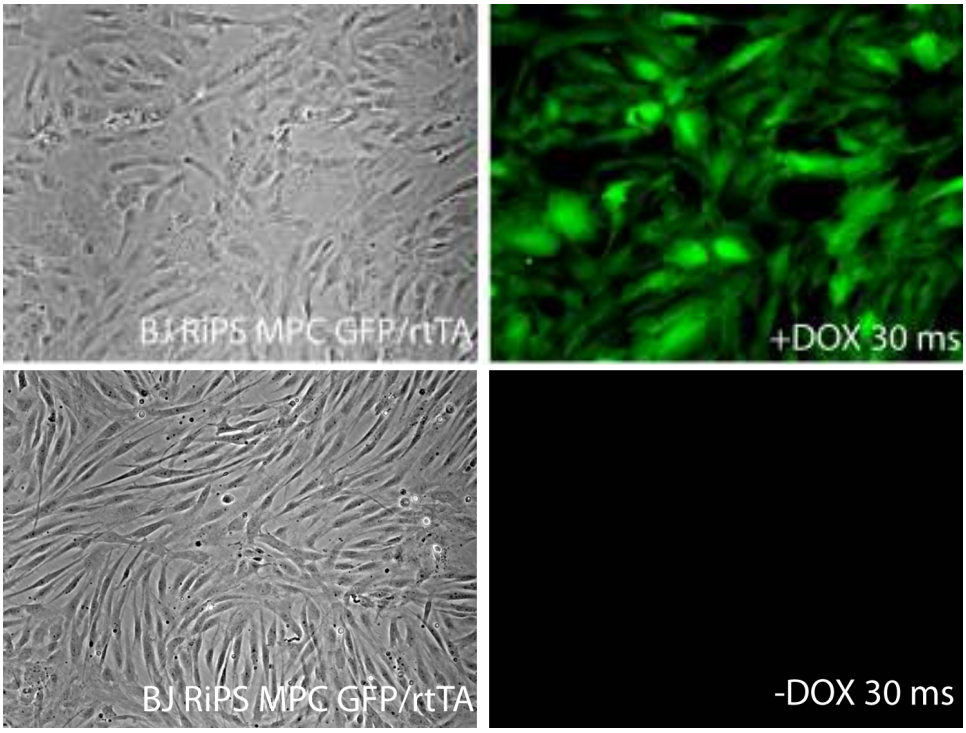


Figure 3-52 The inducible lentiviral system; Bj RiPS MPC transduced with a doxycycline-inducible EGFP virus and a virus constitutively expression rtTA. 95% of cells cultured with doxycycline showed robust expression of EGFP, whereas virtually no expression of EGFP was detected in infected cells cultured without doxycycline. Note the identical exposure time in the immunofluorescence images.

Differentiation of mesenchymal progenitor cells into adipocytes.

Next, we attempted to derive adipocytes from pluripotent cells according to the experimental scheme illustrated in Figure 4-53.

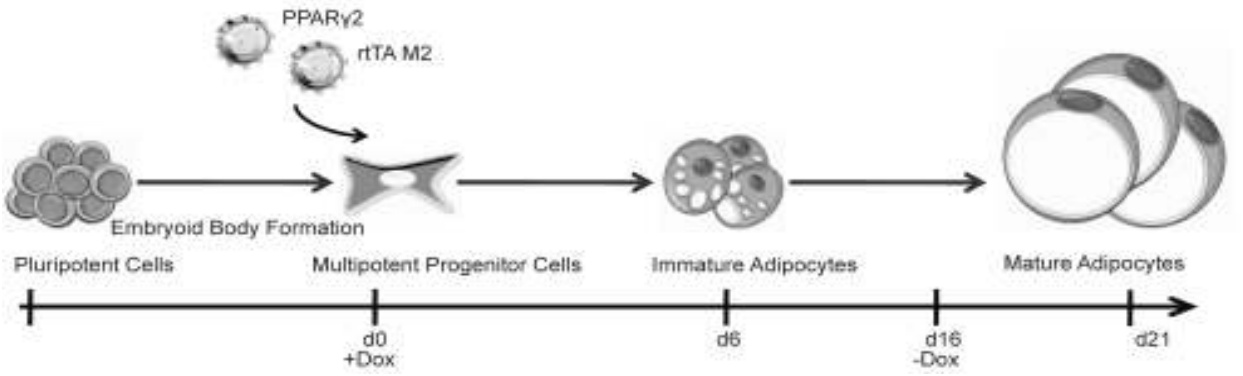


Figure 3-53 Experimental scheme depicting the differentiation of progenitor cells into adipocytes. Pluripotent cells were differentiated into MPCs. MPCs or ADSV cells are transduced with the doxycycline inducible PPARγ and rtTA M2.

Briefly, MPCs were obtained from EBs through plating and repeated passaging. Following infection, MPCs (or ADSVs) were cultured in adipogenic medium containing doxycycline for 16 days. Doxycyline was thereafter withdrawn and the cells were cultured for 5 more days in adipogenic medium. With this experimental setup, we consistently obtained a very high yield of cells with the typical morphological features of adipocytes, a large monoloccular lipid droplet.

Brightfield and Immunocytochemistry

To further corroborate and extend our analysis of adipogenic marker expression in our differentiation cultures we perfomerd immunocytochemistry experiments using a panel of antibodies against FABP4, PPARγ, and C/EBPα. Morpholgically, adipocytes generated by PPARγ overexpression exhibited a morphology characteristic of mature adipocytes. Many cells appear to be monoloccular or possess one large, predominant lipid droplet and a few surrounding small droplets as shown in (Figure 4-54).

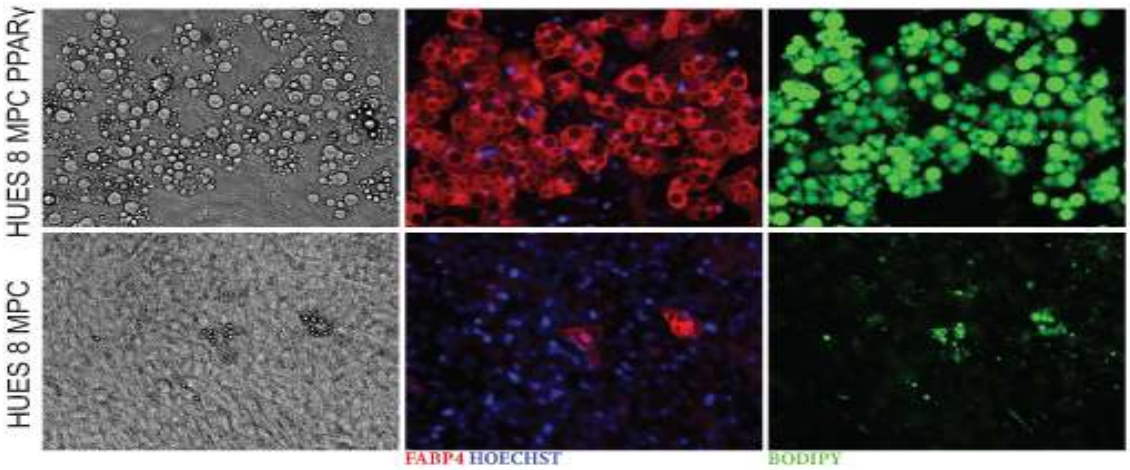


Figure 3-54 In vitro differentiation of transduced HUES 8 MPC (upper panel) and untransduced control HUES 8 MPC (bottom panel). From left to right: Brightfield image showing the morphology of the differentiated cells; Corresponding immunostaining for FABP4 (red) and co-stain HOECHST (blue); staining with neutral lipid dye Bodipy (green) and co-stain HOECHST (blue).

Staining for fatty acid binding protein 4 (FABP4) shows the typical appearance. In comparison the untransduced HUES 8 MPC differentiated into fewer and smaller adipocytes that exhibited a more immature morphology (Bottom panel).

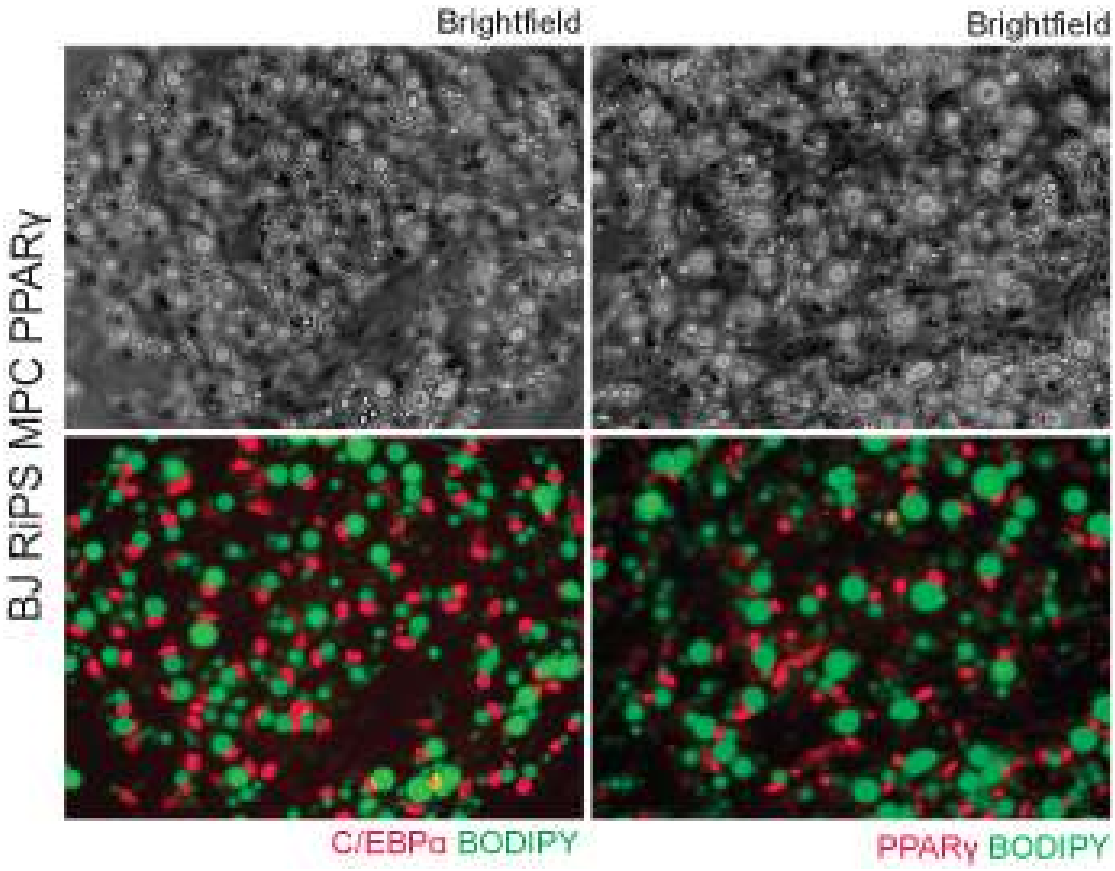


Figure 3-55 *In vitro* differentiation of transduced BJ RiPS #1.1 MPC. Top panel Brightfield image showing the morphology of the differentiated cells; Bottom panel left: Corresponding immunostaining for C/EBPα (red) and co-stain with neutral lipid dye Bodipy (green). Bottom panel right: Corresponding immunostaining for PPARγ (red) and co-stain with neutral lipid dye Bodipy (green)

Figure 4-55 shows staining for PPARγ and C/EBPα of BJ RiPS MPC PPARγ. All cells were co-stained with Bodipy neutral lipid dye. Again the brightfield pictures as well as the Bodipy stain reveal the characteristic mature monoloccular appearance of the adipocytes. Most of the cells stained positive for PPARγ in the absence of doxycycline as well as positive for C/EBPα.

Long Term Culture

Based on the data presented up to this point, it would be conceivable that overexpression of PPARγ would lead cells to temporarily adopt some characteristics of

adipocytes, but that they later, when transgene expression is lost, would revert back to a non-adipocyte fate. To rule out this possibility we performed experiments aimed at showing that adipocyte marker expression is independent of continued exogenous expression of PPARγ. To this end, we performed long term culture experiments where doxycycline was withdrawn day 16, after which the obtained adipocytes were cultured for up to 4 additional weeks. Immunocytochemistry revealed that differentiated cells maintained expression of C/EBPα and BODIPY staining lent further support to the notion that cells retained their adipocyte identity independent of transgene expression (Figure 4-56). Thus, our data argue that a pulse of PPARγ expression is sufficient to permanently switch the fate of MPCs to the adipocyte lineage.

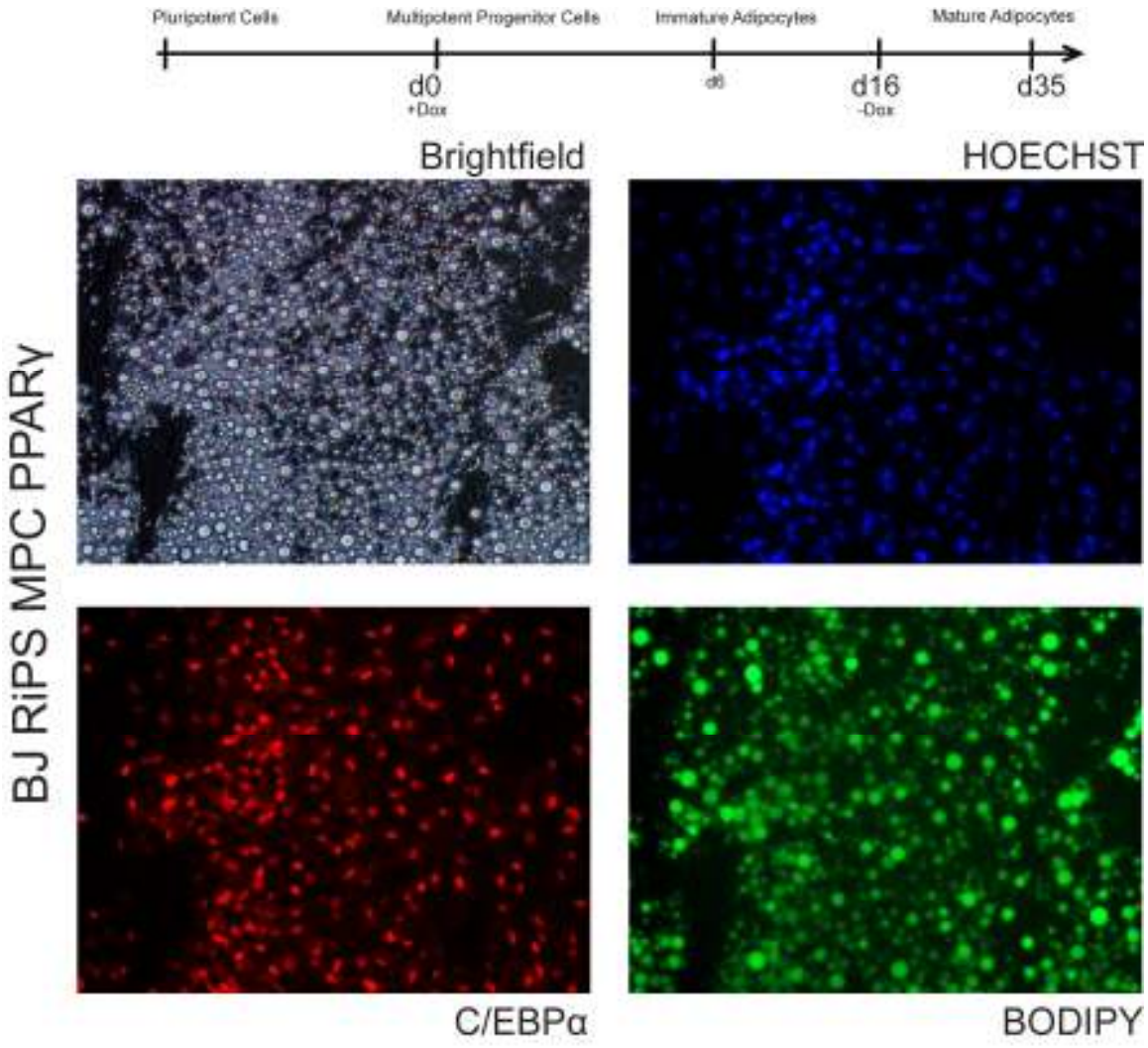


Figure 3-56 *In vitro* differentiation of transduced BJ RiPS MPC 16 days +DOX and additional 19 days –DOX. Top panel left: Brightfield image showing the morphology of the differentiated cells; Top panel right: nuclear stain for HOECHST; Bottom panel left: Corresponding immunostaining for C/EBPα (red); Bottom panel right: Staining of the neutral lipid using Bodipy (green).

Oil-Red-O staining/Quantification

To quantify the amount of lipid accumulation in our cultures, cells were stained with Oil-Red-O after 21 days of differentiation (Figure 4-57 - upper panel).

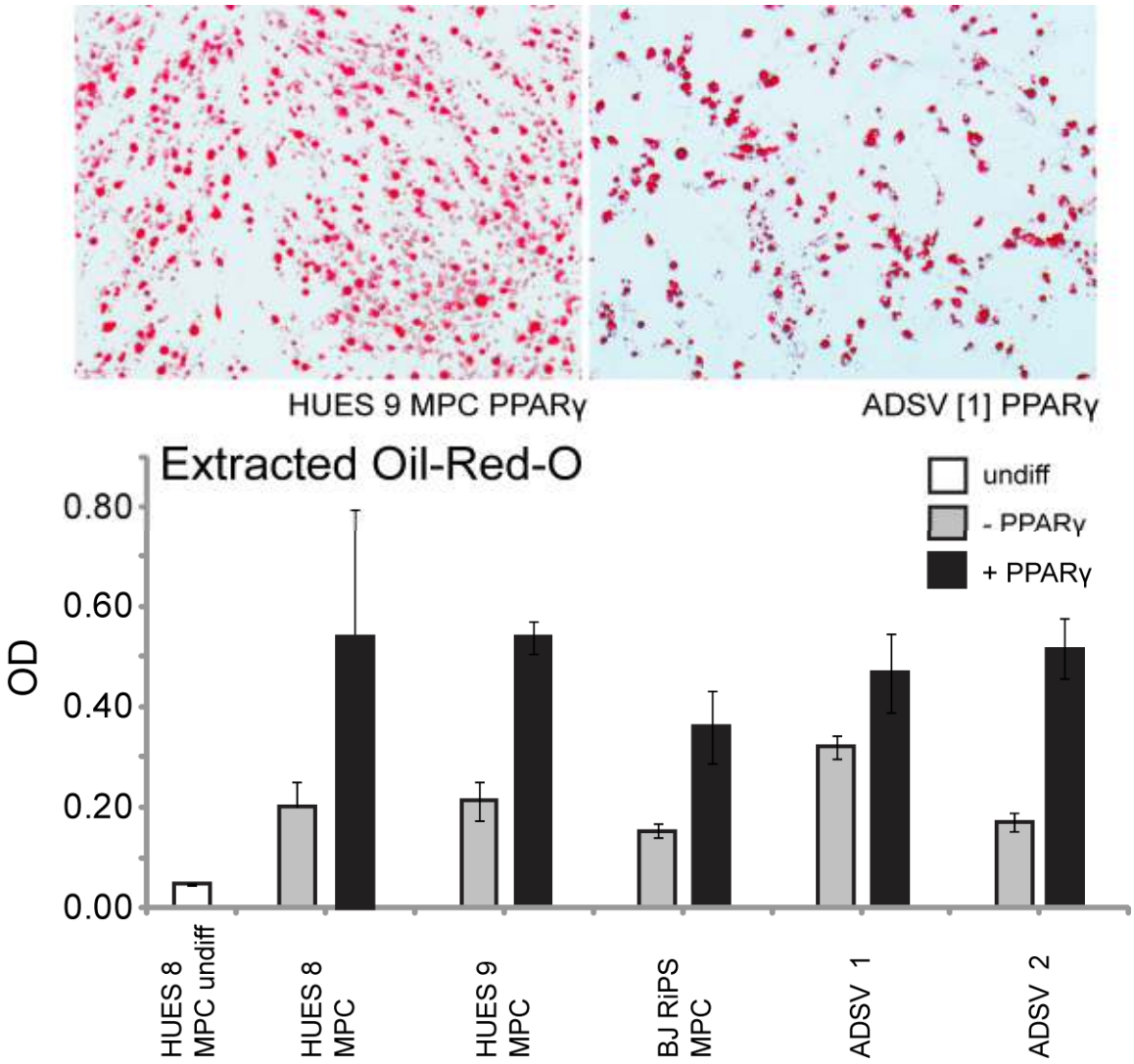


Figure 3-57 OIL-Red-PICTURES of differentiated cells before extraction and quantification of OIL-RED-O by OD measurement after extraction.

This clearly demonstrates a higher differentiation to the adipocyte fate of cells subjected to exogenous PPARγ during differentiation. To quantitate the amount of dye absorbed in the different culture conditions, Oil-Red-O was extracted and measured spectrophotometrically for each sample (Figure 4-57 - bottom panel). Again, this showed that PPARγ –boosted cultures retain more Oil-Red-O, reflecting their increased propensity to adopt the adipocyte fate.

Pluripotent cell-derived adipocytes express mature markers:

Analysis of gene expression

To characterize the adipocyte-like cells obtained through inducible PPARγ expression molecularly, we initially performed semi quantitative RT-PCR assays, . comparing the expression level of several adipogenic in pluripotent cells, undifferentiated MPCs and ADSV as well as the corresponding differentiated lines that were either transduced with PPARγ or differentiated without the overexpression of the transgene (Figure 4-58).

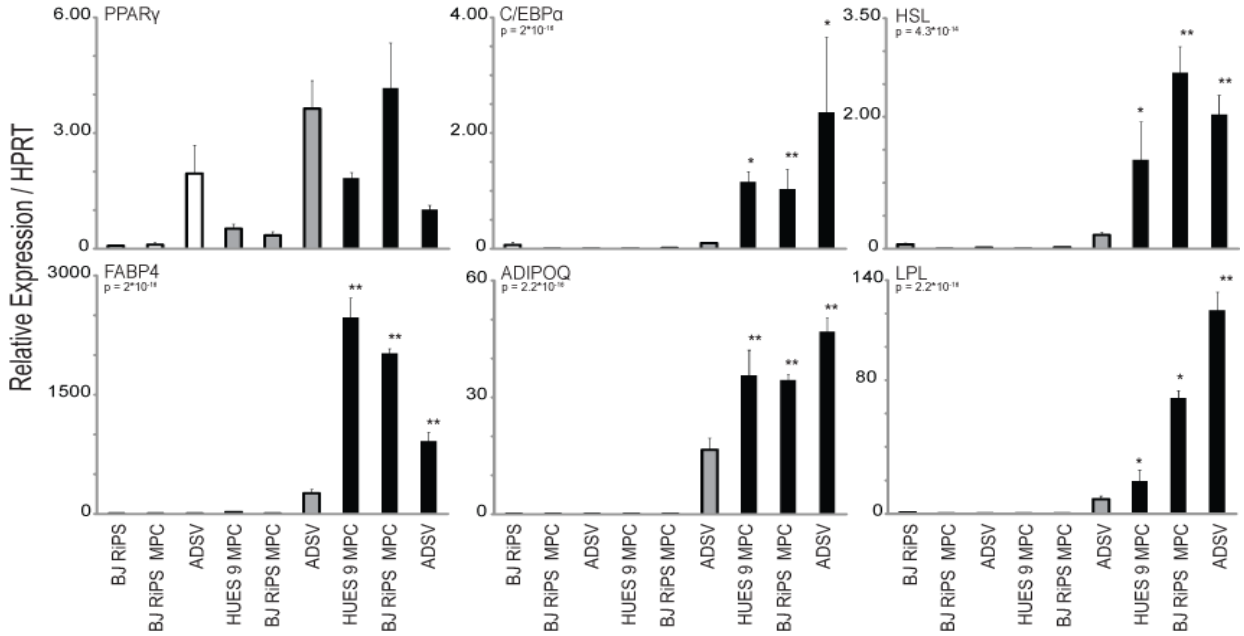


Figure 3-58 qRT-PCR Panel showing the adipogenic marker expression of PPARγ, C/EBPα, FABP4, ADIPOQ, HSL and LPL. Data represents 3 biological replicates and is shown as relative expression to the housekeeping gene HPRT. Grouped in white bars are untransduced and undifferentiated control cells. Shown in grey are untransduced but differentiated lines and in black are different cell lines that were transduced and differentiated under standard conditions. P values at the bar graphs represent two tailed student t-test between the transduced and non-transduced differentiated cell expression values of each cell line. *p < 0.05; **p < 0.01. P values shown under the gene name represent Annova analysis between all samples (including lines not shown in this graph) in the two groups of untransduced and transduced cells.

Adipogenic marker gene expression was absent in undifferentiated cell lines with the exception of PPARγ, which was detected in untreated ADSV cell lines. As shown in Figure 4-58, all differentiated lines expressed PPARγ 2-4.5 fold higher expression levels than the housekeeping gene HPRT. Doxycycline was withdrawn 5 days prior to analysis. This fact, together with the documented tight control of transgene expression, supports the notion that the detected PPARγ mRNA represents transcription from the endogenouss PPARγ gene rather than leakage from the viral transgene. Levels of C/EBPα, another transcription factor important for adipogenesis, were low in untransduced cells but strongly expressed in the transduced cells (Figure 4-58 Top middle). Transcription factors are frequently expressed at lower levels than structural genes and cell-type specific enzymes. In keeping with this, the differences in adipocyte-specific gene expression was even more striking when we extended out analysis to adipocyte-specific molecules involved in lipid transport and metabolism. Fatty acid binding protein 4 (FABP4) is an adipocyte-specific member of a family of carrier proteins for fatty acids and other lipid derived molecules. qPCR analysis revealed up to 2500 fold higher FABP4 expression than the housekeeping gene in cells differentiated by PPARγ overexpression. Robust expression of transcripts encoding the mature adipocyte markers Adiponektin (ADIPOQ), hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) were also detected in doxycycline-induced cultures. We also compared the expression levels of adipocyte-specific genes in adipocytes derived from pluripotent cells by PPARγ overexpression with that seen in human primary fat. In general, adipogenic genes exhibited even higher levels of expression in primary fat (Figure 4-59). Importantly though adipocyte-specific genes in adipocytes derived from transduced MPCs exhibit significant expression of genes exclusively expressed in mature adipocytes.

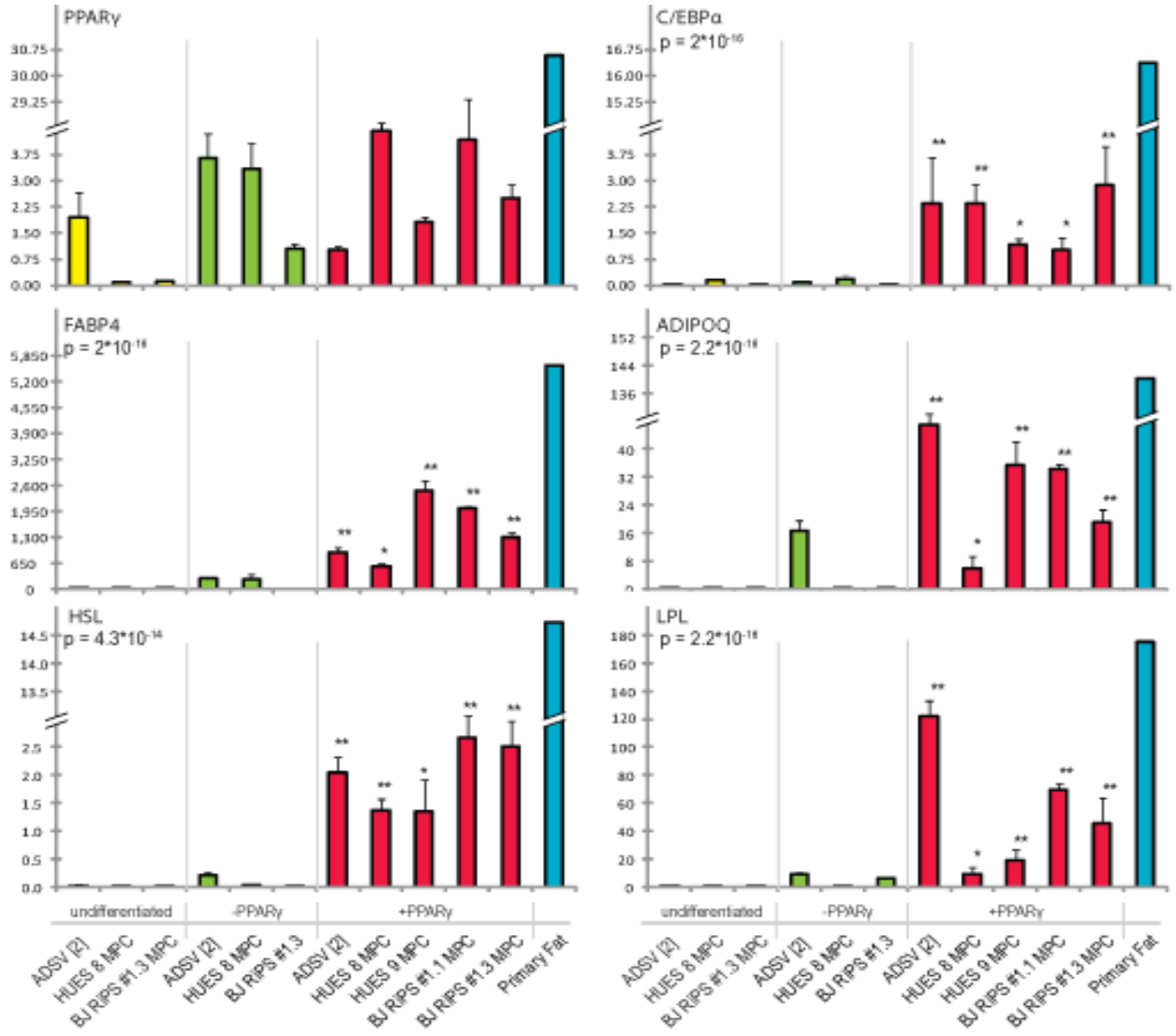


Figure 3-59 qRT-PCR analysis of the adipogenic marker expression of PPARγ, C/EBPα, FABP4, ADIPOQ, HSL and LPL. Data represents 3 biological replicates and is shown as relative expression to the housekeeping gene HPRT. Grouped in yellow bars are untransduced and undifferentiated control cells. Shown in green are untransduced but differentiated lines and in red are different cell lines that were transduced and differentiated under standard conditions. Blue and in some graphs on a separated scale is the expression level of primary fat samples harvested from the pannus of a patient that underwent elective surgery. P values at the bar graphs represent two tailed student t-test between the transduced and non-transduced differentiated cell expression values of each cell line. *p < 0.05; **p < 0.01. P values shown under the gene name represent

annova analysis between all samples in the two groups of untransduced and transduced cells.

Efficiency of differentiation

Caution is warranted when determining the percentage of cells labeled with BODIPY or Oil-Red-O when quantifying the efficiency of adipocyte differentation. This is due to the fact that nuclei of different cells can be positioned very close to each other, rendering it impossible to determine if an overlaying Oil-Red-O or BODIPY stain belongs to either one or both nuclei. To determine the overall efficiency of differentiation we co-stained adipocytes derived from three different lines with C/EBPα and the nuclear dye HOECHST. We used the image analysis software ImageJ to determine threshold levels to count the number of positive stained cells. The overall differentiation efficiency of the transduced MPCs was between 80-88% (Figure 4-60).

Differentiated Cell Line	% of C/EBPα	STDEV
HUES 9 MPC untransduced	9 %	+/- 2
HUES 9 MPC PPARγ	88 %	+/- 8
BJ RiPS PPARγ	87 %	+/- 6

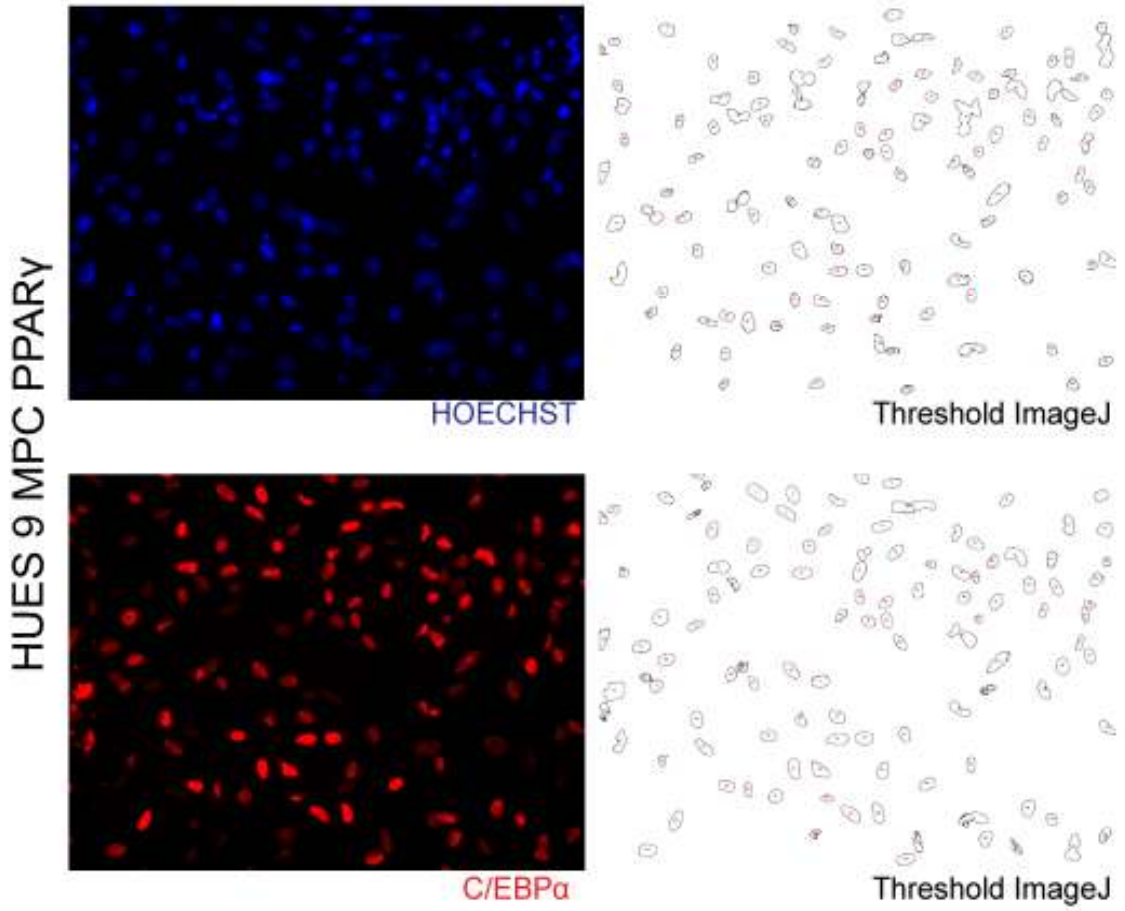


Figure 3-60 Table Differentiation efficiency as determined by a ratio of nuclear HOECHST stain and C/EBPα stain in differentiated adipocytes (Image J data analysis). All fluorescence pictures were thresholded using Image J analysis software.

Pluripotent cell-derived adipocytes exhibit mature functional properties

Glycerol Release assay

To show functional properties of the hPSC-derived adipocytes we examined release of glycerol in response to beta-adrenergic stimulus, a key property of adipocytes. Glycerol release was measured using a colorimetric assay kit after 4h of beta-adrenergic stimulation (Figure 4-61).

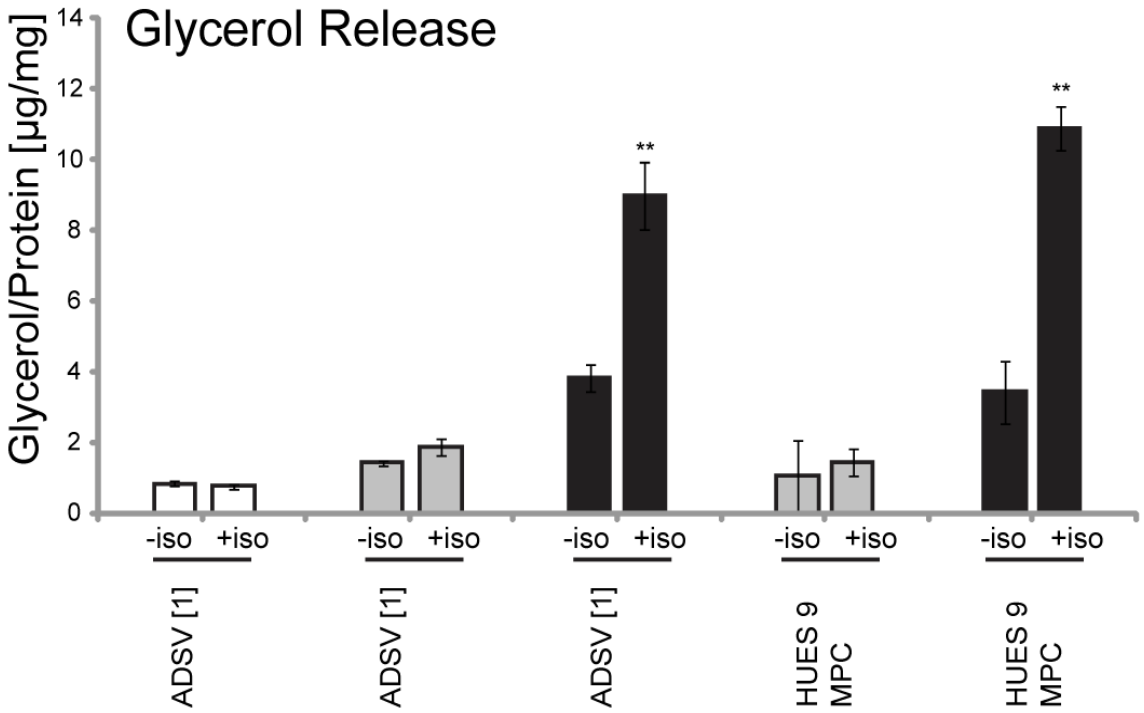


Figure 3-61 Lipolysis - Glycerol release assay; measurement of glycerol in the supernatant of transduced and differentiated cells with and without beta adrenergic stimulus and normalized to extracted whole cell protein.

A strong, statistically significant response to beta adrenergic stimulus was observed. These data indicate that hPSC-derived adipocytes exhibit a fully functional physiologic response characteristic of mature adipocytes, and that they consequently can be used in metabolic assays in response to chemical stimulus.

Multiplex analysis

To further demonstrate mature adipocyte function we analyzed the release of adiponectin by hPSC-derived adipocytes. To this end, we collected medium after 24 hours of incubation and performed an adiponectin enzyme immunoassay. We found

high levels of secreted adiponectin in all PPARγ transduced cell lines including several differentiated hPSC derived MPC lines as well as in the ADSV lines (Figure 4-62).

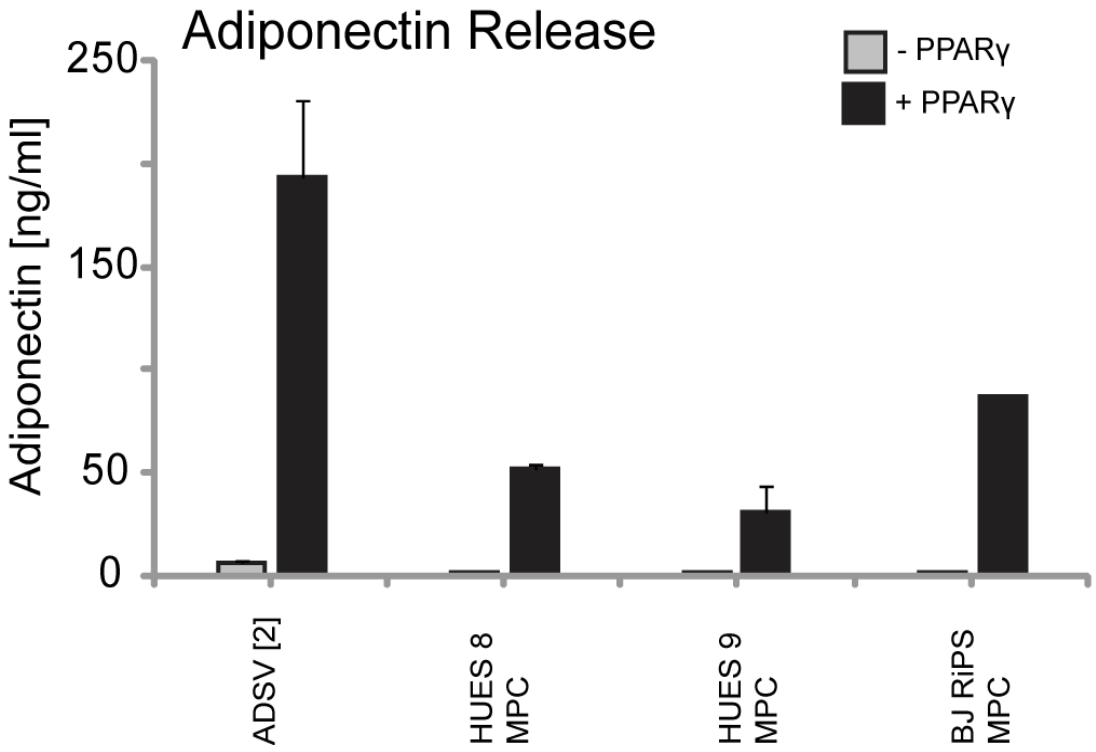
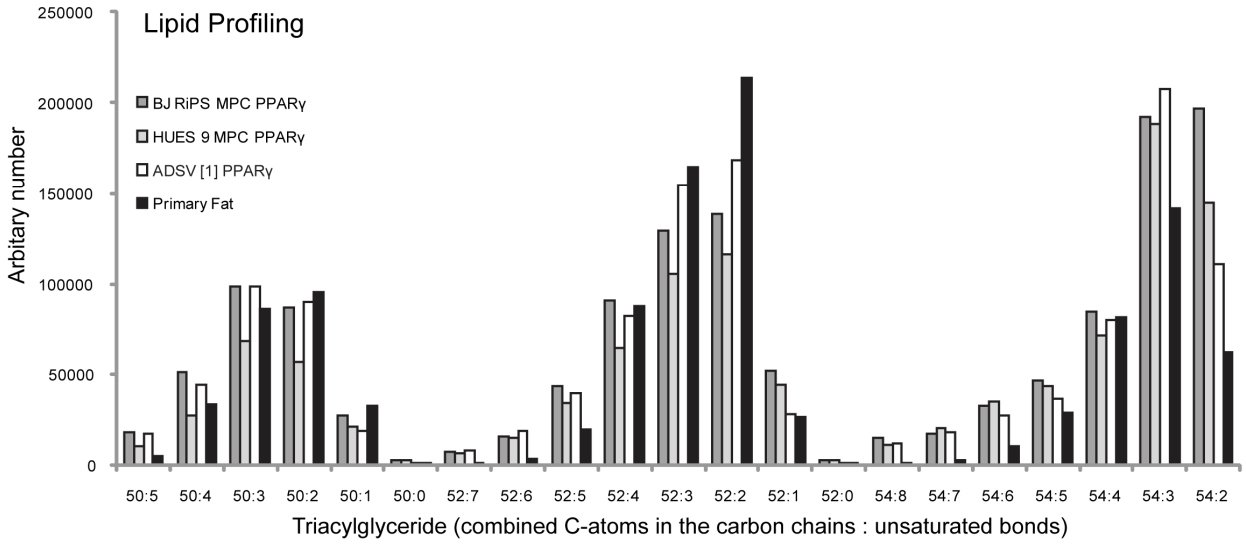


Figure 3-62 ELISA - Analysis of secreted adipokines in the supernatant of transduced and differentiated cells, incubated for 24 h.

Lipid analysis

We sought to characterize the lipid composition of the in vitro derived adipocytes from hPSCs as well as from the ADSV fraction and compare the results with lipid extracted from primary human fat. The lipid profiling of the PPARγ transduced lines showed striking similarities to the pattern found in primary fat tissue. The profile was very consistent between different transduced pluripotent lines as well as transduced ADSVs lines (Figure 4-63).



3-63 Lipid profiling of extracted lipids from transduced and differentiated cells and compared to the pattern present in primary human fat.

Shown in figure 4-63 is a core set of analyzed triglycerides that contains the most abundantly lipids. We did however find that the in vitro derived adipocytes contain more short chain fatty acids than primary adipocytes likely attributed to the de-novo lipogenesis present in the *in-vitro* system (Supplemental figures 4-64). Further the lipid pattern was not completely recapitulated in untransduced and not at all in undifferentiated cells.

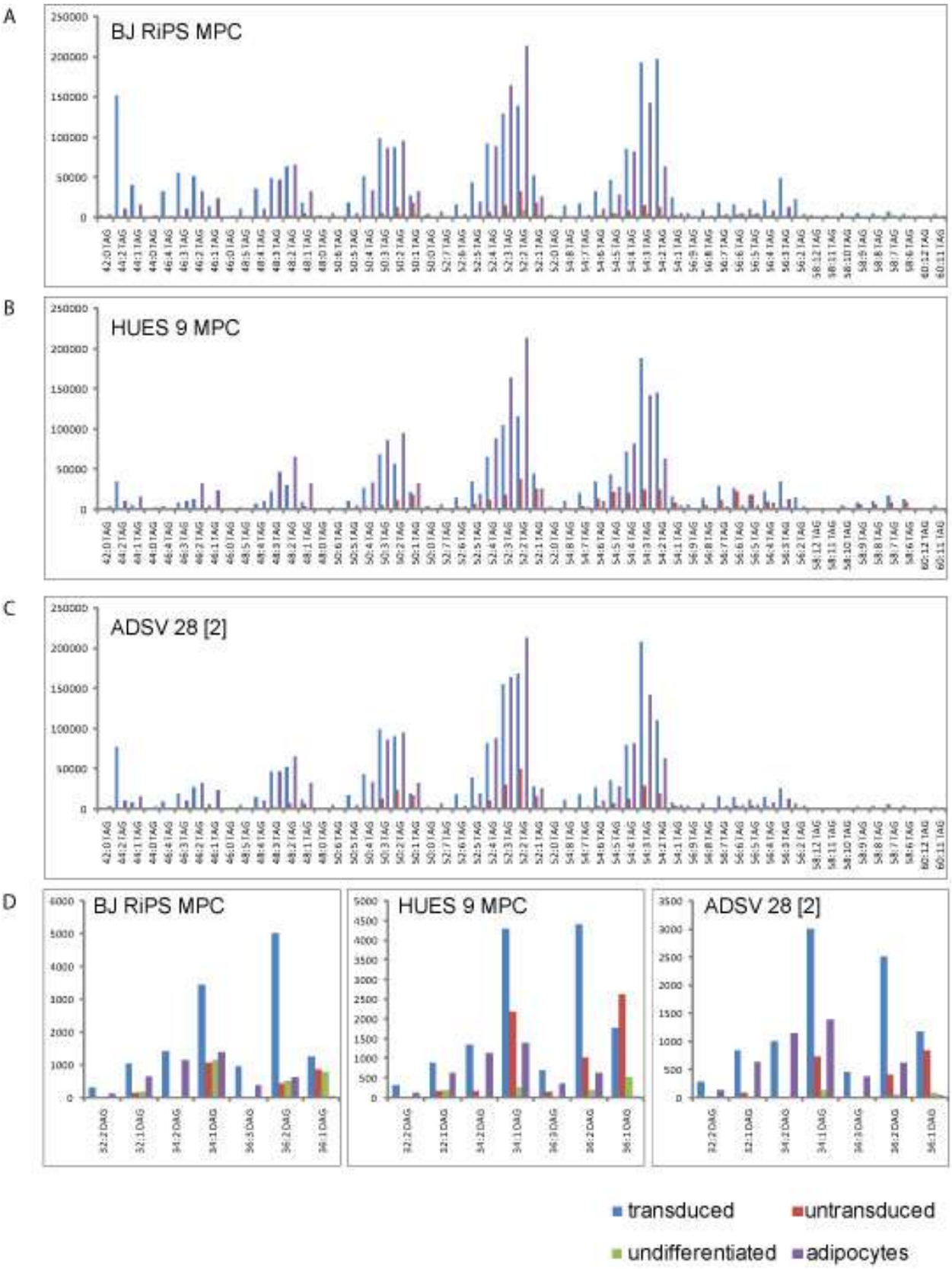

A
BJ RiPS MPC
B
HUES 9 MPC
C
ADSV 28 [2]
D
BJ RiPS MPC
HUES 9 MPC
ADSV 28 [2]
transduced
untransduced
undifferentiated
adipocytes

Figure 3-64 Lipid profiling of extracted lipids from 3 different human cell lines. Data from undifferentiated, untransduced/differentiated and transduced/differentiated cells were compared to the pattern present in primary human fat. The three upper panels show the combined C-atoms of the Triacylglyceride fatty acid carbon chains:number unsaturated bonds on the X-axis; Bottom panel show the combined C-atoms of the Diacylglycerides fatty acid carbon chains:number unsaturated bonds on the X-axis.

Taken together, we show that our differentiation protocol, where pluripotent cells are subjected to a pulse of exogenous PPARγ expression during differentiation, results in a high yield of cells that exhibit the morphology of mature adipocytes. Furthermore, the obtained cells express key markers of adipocytes, both at the mRNA and protein level. Finally, assays of induced lipolysis and secreted proteins as well as lipid content profiling gave independent support that our differentiation protocol can be used to obtain cells that functionally are highly similar to adipocytes found in primary fat. In sum, our results show that pluripotent human cells can be differentiated to cultures of adipocytes that should prove to be an ideal cellular source for different in vitro analyses of fat cells.

3.3.4 Discussion:

Low efficiency of differentiation, inadequate characterization and a lack of reproducibility between different initial cell lines used were the three significant flaws in previously published attempts to generate adipocytes. We have shown that via and EB intermediate it is possible to efficiently generate MPCs from hPSCs. Further, we have demonstrated that these mesenchymal progenitor cells (MPCs) can be efficiently and robustly differentiated into adipocytes through the inducible overexpression of PPARγ. Moreover, we have performed a detailed analysis of the resulting adipocytes and have found that they possess the molecular and functional characteristics of mature adipocytes.

We have used a comparatively simple protocol that involves an EB formation step but not flow sorting to produce MPCs from pluripotent cells. The MPCs almost uniformly expressed the CD73 antigen. Importantly, when transduced with PPARγ these MPCs displayed a dramatic increase in the efficiency of differentiation to adipocytes. Thus, we

chose not to extensively characterize the intermediate MPC population. Overall, we have established a simplified approach for generating MPCs that are capable of robustly and efficiently differentiating to adipocytes.

In contrast to former experiments where ectopic overexpression of PPARγ was applied to a variety of mouse and human cell types we have utilized a model of induced transgene expression. After doxycycline was withdrawn and subsequent exogenous PPARγ expression halted the adipocyte identity of the cell was established and maintained by endogenous transcription networks. When inducible PPARγ overexpression was applied to hPSC-derived MPCs and ADSV cells, cell types known to be prone to adipogenic differentiation, we observed differentiation efficiency to adipocytes of up to 88%. Currently, this is the most efficient hPSCs differentiation protocol reported.

Throughout this study we compared pluripotent derived adipocytes to primary ADSV cells. We chose to show the differentiation results of untransduced ADSVs as well as PPARγ transduced ADSVs and primary human tissue. Pluripotent and ADSVs that were differentiated under the influence of PPARγ showed a very similar morphology, marker expression and in general performed very similar in most of the assays performed.

Overall, we found that cells which were exposed to PPARγ overexpression for 16 days consistently displayed a more mature adipocyte identity than cells only exposed to adipogenic media only. For instance, while PPARγ transduced cells often appear as monoloccular or at least have one dominant large lipid droplet with a few small lipid droplets, the untransduced pluripotent derived adipocytes as well as the ADSV derived adipocytes appear to be more multiloccular and only a few cells exhibit a large single lipid droplet. The molecular characterization by qRT-PCR revealed that the PPARγ transduced and differentiated cells express adipocyte-specific genes at high levels. They follow the molecular signature of primary human fat tissue. In contrast, the untransduced cells showed adipogenic marker expression at significantly lower levels . Mature markers were either undetectable or expressed at very low levels in untransduced cells by comparison. These data demonstrate that untransduced cells not

only less efficiently differentiated to adipocytes but also failed to mature during the differentiation process.

In general, human primary fat tissue showed a higher expression of all adipocyte specific genes than differentiated cells. We chose the housekeeping gene HPRT as a relative control because we observed very little to no level of change between undifferentiated and differentiated cell lines as well as between lines *in vitro*. The only exception was primary fat where we consistently observed a lower expression level of the HPRT gene. As all data was normalized to HPRT expression, this may help explain the difference in adipocyte specific genes observed in primary fat as compared to our differentiated cell lines.

We also performed a series of functional assays of our hPSC-derived adipocytes. These functional analyses included an enzymatic immunoassay of the adipokine Adiponectin, a glycerol release assay designed to measure lipolysis in response to β-adrenergic stimulus, and global lipid profiling by mass spectroscopy. High levels of released adiponectin were found in all transduced and differentiated lines. Comparative studies of differentiated MPCs and ADSVs also showed strikingly similarities between the two populations in the ability of the cells to release glycerol and thus breakdown fat in a catabolic process called lipolysis. Functional equivalency of human cell derived and primary adipocytes were demonstrated by near identical lipid profile analysis. A quantification of the starting amount of lipid was not possible with our method and so while we cannot compare the amount of stored lipids we demonstrated that the transduced differentiated cells showed a very similar distribution of triglycerides with the exception of the triglyceride fraction that contained very short chains. Here the differentiated cells showed a higher ratio that is likely attributed to the de-novo lipogenesis in the *in vitro* culture.

The high efficiency and reproducibility of our protocol combined with the functional equivalency of the human cell derived adipocytes are all necessary features needed for a reliable source of adipocytes for downstream metabolic assays such as those used to study the storage and release of fat.

The possibilities to study human disease with distinct genetic backgrounds through hiPS cells are responsible for the enormous interest that these cells draw in the public opinion as well as in the scientific community. Unlike any somatic cell model like ADSV cells, hiPS cells self-renew and thus vast numbers of cells with the same genetic background can be generated in order to study metabolism or disease development in the same background. Further recent advantages in manipulating the genome of human cells in the pluripotent stage through homologous recombination and zinc-finger targeting rendering these cells ideal candidates for any in vitro study of human development or disease.

This inducible and highly efficient system to differentiate human pluripotent cells into adipocytes is a very attractive model to investigate human adipocyte metabolism in normal and disease state. By providing almost a homogenous and replenishable population of adipocytes with the same background this differentiation method allows for large scale studies like chemicals screens to find molecules that alter adipocyte function and physiology and it will ultimately help to understand the role of adipocytes in human disease.

3.3.5 Experimental Procedures:

Maintenance of pluripotent cells, differentiation into multipotent progenitor cells, differentiation protocol, derivation of adipose derived stromal cells (ask Greg) ADSC, maintenance of ADSC

hES and hiPS cells were cultured feeder free on geltrex (Invitrogen) in chemical defined medium mTESR1 (Stem Cell Technologies). To induce differentiation of hES and hiPS into embryoid bodies (EB), hES were disaggregated, using Dispase, into small clumps containing 5-10 cells, and transferred to low-adhesion plastic 6-well dishes (Costar Ultra Low Attachment; Corning Life Sciences) in growth medium containing DMEM, 15% FBS, 1% Glutamax. Half-medium changes were performed any other day. After 7 days in suspension culture, EBs were collected and replated on gelatin coated 6-well dishes in medium containing DMEM, 10% FBS, 1% Glutamax. 1 well was replated into 1 well. EBs attached in 24 hours and fibroblast cells grow out of them. After about 5 days they

cover the well. Cells were trypsinized (0.25% Trypsin) and replated on cell culture dishes containing a MSC growth medium (1:3 split ratio). DMEM, 15% FBS, 1% Glutamax, 2.5 ng/ml bFGF. Cells were consequently passaged in a 1:3 split ratio and cells were used for differentiation experiments. Adipogenic differentiation was carried out for 21 days using differentiation medium termed A2 (DMEM, 7.5% KOSR, 7.5% human plasmanate, 0.5% non essential amino acids, 1% Penicillin, Streptomycin, 0.1 µM dexamethasone, 10 µg/ml Insulin (Sigma), 0.5 µM Rosiglitazone). A2 medium was supplemented for 16 days with doxycyline 700 ng/ml. Afterwards differentiation of was continued in the absence of doxycline until day 21.

Cloning of transgene(s)

pLV-tetO-attR1/R2: pLV-tetO (Stadtfeld 2008) was linearized with EcoRI followed by treatment with DNA Polymerase I, Large (Klenow) fragment to fill-in and blunt the 5' overhangs. The resulting fragment was ligated with the Reading Frame A Gateway cassette (Invitrogen).

pENTR-PPARG2: To generate pENTR-PPARG2, the human PPARG2 transcript was amplified from (Plasmid 11439: pBabe bleo human PPAR gamma 2 (Addgene) and recombination sites were added. PPARG2 attB1 and PPARG2 attB2r: The PPARG2 attB1 primer inserts a CCACC Kozak consensus immediately 5' to the initiating ATG. The attB1/attB2r flanked PPARG2 PCR product was recombined into pDONR221 (Invitrogen) with BP Clonase.

pLV-tetO-PPARG2: pENTR-PPARG2 and pLV-tetO-attR1/R2 were recombined with LR Clonase II as described by the manufacturer's recommendations (Invitrogen).

All clones were confirmed by restriction digest screening and sequencing.

Production of virus:

A third-generation, Tat-free packaging system (Tiscornia et al., 2006) was used to produce recombinant lentivirus. The vectors either rtTA M2 or TET-CMV-PPARγ together with the two packaging plasmids pMDL, pREV and the plasmid coding for VSV-G envelope were transfected into 293T(HEK) cells using Calcium chlorate as

previously described (al Yacoub et al., 2007). In brief, we transfected HEK293 T cells at subconfluency, discarded medium after 24 hours and collected viral containing supernatant after 48, and 72 h. Viral supernatant was filtered through a 0.45-µm filters and used directly for the transduction of cells.

Transduction direct and indirect method:

Cells were transduced using lentiviral supernatant 24h after passaging. Cells were incubated at 37^0C for 4h. Viral supernatant was removed, cells were washed with PBS and maintenance medium was added. Cells were subsequently passaged or used directly in differentiation experiments. 700 ng/ml Doxycycline was added to induce transgene expression.

Flow cytometry analysis:

All cells were trypsinized, washed with PBS and centrifuged at 1000 RPM for 5 minutes. Cells were counted and $1x10^6$ cells were transferred into polyprobylene tubes. Staining for CD73 antigen (PE-CD73, Ecto-5´-nucleotidase AD2, BD Pharmingen) was performed using 20 ul of the conjugated antibody per 10,000 cell events per assay were acquired on a FACsCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar).

Immunocytochemistry

Immunostaining was performed using the following antibodies: a-FABP4, a-Perilipin, a-C/EBPα, a-PPARγ and Alexa Fluor secondary antibodies. Lipid droplets were stained using Bodipy in 1:20000 dilutions. Hoechst stain was used 1:5000 to show cell nuclei.

RNA extraction, cDNA synthesis and qRT PCR

Total RNA from human cell lines and human fat was extracted with Trizol® (Invitrogen) and 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). 1 µg of total RNA was converted to cDNA using the Superscript First-Strand Kit (Invitrogen). RT-PCR was performed using a

Realplex Mastercycler (Eppendorf) with the Quantifast SYBR® Green PCR mix (Qiagen) with 1µl cDNA per reaction. Primer sequences are listed in supplementary table.

Microarray:

RNA Prep, Bioanalyzer, acquire information about machine from Broad facility. Frank will write a method for the analysis.

Lipolysis Assay:

To measure lipolysis activity, day 21 adipocytes, differentiated from both pluripotent cells and human ADSVC cells, were starved in DMEM containing 1% FBS for 1 hour. Then, cells were incubated in HBSS (Hank's Balanced Salt Solution) with 2% fatty acid free-BSA alone or with 10 µM Isoproterenol for 4 hours. The culture media were collected for glycerol measurement using a colorimetric assay kit (Sigma). Protein concentration used to normalize glycerol contents was measured using the Bradford protein assay (BioRad). Glycerol release is expressed in µg/mg. Data are presented as mean ± SEM of triplicates.

Quantification of Oil-red-O:

Oil-Red-O staining was carried out as previously reported and Oil-Red-O was extracted using 100%Isopropanol. Extracted Oil-Red-O was collected in triplicates and absorption was read at 500 nm absorption. Measured absorption was normalized to extracted protein values from the same experimental setups. All procedures were carried out on ice. 10 ml of buffer whole cell lysate buffer (WCL) were mixed with 1 protease inhibitor tablet. Each well was incubated with 500 ul of the buffer, cells were detached with a cell scraper and the suspension collected in an Eppendorf tube. The suspension was homogenized (tissue tearer) and incubated at 4C for 20 minutes (rocking). Suspension was centrifuged at Max Speed at 4C for 10 minutes; the supernatant was collected and stored at -80C. Protein concentration was measured using a Bradford assay.

Multiplex analysis:

Multiplex analysis. Cell culture supernatants were analyzed for 3 adipokines (adiponectin, leptin, and TNFalpha) using the Protein Multiplex Immunoassay kit (Millipore Corporation) as per the manufacturer's protocol. Briefly, the samples and kit controls were loaded onto a Millipore Multiscreen BV 96-well filter plate. Serial dilutions of adipokine standards were prepared in parallel and added to the plate. Millipore's Multiplex beads were vortexed and sonicated for 30s and 25µl was added to each well. Samples were incubated on a plate shaker at 600 revolutions/min in the dark at 4°C overnight. The plate was applied to a Millipore Multiscreen Vacuum Manifold and washed twice with 200µl of wash buffer. 50µl of biotinylated Human Adipocyte Detection Antibodies was added to each well. The plate was incubated on a plate shaker at 600 revolutions/ min in the dark at room temperature for 1 h. 50µl of streptavidin-phycoerythrin was added directly to each well. The plate was incubated on a plate shaker at 600 revolutions/min in the dark at room temperature for 30 min. The plate was applied to the vacuum manifold, washed twice with 200µl of wash buffer, and each well was resuspended in 100µl sheath fluid and shaken for 5 min.The assay plate was transferred to the Luminex 200 instrument for analysis. Cytokine concentrations were calculated using Upstate Beadview software with a five parameter curve-fitting algorithm applied for standard curve calculations.

Metabolic Panel:

All experiments were carried out using 3 biological replicates of cells grown on 10 cm plates. Medium was replaced 2 hours before the extraction of the lipids. 3 ml of ice cold isopropanol (HPLC grade) was added and cells were scraped using a cell scraper and transferred into 15 ml centrifuge tubes. The extracts were incubated for 1h at 4°C, then vortexed and centrifuged at 3500 RPM for 10 min. The supernatant was transferred to a new 15 ml centrifuge tube and stored at -20°C.

All data was acquired using a Applied Biosystems QSTAR XL hybrid quadrupole/time-of-flight mass spectrometer (Applied Biosystems/Sciex, Foster City, CA). MultiQuant software (version 1.1, Applied Biosystems/Sciex, Foster City, CA) was used for

automated peak integration and peaks were manually reviewed for quality of integration. Internal standard peak areas were monitored for quality control and used to normalize analyte peak areas.

4 Discussion

Thomson and colleagues were the first to derive human embryonic stem cells (Cowan et al., 2004; Thomson et al., 1998). This discovery unveiled wide ranging potential not only therapeutically in the utilization of these cells for regenerative medicine and disease modeling but also providing a cell based system for modeling the study of early human development. Studies based on the hypothesis that ES cell specific transcription factors might induce pluripotency in somatic cells led to the discovery of transcription-factor-mediated reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Soon after these results were reproduced simultaneously by several groups and since then many publications have gone on to address specific questions surrounding the central concept of cellular reprogramming. Statistical studies of this reprogramming process have reported a very low success rate of 0.01-0.1%. One of the goals in the field of iPS cell study has been to increase this efficiency by gaining a better understanding of the underlying events that facilitate reprogramming. Another hindrance in the advancement of cellular reprogramming from the laboratory to the clinic is the safety concerns surrounding the genomic integrations of the transgenes used to facilitate the process.

Understanding reprogramming and improving the methodology is crucial if the potential of cellular therapies are to become a clinical reality. Reprogramming of somatic cells to pluripotent state has shown that cell fate is not terminal and is held in place by an established network of transcription factors that is regulated by epigenetic barriers. Waddington's depiction of an epigenetic landscape describes how a cell loses pluripotency in a stepwise manner when acquiring a new cell fate but the mechanisms that guide this differentiation are poorly understood. In recent months many studies of lineage reprogramming have emerged and it has been hypothesized that lineage reprogramming between closely related cell types is easier as they present less prohibitive epigenetic barriers (Selvaraj et al., 2010). The suggestion that a patient-specific terminal differentiated cell could be used to generate abundant amounts of a specific cell type by direct conversion provides a unique advantage as it eliminates the pluripotent stage in the reprogramming and the risks that associated with stem cell

therapy. However, the disadvantage of direct conversion is that somatic cells cannot be propagated for a long time and genetic modifications such as homologous recombination are currently only practical in pluripotent cells. As a result the strategy for cell-based therapy chosen will strongly depend on the experimental plan, the availability of differentiation protocols, the type of cell population initially harvested, the necessity for genomic modifications and the quantity of cells required for treatment at the end of the reprogramming process.

Like ES cells, iPS cells can differentiate into virtually any cell type making them the most attractive cell type to model human disease. Although the differentiation of ES/iPS cells into terminal differentiated cells of interest has been shown in principle, most protocols are unreliable and yield only a small successful conversion rate.

This thesis makes several relevant contributions towards the therapeutic utilization of ES/iPS cells and has addressed the following key questions. How similar are ES cells and IPS cells? Are all cell types amenable to reprogramming and in particular can keratinocytes be used in the process? Can an inducible lentiviral system be used to overexpress transcription factors and force a new cell identity in an attempt to either reprogram cells or differentiate pluripotent cells to become adipocytes? Can a combination of overexpressed transcription factors and the right differentiation conditions lead to highly efficient differentiation of pluripotent cells? The work also effect bigger questions concerning the universality of reprogramming. Is it possible to change the fate of every cell type by the expression of the right set of transcription factors without going through a pluripotent stage? What are the mechanisms that govern and guide differentiation and determine cellular fate?

4.1 To generate human induced pluripotent stem cells from somatic cells.

During the early stages of embryonic growth the embryo passes through an important developmental stage called gastrulation. This process results in the derivation of three types of germ cell linages, termed mesoderm endoderm and ectoderm. In all initial studies generating induced pluripotent stem cells human fibroblasts were used as the starting cell material for reprogramming. Fibroblasts are derived only from mesoderm,

raising the question of whether cells from the ectodermal lineage could also be reprogrammed by the four factors OCT4, SOX2, cMYC, KLF4. In addition all three initial publications used constitutively expressed retroviruses to carry out the reprogramming process which resulted in residual transgene expression after the iPS cell stage was reached.

To address the issue of residual exogenous expression we cloned Tet-inducible lentiviruses that overexpress the factors OCT4, SOX2, cMYC, KLF4 and NANOG dependant on the presence of rtTA M2 and doxycycline. Using an inducible system allows for the withdrawal of doxycycline when pluripotency is reached and termination of exogenous expression of the reprogramming factors. In the absence of doxycycline we were able show that the iPS cells generated were transgene independent and had established a stable state that is now governed by endogenous expression of the pluripotency network. This work is important, as residual overexpression of the transgenes has been implicated in skewed cellular differentiation patterns.

As previously discussed reprogramming is a very inefficient process, our system allows for the derivation of secondary hiPS cells at about a 100 fold higher efficiency. Secondary hiPS cells were generated after primary iPS cells were terminally differentiated into fibroblasts, these cells were placed on a feeder layer of MEF's and the inducible transgenes were reactivated when doxycycline was added. Proof of principle of this technology was shown when all 19 students of the undergraduate Harvard SCRB 165 class successfully utilized this procedure to derive secondary iPS (unpublished data). Human fibroblast cells have been initially used in reprogramming experiments; in particular the neonatal BJ fibroblast line has been the line of choice for many. It is unclear whether or not BJ fibroblasts are easier to reprogram than other cells or if they are used because of historic comparative reasons (Cowan et al., 2005). Given that fibroblast cells had been reprogrammed we set out to answer the question if keratinocytes could be reprogrammed as well. Keratinocytes are an ectodermal cell line. The culture of keratinocytes requires a serum free growth medium which has the advantage that it select against other cell types. Keratinocytes can also be harvested very easy from human patients by minimum invasive skin punch biopsies and even from

hair-pulls. We were the first to report successful iPS derivation from keratinocyte lines, as discussed earlier this is an important conclusion because these cells are from a different germ layer ectoderm and show that reprogramming is not restricted to the cells of mesodermal origin. Interestingly keratinocytes reprogrammed faster than fibroblast cell lines. Keratinocytes and hESC represent an epithelial cell type and we hypothesized that they reprogram faster because they don't have to undergo a mesenchymal-epithelial transition like the fibroblast cells (Yang and Weinberg, 2008). In addition a series of studies have demonstrated that reprogramming is a stochastic process and according to this model eventually every cell could be reprogrammed as long as the right growth conditions, continuous expression of the reprogramming factors are applied (Hanna et al., 2009; McAdams and Arkin, 1997; Yamanaka, 2009). We hypothesized, that in our study the increase cell division rate lead to altered kinetics and an increase incidence of successful reprogramming (Banito et al., 2009).

4.2 To generate integration free human induced pluripotent stem cells using modified RNA

There are a number of different methods used to derive hiPS which in general fall into two categories, depending on whether the transgenes used integrates into the genome or not. Genomic integration of the reprogramming factors is sub- optimal as the resulting IPs cells will carry these exogenous viral transgenes and the associated potential complications as previously discussed. Alternatively, methodology such as protein or non-integrating viruses has been used to carry out IPS generation and while these protocols do not involve chromosomal integration the efficiency of reprogramming is extremely low and not robust enough for practical application. Attempts have been made to solve this problem by using the CRE-mediated excision of integrated vectors however this bares the risk of genomic rearrangements or incomplete excision. In addition nucleosomes and transfection of DNA based vectors have been used to reprogram the cells but they also harbor the risk of random integration and insertional mutagenesis. The protocol described in aim 2 (Warren et al., 2010) using modified RNA does not involve genomic integration of the transgene, in addition it also has a

comparatively high rate of efficient reprogramming of somatic cells and thus overcomes these obstacles.

In collaboration with the Rossi lab we have developed a protocol using modified RNA and the interferon inhibitor B18R, which increases reprogramming efficiency by preventing the innate cellular anti-viral response that accompanies repeated transfection with RNA. The efficiency of IPs generation using this technique was significantly higher than standard iPS derivation and substantially higher than any other non-integrating derivation method. This is particular important as it is the first iPS derivation method that has the potential to become a standard method for the field of regenerative medicine and an inevitable step towards therapeutic use of hiPS. Interestingly the same methodology can be applied towards the differentiation of IPs cells to a specific a cell type of interest. The optimization of the transfection protocol and the amount of RNA used was the biggest obstacle in making this technology work and so far every cell type that we tested has a different response to RNA concentration and transfection reagent. While this technology is still in its infancy it has the potential to be very important for the field at large. The precise dosing and temporal control that is possible using modified RNAs allows for a detailed analysis of the stochiometry involved in lineage reprogramming or conversely differentiating cells. These implications render this technique not only useful for the future as an applied science but will also help to shed light on the mechanisms that guide cell fate.

4.3 To differentiate human pluripotent stem cells into adipocytes

Adipocytes have been previously derived from human pluripotent cells but as discussed earlier at a much lower efficiency and with only minimum characterization of the differentiated cells. We tried to recapitulate these results with varied success between attempts and in general an overall low degree of efficiency. In order to establish a more reliable and efficient methodology we attempted to mimic the *in vivo* events surrounding the formation of a mesoderm precursor. While this technique proved reliable it demonstrated an overall differentiation efficiency that was still low. In addition we also observed that the mesodermal precursor cells lose their adipogenic potential with continuous passaging. Prior to our study it was well established, that overexpression of

PPARγ in fibroblast cells leads to a strong accumulation of lipids. Capitalizing on this information we were the first group to utilize PPARγ in an inducible system and demonstrated lipid deposits in the infected cells. Interestingly we showed that cells derived in this manner were independent of transgene expression but did not lose their adipocyte identity when doxycycline was removed and exogenous PPAR expression stopped. This is the first example of adipocyte generation from human pluripotent cells with such a high efficiency and reproducibility. Furthermore we have characterized the cells and were able to show the presence of differentiated adipocyte markers and lipid storage. Taken together this data demonstrates that these cells are capable of functioning as mature fat cells and allows for the possibility of large-scale application such as investigating the role of the adipocytes in the development of metabolic syndromes. As such, our technology is being used by a large pharmaceutical company in the study of human adipocytes and their role in inflammation when co-cultured with macrophages.

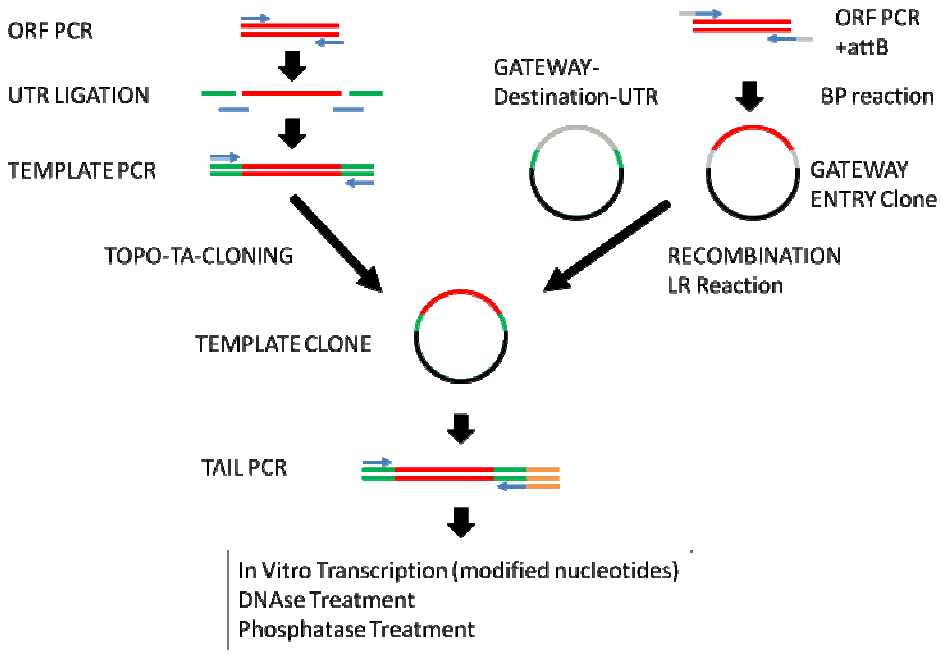
As discussed earlier fat is derived in the two different forms of brown and white, in addition both these subtypes of fat are found in depots through the body. An expanding area of study in the adipocyte field is concerned about the difference between these two populations and what causes the existing differences between white fat populations at different depot locations. Interestingly several studies have shown that an increase in visceral fat will result in an increase in the risk of metabolic syndromes and related diseases when compared to increases in subcutaneous fat. The amount of low grade inflammation differs between various fat depots as well as the amounts of secreted adipokines. Only very recently have we begun to appreciate the differences between various white fat depots. ADSV harvested from distinct fat tissue depots will harbor a characteristic epigenetic pattern that will guide the behavior of these cells. The same is true for other cells of the same origin. It will be almost impossible to generate adipocytes from somatic cells that are not influenced by the epigenetic memory of its origin and likewise it would be impossible to harvest starting cells from any tissue depot that will not harbor a certain epigenetic state of interest. Human pluripotent cells however have the advantage of an epigenetic naïve state. Despite initial studies showing that iPS retain their epigenetic memory; further studies have proven that this

epigenetic memory is not retained over the long-term culture and that these cells are returned to a naïve state. With a guided differentiation approach and the right cues it should be possible to model any fat depot in the human. Subsequently these *in vitro* differentiated cells could be used to address disease modeling.

4.4 Future Perspectives

The work that we have performed thus far lays the groundwork for several future experiments. The first project, the generation of human iPS cells and ADMSCs from patient lipo-aspirate material, is already underway, with patients being recruited from Massachusetts General Hospital (MGH). These patients have morbid obesity, diabetes type II and or cardiovascular disease, obese patients that do not exhibit co-morbid diseases as well as healthy control patients. We expect this project to yield an *in vitro* disease model system of different subtypes of obesity.

As our ultimate goal is to directly impact patient care, our second project is to translate all our iPS-derivation and differentiation protocols from lenti- and retrovirus-based gene delivery systems to modified RNA-based systems. Major benefits include precise transcription factor dosing, equivalent expression in all cells, and lack of genomic integration. The greatest obstacle in this project is the optimization of the RNA-based systems, but this is not insurmountable. In terms of progress, we have designed new Gateway destination vectors that allow us to rapidly generate new template clones for the IVT reactions (Figure 5-1). In prior projects we have already created entry clones for many different transcription factors that are known or are suggested to play a role in reprogramming, adipogenesis, liver, pancreas and kidney development.



4-1 Generation of IVT constructs with two strategies, modified from (Warren et al., 2010). In terms of directed, *in vitro* pluripotent cell differentiation, we aim to develop several differentiation strategies utilizing these RNA-overexpression vectors. The work we have done showing RNA-mediated overexpression of MyoD shows that modified RNA is a viable approach to accomplish this goal.

Using these strategies, we plan to differentiate pluripotent cells into adipocytes, β-pancreas cells, and hepatocytes from patient-specific iPS. These human- and cell-type-specific models of disease will allow us to uncover novel disease mechanisms, which will contribute to the development of new therapeutic strategies.

5 Summary

In a series of groundbreaking papers, (Maherali et al., 2007b; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007) showed that ectopic expression of the transcription factors Oct4, Sox2, c-Myc and Klf4 in murine somatic cells reprogrammed the cells to a pluripotent stage. Because these iPS cells were molecularly and functionally equivalent to embryonic stem cells, their potential as a tool for studying disease phenotypes was immediately apparent.

It was also clear at the time that if the technology could be translated into humans, human iPS cells would be a source of patient-matched cells for transplantation and the study of complex human disease. Furthermore, unlike human embryonic stem cells, iPS cells could be derived without destruction of embryos.

Thus, at the time I started working on iPS cells, several key outstanding issues remained. Would it be possible to derive iPS cells from humans? Are all cell types equally amenable to reprogramming? Could iPS cells be derived without integrating the overexpression vectors, thus avoiding the risk of integrational mutagenesis? How efficiently could iPS cells be differentiated into varying somatic cell types, and what protocols would be needed?

The three studies presented in this thesis address these questions. They thus represent important contributions to the field of stem cell biology. Our first study was the first to show that multiple sources of human somatic cells could be reprogrammed into iPS cells using the same set of transcription factors. We identified keratinocytes as a new and more suitable source of starting cells. Keratinocytes can be reprogrammed more quickly and with higher efficiency than fibroblast lines. They are also easily obtained by hair-pulls and skin punch biopsies.

In the same study we were the first to develop an inducible system of iPS reprogramming. Such a system allows for the study of reprogramming events in a more homogenous cell population, where all cells expressing the transgenes, and we observed a 100-fold increase in reprogramming efficiency.

Our second study advanced the field of stem cell biology by using virus-free reprogramming based on serial transfection of modified RNAs. The protocol represents a significant contribution to the field of regenerative medicine because of its relative simplicity and effectiveness. Thus, it has the potential to become the standard for human iPS derivation. Moreover, the efficiency of our iPS derivation was higher than in any prior used method. This is particularly important as other non-integrating derivation methods are very inefficient and thus impractical.

Our third study moved from the derivation of iPS cells to their experimental applications. We differentiated, or programmed, adipocytes from pluripotent cells through the inducible overexpression of PPARγ. This differentiation is both robust and highly efficient. Moreover, our derived adipocytes do not require prolonged transgene expression. Prior to our work, few studies showed that adipocytes could be derived from pluripotent cells and these studies provided very limited characterization of the differentiated cells. We characterized the cells extensively, showing expression of nearly all mature adipocyte markers. We also showed that these cells are functional in that they release adiponectin and glycerol in response to beta-adrenergic stimuli. The rational manipulation of adipose physiology and the use of *in vitro* derived adipocytes for drug screening is a promising avenue towards understanding obesity and, ultimately, developing therapies for associated pathological conditions such as cardiovascular disease and stroke.

In conclusion, this thesis provides novel biological insights and experimental procedures that facilitate reprogramming of human somatic cells to pluripotency as well as the differentiation or programming of pluripotent cells to a mature adipocyte state. This may ultimately help us to gain insights into disease mechanisms and makes a step towards the final goal of regenerative medicine.

6 Zusammenfassung

Eine Serie von innovativen Publikationen (Maherali et al., 2007b; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007) hat gezeigt, dass ektopische Expression der Transkriptionsfaktoren Oct4, Sox2, c-MYC und Klf4 somatische Zellen von Mäusen in einen pluripotenten Zustand reprogrammieren können. Diese induzierten pluripotenten Stammzellen teilen alle wichtigen Eigenschaften mit embryonalen Stammzellen und sind funktionell und molekular als gleichwertig zu betrachten. Es war sofort klar, dass diese Technologie ein grosses Potential für die medizinische Forschung hat, sollte es gelingen die Ergebnisse im humanen System zu etablieren. Im Gegensatz zur Herstellung von humanen embryonalen Stammzellen, müssten bei der Gewinnung von patientenspezifischen humanen iPS-Zellen keine Embryonen zerstört werden. Humane iPS Zellen könnten als Quelle für regenerative Therapien in Frage kommen und möglicherweise das Problem der Immunabstossung umgehen. Kurzfristig jedoch realistischer ist es, das Potential der Zellen für die *in vitro* Untersuchung der Pathogenese von diversen Krankheiten einzusetzen.

Am Beginn meiner Doktorarbeit gab es viele offene Fragen. Wäre es möglich iPS Zellen von humanen Zellen zu generieren? Können alle somatischen Zellen durch Reprogrammierung in einen pluripotenten Zustand überführt werden? Da einige der eingesetzten Reprogrammierungs-Vektoren bekannte Onkogene sind und zusätlich Mutationen durch genomische Integration verursacht werden, gab es die wichtige Frage, ob iPS Zellen integrationsfrei generiert werden können? Die wichtigste Eigenschaft von pluripotenten Stammzellen ist das inhärente Potential jede Zelle des Körpers zu bilden jedoch sind bestehende Protokolle meist technisch anspruchsvoll und ineffizient. Ist es möglich pluripotente Zellen einheitlich zu erwünschten Zellen zu differenzieren (programmieren), indem man die notwendigen Transkriptionsfaktoren überexprimiert und damit Analyse und Nutzung der Zellen optimiert werden? Die drei Manuskripte, die in dieser Dissertation vorgestellt werden, versuchen einige dieser Fragen beantworten.

In der ersten Studie konnten wir zeigen, dass verschiedene humane somatische Zellen zu pluripotenten Zellen reprogrammiert werden können. Wir haben als erste Gruppe demonstriert, dass Keratinozyten als geeignete Startzellpopulation dienen können, da sie schneller und effizienter als Fibroblasten zu reprogrammieren sind. Die Gewinnung von Keratinozyten ist minimal invasiv, aus Haarwurzeln oder Haut-Biopsien möglich. Weiterhin haben wir ein induzierbares sekundäres System fuer die Reprogrammierung von somatischen Zellen entwickelt. Durch die Reaktivierung der Transgene können differenzierte iPS Zellen erneut zu nun sekundären iPS Zellen reprogrammiert werden. Das erlaubt die Analyse von Reprogrammierungs-Mechanismen in einer homogenen Zellpopulation.

In der zweiten Studie haben wir ein integrationsfreies Reprogrammierungs-Protokoll entwickelt, welches eine wiederholte Transfektion von modifizierten RNA's beinhaltet. Das Protokoll ist ein signifikanter Beitrag für das Feld der regenerativen Medizin weil es einfach aber hocheffektiv ist. Dies ist insbesondere von Bedeutung, da andere nicht-integrative Methoden technisch kompliziert sehr ineffizient oder nicht vollständig integrationsfrei sind.

In der dritten Studie nutzten wir die generierten iPS Zellen und differenzierten diese durch die vorübergehende Überexpression des Transkriptionsfaktors PPARγ zu Adipocyten. Vorherige Studien haben gezeigt, dass eine geringe Fraktion der pluripotenten Zellen prinzipiel zu Adipocyten differenziert werden können. Die Charakterisierung dieser Zellen war jedoch minimal. Mit unserem Protokoll sind wir in der Lage 80-85% der Zellen zu Adipocyten zu differenzieren. Unsere ausführliche Charakterisierung zeigten funktionell Lipolyse und Sekretion von Adiponektin sowie molekular die Markerexpression von ausgereiften Adipocyten.

Zusammenfassend lieferte diese Doktorarbeit neuartige biologische Erkenntnisse über die Faktoren-vermittelte Reprogrammierung von humanen somatischen Zellen zu iPS und deren Transkriptionsfaktor vermittelte Differenzierung zu Adipozyten. Die Studie von patientenspezifischen Adipozyten mag zu Verständniss von pathologischen Mechanismen und letztendlich zu neuen Therapien der assozierten Folgeerkrankungen wie Typ-2-Diabetes, Herz-Kreislauf-Erkrankungen und Schlaganfall führen.

7 Abbreviation list

ADA-SCID - adenosine deaminase deficiency-related severe combined immunodeficiency

AHA - American heart association

ALS - amyotrophic lateral sclerosis

BAT – Brown adipose tissue

BMD - Becker muscular dystrophy

BMPs - bone morphogenic proteins

DMS - type III, Duchenne

DS - Down syndrome/trisomy 21

ESC - embryonic stem cells

FD - familial dysautonomia

FISH - fluorescence in situ hybridization

GABA - gamma-aminobutyric acid

GD - Gaucher disease

HD - Huntington disease

HDL - high density lipoproteins

hESC - human embryonic stem cells

hiPS - human induced pluripotent stem cells

HUES - Harvard University embryonic stem cell lines

IBMX - 3-Isobutyl-1-methylxanthin

ICM - inner cell mass

iPS - induced pluripotent stem cells

IVF - in vitro fertilization

JDM - juvenile-onset, type 1 diabetes mellitus

LDL - low density lipoproteins

MEFs - mouse embryonic fibroblasts

mESC - mouse embryonic stem cells

miPS - mouse induced pluripotent stem cells

MNs - motor neurons

MPCs - mesenchymal precursor cells

MSCs - mesenchymal stem cells

PCR - polymerase chain reaction

PD - Parkinson disease

PGD - preimplantation genetic diagnosis

PPARγ2 - peroxisome proliferator-activated receptor γ2

RA - retinoic acid

SCNT - somatic cell nuclear transfer

SBDS - Shwachman-Bodian-Diamond syndrome

Shh - Sonic HedgeHog

SOD1 - super oxide dismutase

TF - Transcription Factor

TRE - tetracycline response element

WAT – White adipose tissue

ZFNs - zinc-finger nucleases

8 Publication record

1.) Maherali, N.[*], **Ahfeldt, T.**[*], Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. (2008).
A high-efficiency system for the generation and study of human induced pluripotent stem cells.
Cell Stem Cell 3, 340-345. ([*] These authors contributed equally as first authors)

2.) Park, I.H., Arora, N., Huo, H., Maherali, N., **Ahfeldt, T.**, Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., and Daley, G.Q. (2008a).
Disease-specific induced pluripotent stem cells.
Cell 134, 877-886.

3.) Eminli, S., Foudi, A., Stadtfeld, M., Maherali, N., **Ahfeldt, T.**, Mostoslavsky, G., Hock, H., and Hochedlinger, K. (2009).
Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells.
Nat Genet *41*, 968-976.

4.) Lau F., **Ahfeldt.T.**, Osafune K., Akutsu H, Cowan CA. (2009).
Induced pluripotent stem (iPS) cells: an up-to-the-minute review.
F1000 Biology Reports 2009 *1:84*.

5.) Musunuru, K., Strong, A., Frank-Kamenetsky, M., Lee, N.E., **Ahfeldt, T.**, Sachs, K.V., Li, X., Li, H., Kuperwasser, N., Ruda, V.M.*, et al.* (2010).
From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus.
Nature *466*, 714-719.

6.) Luigi Warren, Philip D. Manos**, Tim Ahfeldt**, Yuin-Han Loh, Hu Li, Frank Lau, Wataru Ebina, Pankaj Mandal, Zachary D. Smith, Alexander Meissner, George Q. Daley, Andrew S. Brack, James J. Collins, Chad Cowan, Thorsten M. Schlaeger, Derrick J. Rossi

Highly efficient reprogramming to pluripotency and directed differentiation of human cells using synthetic modified mRNA

Cell Stem Cell (in press)

7) **Tim Ahfeldt**, Youn-Kyoung Lee, Adam Kaplan, Dave Lum, Robert Schinzel, Frank Lau, Rahul Deo, Jennifer Shay, Greg Mowrer, Rhee Eugene, Robert Gerszten and Chad A. Cowan

Programming human embryonic stem cells and human induced pluripotent stem cells into adipocytes

Cell Stem Cell (submission to Cell Stem Cells)

9 Erklärung ueber den Eigenanteil an den Publikationen experimentelle Eigenanteil wird im Folgenden dargelegt.

Die vorliegende Doktorarbeit besteht aus drei wissenschaftlichen Veröffentlichungen sowie ergänzenden Daten die nicht in die Originalpublikation eingegangen sind oder diese weiterführen. In den beiden Publikationen in denen ich Erstautor oder geteilter Erstautor bin, war ich an allen experimentellen Aspekten beteiligt und wurde teilweise von anderen Wissenschaftlern in neuen Techniken angelernt oder unterstützt. Weiterhin habe ich Ergebnisse aus einer Publikation aufgenommen an der ich als Autor beteiligt war und die meine zukünftige Arbeit stark beeinflusst. Der von mir zu diesen drei Manuskripten beigetragene experimentelle Eigenanteil wird im Folgenden dargelegt.

Die erste Arbeit ist eine Kollaboration zwischen Prof. Dr. Konrad Hochedlinger's und Chad Cowan's Labor und ich teile mir die Erstautorenschaft mit einer Doktorandin aus Prof. Hochedlinger's Labor. Gemeinsam haben wir die Grundkonzepte und die Experimente entwickelt sowie die Ergebnisse ausgewertet. Mein experimenteller Eigenanteil beinhaltet die Bereitstellung von Studienmaterial, u.a. Kultur von somatischen Zellen, MEFs und hIPS. Ich habe die *in vitro* Differenzierung von iPS, qRT-PCR und Analyse der qPCR-Analyse, die Terratomainjektionen und einen Teil der Immunostainings durchgeführt. Die Hybridisierung der Micorarrays erfolgte im Stowers Institut, ich habe die Daten analysiert.

Die zweite Arbeit entstand als eine Kollaboration zwischen Chad Cowan's und Derrick Rossi's Labor. Derrick Rossi ist ein Experte im Bereich der Immunologie, aber sein Labor hatte zu Beginn des Projekts keine Erfahrung mit hESC oder hIPSC. Im Laufe der Experimente wurden noch zwei weitere Labore, das von George Daley und Thorsten Schläger's Labor involviert. In dieser Arbeit bin ich nicht Erstautor, war aber an der Planung und Durchführung der Experimente von Beginn an beteiligt und habe wichtige Schritte selbst durchgeführt. Mein experimenteller Eigenateil bestand in der Optimisierung der Transfektionsbedingungen in verschiedene somatische Zelllinien. Weiterhin war ich an der Kultur der humanen RiPS Zellen beteiligt und habe die *in vitro*

Differenzierungen, Immunostainings und histologische Analyse der Terratomas durchgeführt. In weiterführenden Experimenten habe ich auch die Konstruktion von IVT templates sowie die Produktion der modifizierten RNA übernommen.

In der dritten Arbeit bin ich Erstautor und habe die die Grundkonzepte und die initialen Grundideen der Experimente entwickelt. Die Planung und die Etablierung des experimentellen Aufbaus, sowie die Auswertung der Ergebnisse und die Verfassung der Manuskripte erfolgte durch meine Person, mit Unterstützung von Prof. Dr. Chad Cowan. Er betreute die Arbeit und half durch kritische Überarbeitung der Manuskripte. Ich wurde bei der Durchführung einzelner Experimente von Co-Autoren unterstützt. Die Klonierungsstrategie des Gateway FUGW Backbones wurde von Dave Lum (PhD) entwickelt. Bei der Analyse der Microarrays habe ich Hilfe von Frank Lau (MD) und Rahul Deo (MD) erhalten. Bei der “Flow Cytometry” Analyse habe ich Hilfe von der MGH Core Facility erhalten. Für die Durchführung des Glycerol-Release-Assays habe ich Hilfe von Youn-Kyoung Lee erhalten. Bei der Durchführung der Multiplex-Analyse hat Jennifer Shay mich technisch unterstützt. Das Lipid Profiling erfolgte durch Rhee, Eugene P. (MD), ich habe die Analyse der Daten durchgeführt.

10 Acknowledgements

I would like to thank my Prof. Dr. Dr. Ulrike Beisiegel for being a great teacher during my undergraduate studies and supervising me through my diploma-thesis as well as my doctoral thesis. Her help and commitment made it possible to work on my thesis abroad in collaboration with the Harvard University. I am grateful for the commitment of Prof. Dr. Thorsten Burmester to be my second dissertation advisor

I highly appreciate the strong support and mentorship of my Professor Chad Cowan all through my time in Boston. I want to thank all the members of the Cowan laboratory for providing a very good working atmosphere and new friendships. Special thanks to Robert Schinzel, my bay mate and friend for almost two decades; Dave Lum for his extensive help with molecular cloning; Frank Lau, Rahul Deo and Teresa Holm for critical reading and their bigger view on science, Kiran Misunuru for being such a great example of a scientist and his help with troubleshooting, Emil Hanson for critical reading of the manuscript and his passion about science. Michael Henderson, Jimmy and Kevin Hom for their technical help as undergraduate students and for the fun they brought to the lab; Jennifer Shay for being a great lab manager, Adam Kaplan and Greg Mowrer the two most amazing technicians I had the pleasure of working with.

Thanks to my partner April Cook, for reading the manuscript and discussions about my project during my thesis. Finally, I would like to thank my family and my friends for their support during my studies.

11 Bibliography

Akutsu, H., Cowan, C.A., and Melton, D. (2006). Human embryonic stem cells. Methods Enzymol *418*, 78-92.

al Yacoub, N., Romanowska, M., Haritonova, N., and Foerster, J. (2007). Optimized production and concentration of lentiviral vectors containing large inserts. J Gene Med *9*, 579-584.

Audouy, S., and Hoekstra, D. (2001). Cationic lipid-mediated transfection in vitro and in vivo (review). Molecular membrane biology *18*, 129-143.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev *17*, 126-140.

Babaie, Y., Herwig, R., Greber, B., Brink, T.C., Wruck, W., Groth, D., Lehrach, H., Burdon, T., and Adjaye, J. (2007). Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem cells (Dayton, Ohio) *25*, 500-510.

Banito, A., Rashid, S.T., Acosta, J.C., Li, S., Pereira, C.F., Geti, I., Pinho, S., Silva, J.C., Azuara, V., Walsh, M.*, et al.* (2009). Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev *23*, 2134-2139.

Barberi, T., and Studer, L. (2006). Mesenchymal cells. Methods Enzymol *418*, 194-208.

Barberi, T., Willis, L.M., Socci, N.D., and Studer, L. (2005). Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Med *2*, e161.

Billon, N., Iannarelli, P., Monteiro, M.C., Glavieux-Pardanaud, C., Richardson, W.D., Kessaris, N., Dani, C., and Dupin, E. (2007). The generation of adipocytes by the neural crest. Development (Cambridge, England) *134*, 2283-2292.

Boillee, S., Vande Velde, C., and Cleveland, D.W. (2006). ALS: a disease of motor neurons and their nonneuronal neighbors. Neuron *52*, 39-59.

Botstein, D., and Risch, N. (2003). Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. Nat Genet *33 Suppl*, 228-237.

Boyer, L.A., Mathur, D., and Jaenisch, R. (2006). Molecular control of pluripotency. Current opinion in genetics & development *16*, 455-462.

Briggs, R., and King, T.J. (1952). Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. Proc Natl Acad Sci U S A *38*, 455-463.

Capowski, E.E., Schneider, B.L., Ebert, A.D., Seehus, C.R., Szulc, J., Zufferey, R., Aebischer, P., and Svendsen, C.N. (2007). Lentiviral vector-mediated genetic modification of human neural progenitor cells for ex vivo gene therapy. Journal of neuroscience methods *163*, 338-349.

Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A., and Bhatia, M. (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood *102*, 906-915.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Chan, E.M., Ratanasirintrawoot, S., Park, I.H., Manos, P.D., Loh, Y.H., Huo, H., Miller, J.D., Hartung, O., Rho, J., Ince, T.A.*, et al.* (2009). Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. Nature biotechnology *27*, 1033-1037.

Chang, C.-W., Lai, Y.-S., Pawlik, K.M., Liu, K., Sun, C.-W., Li, C., Schoeb, T.R., and Townes, T.M. (2009). Polycistronic Lentiviral Vector for "Hit and Run" Reprogramming of Adult Skin Fibroblasts to Induced Pluripotent Stem Cells. Stem Cells *27*, 1042-1049.

Cook, A.a.C., C. (2009). Adipose (StemBook, ed. The Stem Cell Research Community).

Cowan, C.A., Atienza, J., Melton, D.A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science *309*, 1369-1373.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D.*, et al.* (2004). Derivation of embryonic stem-cell lines from human blastocysts. The New England journal of medicine *350*, 1353-1356.
D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K., and Baetge, E.E. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nature biotechnology *24*, 1392-1401.
Daley, G.Q., and Scadden, D.T. (2008). Prospects for stem cell-based therapy. Cell *132*, 544-548.
Dani, C. (1999). Embryonic stem cell-derived adipogenesis. Cells Tissues Organs *165*, 173-180.
Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987-1000.
Despres, J.P., and Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. Nature *444*, 881-887.
Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA. Science (New York, NY *303*, 1529-1531.
Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R.*, et al.* (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science *321*, 1218-1221.
Eckel, R.H., Kahn, R., Robertson, R.M., and Rizza, R.A. (2006). Preventing cardiovascular disease and diabetes: a call to action from the American Diabetes Association and the American Heart Association. Diabetes Care *29*, 1697-1699.
Elango, N., Elango, S., Shivshankar, P., and Katz, M.S. (2005). Optimized transfection of mRNA transcribed from a d(A/T)100 tail-containing vector. Biochemical and Biophysical Research Communications *330*, 958-966.
Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.
Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci *85*, 348-362.
Gesta, S., Tseng, Y.H., and Kahn, C.R. (2007). Developmental origin of fat: tracking obesity to its source. Cell *131*, 242-256.
Glazier, A.M., Nadeau, J.H., and Aitman, T.J. (2002). Finding genes that underlie complex traits. Science (New York, NY *298*, 2345-2349.
Green, H., and Kehinde, O. (1975). An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell *5*, 19-27.
Gurdon, J.B. (1962). Adult frogs derived from the nuclei of single somatic cells. Dev Biol *4*, 256-273.
Gurdon, J.B., and Melton, D.A. (2008). Nuclear reprogramming in cells. Science *322*, 1811-1815.
Gurdon, J.B., and Uehlinger, V. (1966). "Fertile" intestine nuclei. Nature *210*, 1240-1241.
Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine, E.J., Cassady, J.P., Foreman, R.*, et al.* (2008). Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell *133*, 250-264.
Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C.J., Creyghton, M.P., van Oudenaarden, A., and Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. Nature *462*, 595-601.
Hanna, J., Wernig, M., Markoulaki, S., Sun, C.W., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M.*, et al.* (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science (New York, NY *318*, 1920-1923.
Hochedlinger, K., Yamada, Y., Beard, C., and Jaenisch, R. (2005). Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell *121*, 465-477.

Holtkamp, S., Kreiter, S., Selmi, A., Simon, P., Koslowski, M., Huber, C., Tureci, O., and Sahin, U. (2006). Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood *108*, 4009-4017.
Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.-K., Schlee, M*., et al.* (2006). 5'-Triphosphate RNA Is the Ligand for RIG-I. Science (New York, NY *314*, 994-997.
Howard, B.V., Ruotolo, G., and Robbins, D.C. (2003). Obesity and dyslipidemia. Endocrinol Metab Clin North Am *32*, 855-867.
Hu, B.Y., Weick, J.P., Yu, J., Ma, L.X., Zhang, X.Q., Thomson, J.A., and Zhang, S.C. (2010). Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proceedings of the National Academy of Sciences of the United States of America *107*, 4335-4340.
Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E., and Melton, D.A. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nature biotechnology *26*, 795-797.
Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K*., et al.* (2009). A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell *5*, 491-503.
Jia, F., Wilson, K.D., Sun, N., Gupta, D.M., Huang, M., Li, Z., Panetta, N.J., Chen, Z.Y., Robbins, R.C., Kay, M.A*., et al.* (2010). A nonviral minicircle vector for deriving human iPS cells. Nature methods *7*, 197-199.
Kadowaki, T., and Yamauchi, T. (2005). Adiponectin and adiponectin receptors. Endocr Rev *26*, 439-451.
Kahn, S.E., Hull, R.L., and Utzschneider, K.M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature *444*, 840-846.
Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature *458*, 771-775.
Kariko, K., Buckstein, M., Ni, H., and Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity *23*, 165-175.
Kariko, K., Muramatsu, H., Welsh, F.A., Ludwig, J., Kato, H., Akira, S., and Weissman, D. (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther *16*, 1833-1840.
Kariko, K., and Weissman, D. (2007). Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development. Current opinion in drug discovery & development *10*, 523-532.
Kawai, T., and Akira, S. (2007). Antiviral signaling through pattern recognition receptors. Journal of biochemistry *141*, 137-145.
Kawamura, T., Suzuki, J., Wang, Y.V., Menendez, S., Morera, L.B., Raya, A., Wahl, G.M., and Belmonte, J.C. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature *460*, 1140-1144.
Kim, D., Kim, C.-H., Moon, J.-I., Chung, Y.-G., Chang, M.-Y., Han, B.-S., Ko, S., Yang, E., Cha, K.Y., Lanza, R*., et al.* (2009a). Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins. Cell Stem Cell *4*, 472-476.
Kim, D., Kim, C.H., Moon, J.I., Chung, Y.G., Chang, M.Y., Han, B.S., Ko, S., Yang, E., Cha, K.Y., Lanza, R*., et al.* (2009b). Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell *4*, 472-476.
Kingsmore, S.F., Lindquist, I.E., Mudge, J., Gessler, D.D., and Beavis, W.D. (2008). Genome-wide association studies: progress and potential for drug discovery and development. Nature reviews *7*, 221-230.

Kopelman, P.G. (2000). Obesity as a medical problem. Nature *404*, 635-643.
Kopp, J.L., Ormsbee, B.D., Desler, M., and Rizzino, A. (2008). Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem cells (Dayton, Ohio) *26*, 903-911.
Kroon, E., Martinson, L.A., Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., Cunningham, J.*, et al.* (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nature biotechnology *26*, 443-452.
Lakey, J.R., Mirbolooki, M., and Shapiro, A.M. (2006). Current status of clinical islet cell transplantation. Methods Mol Biol *333*, 47-104.
Lee, G., Papapetrou, E.P., Kim, H., Chambers, S.M., Tomishima, M.J., Fasano, C.A., Ganat, Y.M., Menon, J., Shimizu, F., Viale, A.*, et al.* (2009). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature *461*, 402-406.
Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C.-C., and Kain, S.R. (1998). Generation of Destabilized Green Fluorescent Protein as a Transcription Reporter. J Biol Chem *273*, 34970-34975.
Lowell, B.B., and Flier, J.S. (1997). Brown adipose tissue, beta 3-adrenergic receptors, and obesity. Annu Rev Med *48*, 307-316.
Lowry, W.E., Richter, L., Yachechko, R., Pyle, A.D., Tchieu, J., Sridharan, R., Clark, A.T., and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. Proceedings of the National Academy of Sciences *105*, 2883-2888.
Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. (2008). A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell *3*, 340-345.
Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminili, S., Arnold, K., Stadtfeld, M., Yacechko, R., Tchieu, J., Jaenisch, R.*, et al.* (2007a). Cell Stem Cell *1*, 55-70.
Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R.*, et al.* (2007b). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell *1*, 55-70.
Martin, N., Boomsma, D., and Machin, G. (1997). A twin-pronged attack on complex traits. Nat Genet *17*, 387-392.
McAdams, H.H., and Arkin, A. (1997). Stochastic mechanisms in gene expression. Proc Natl Acad Sci U S A *94*, 814-819.
Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature *454*, 49-55.
Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.
Miura, K., Okada, Y., Aoi, T., Okada, A., Takahashi, K., Okita, K., Nakagawa, M., Koyanagi, M., Tanabe, K., Ohnuki, M.*, et al.* (2009). Variation in the safety of induced pluripotent stem cell lines. Nature biotechnology *27*, 743-745.
Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nature biotechnology *26*, 101-106.
Nallagatla, S.R., and Bevilacqua, P.C. (2008). Nucleoside modifications modulate activation of the protein kinase PKR in an RNA structure-specific manner. RNA (New York, NY *14*, 1201-1213.
Nallagatla, S.R., Toroney, R., and Bevilacqua, P.C. (2008). A brilliant disguise for self RNA: 5'-end and internal modifications of primary transcripts suppress elements of innate immunity. RNA biology *5*.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.
Niwa, H. (2007). How is pluripotency determined and maintained? Development (Cambridge, England) *134*, 635-646.
Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet *24*, 372-376.
Ogden, C.L., Flegal, K.M., Carroll, M.D., and Johnson, C.L. (2002). Prevalence and trends in overweight among US children and adolescents, 1999-2000. Jama *288*, 1728-1732.
Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313-317.
Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. Science (New York, NY *322*, 949-953.
Olivier, E.N., Rybicki, A.C., and Bouhassira, E.E. (2006). Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. Stem Cells *24*, 1914-1922.
Papapetrou, E.P., Tomishima, M.J., Chambers, S.M., Mica, Y., Reed, E., Menon, J., Tabar, V., Mo, Q., Studer, L., and Sadelain, M. (2009). Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation. Proceedings of the National Academy of Sciences *106*, 12759-12764.
Park, I.-H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008a). Reprogramming of human somatic cells to pluripotency with defined factors. Nature *451*, 141-146.
Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., and Daley, G.Q. (2008b). Disease-specific induced pluripotent stem cells. Cell *134*, 877-886.
Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008c). Reprogramming of human somatic cells to pluripotency with defined factors. Nature *451*, 141-146.
Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. Science *269*, 540-543.
Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006). RIG-I-Mediated Antiviral Responses to Single-Stranded RNA Bearing 5'-Phosphates. Science (New York, NY *314*, 997-1001.
Pickering, S.J., Minger, S.L., Patel, M., Taylor, H., Black, C., Burns, C.J., Ekonomou, A., and Braude, P.R. (2005). Generation of a human embryonic stem cell line encoding the cystic fibrosis mutation deltaF508, using preimplantation genetic diagnosis. Reprod Biomed Online *10*, 390-397.
Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.
Rabinovich, P.M., Komarovskaya, M.E., Wrzesinski, S.H., Alderman, J.L., Budak-Alpdogan, T., Karpikov, A., Guo, H., Flavell, R.A., Cheung, N.K., Weissman, S.M*., et al.* (2008). Chimeric Receptor mRNA Transfection as a Tool to Generate Antineoplastic Lymphocytes. Human gene therapy.
Rabinovich, P.M., Komarovskaya, M.E., Ye, Z.J., Imai, C., Campana, D., Bahceci, E., and Weissman, S.M. (2006). Synthetic messenger RNA as a tool for gene therapy. Human gene therapy *17*, 1027-1035.
Randall, R.E., and Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol *89*, 1-47.
Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., and Spiegelman, B.M. (2002). C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev *16*, 22-26.

Rosen, E.D., and Spiegelman, B.M. (2000). Molecular regulation of adipogenesis. Annual review of cell and developmental biology *16*, 145-171.
Rosen, E.D., and Spiegelman, B.M. (2006). Adipocytes as regulators of energy balance and glucose homeostasis. Nature *444*, 847-853.
Selvaraj, V., Plane, J.M., Williams, A.J., and Deng, W. (2010). Switching cell fate: the remarkable rise of induced pluripotent stem cells and lineage reprogramming technologies. Trends Biotechnol *28*, 214-223.
Shea, K.L., Xiang, W., LaPorta, V.S., Licht, J.D., Keller, C., Basson, M.A., and Brack, A.S. (2010). Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during regeneration. Cell Stem Cell *6*, 117-129.
Si-Tayeb, K., Noto, F.K., Sepac, A., Sedlic, F., Bosnjak, Z.J., Lough, J.W., and Duncan, S.A. (2010). Generation of human induced pluripotent stem cells by simple transient transfection of plasmid DNA encoding reprogramming factors. BMC Dev Biol *10*, 81.
Smith, K.P., Luong, M.X., and Stein, G.S. (2009). Pluripotency: toward a gold standard for human ES and iPS cells. J Cell Physiol *220*, 21-29.
Smith, Z.D., Nachman, I., Regev, A., and Meissner, A. (2010). Dynamic single-cell imaging of direct reprogramming reveals an early specifying event. Nat Biotech *advance online publication*.
Solter, D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat Rev Genet *7*, 319-327.
Stadtfeld, M., Brennand, K., and Hochedlinger, K. (2008a). Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr Biol *18*, 890-894.
Stadtfeld, M., Maherali, N., Breault, D.T., and Hochedlinger, K. (2008b). Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell *2*, 230-240.
Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008c). Induced pluripotent stem cells generated without viral integration. Science (New York, NY *322*, 945-949.
Symons, J.A., Alcami, A., and Smith, G.L. (1995). Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. Cell *81*, 551-560.
Tada, M., Tada, T., Lefebvre, L., Barton, S.C., and Surani, M.A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. The EMBO journal *16*, 6510-6520.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861-872.
Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.
Tchkonia, T., Lenburg, M., Thomou, T., Giorgadze, N., Frampton, G., Pirtskhalava, T., Cartwright, A., Cartwright, M., Flanagan, J., Karagiannides, I.*, et al.* (2007). Identification of depot-specific human fat cell progenitors through distinct expression profiles and developmental gene patterns. Am J Physiol Endocrinol Metab *292*, E298-307.
Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.
Tiscornia, G., Singer, O., and Verma, I.M. (2006). Production and purification of lentiviral vectors. Nat Protoc *1*, 241-245.
Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. (1994). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev *8*, 1224-1234.
Uematsu, S., and Akira, S. (2007). Toll-like Receptors and Type I Interferons. J Biol Chem *282*, 15319-15323.
Urbach, A., Schuldiner, M., and Benvenisty, N. (2004). Modeling for Lesch-Nyhan disease by gene targeting in human embryonic stem cells. Stem cells (Dayton, Ohio) *22*, 635-641.

Utikal, J., Polo, J.M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R.M., Khalil, A., Rheinwald, J.G., and Hochedlinger, K. (2009). Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature *460*, 1145-1148.
Uzri, D., and Gehrke, L. (2009). Nucleotide sequences and modifications that determine RIG-I/RNA binding and signaling activities. J Virol, JVI.02449-02408.
Van den Bosch, G.A., Van Gulck, E., Ponsaerts, P., Nijs, G., Lenjou, M., Apers, L., Kint, I., Heyndrickx, L., Vanham, G., Van Bockstaele, D.R.*, et al.* (2006). Simultaneous Activation of Viral Antigen-specific Memory CD4+ and CD8+ T-cells Using mRNA-electroporated CD40-activated Autologous B-cells. Journal of Immunotherapy *29*, 512-523 510.1097/1001.cji.0000210385.0000248327.0000210381e.
Van Gaal, L.F., Mertens, I.L., and De Block, C.E. (2006). Mechanisms linking obesity with cardiovascular disease. Nature *444*, 875-880.
van Harmelen, V., Astrom, G., Stromberg, A., Sjolin, E., Dicker, A., Hovatta, O., and Ryden, M. (2007). Differential lipolytic regulation in human embryonic stem cell-derived adipocytes. Obesity (Silver Spring) *15*, 846-852.
Van Tendeloo, V.F., Ponsaerts, P., Lardon, F., Nijs, G., Lenjou, M., Van Broeckhoven, C., Van Bockstaele, D.R., and Berneman, Z.N. (2001). Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood *98*, 49-56.
Verlinsky, Y., Strelchenko, N., Kukharenko, V., Rechitsky, S., Verlinsky, O., Galat, V., and Kuliev, A. (2005). Human embryonic stem cell lines with genetic disorders. Reprod Biomed Online *10*, 105-110.
Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Hu, L., Lau, F., Wataru, E., Pankaj, M., Zachary, D., Meissner, A.*, et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells using synthetic modified mRNA. Cell Stem Cell *in press*.
Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S.-i., Muguruma, K.*, et al.* (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotech *25*, 681-686.
Weissman, D., Ni, H., Scales, D., Dude, A., Capodici, J., McGibney, K., Abdool, A., Isaacs, S.N., Cannon, G., and Kariko, K. (2000). HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. J Immunol *165*, 4710-4717.
Weng, L., Dai, H., Zhan, Y., He, Y., Stepaniants, S.B., and Bassett, D.E. (2006). Rosetta error model for gene expression analysis. Bioinformatics *22*, 1111-1121.
Wernig, M., Lengner, C.J., Hanna, J., Lodato, M.A., Steine, E., Foreman, R., Staerk, J., Markoulaki, S., and Jaenisch, R. (2008). A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nature biotechnology *26*, 916-924.
Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature *448*, 318-324.
Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature *385*, 810-813.
Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M.*, et al.* (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature.
Wolvetang, E.J., and Hannan, N. (2008). Adipocyte differentiation in human embryonic stem cells transduced with Oct4 shRNA lentivirus. Stem Cells Dev.
Xiong, C., Xie, C.Q., Zhang, L., Zhang, J., Xu, K., Fu, M., Thompson, W.E., Yang, L.J., and Chen, Y.E. (2005). Derivation of adipocytes from human embryonic stem cells. Stem Cells Dev *14*, 671-675.

Yamanaka, S. (2009). Elite and stochastic models for induced pluripotent stem cell generation. Nature *460*, 49-52.
Yang, J., and Weinberg, R.A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell *14*, 818-829.
Yisraeli, J.K., Melton, D.A., and James, E.D.a.J.N.A. (1989). Synthesis of long, capped transcripts in Vitro by SP6 and T7 RNA polymerases. In Methods in Enzymology (Academic Press), pp. 42-50.
Yoshida, Y., Takahashi, K., Okita, K., Ichisaka, T., and Yamanaka, S. (2009). Hypoxia Enhances the Generation of Induced Pluripotent Stem Cells.
Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, II, and Thomson, J.A. (2009a). Human induced pluripotent stem cells free of vector and transgene sequences. Science *324*, 797-801.
Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I., and Thomson, J.A. (2009b). Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences. Science (New York, NY, 1172482.
Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R*., et al.* (2007a). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917-1920.
Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R*., et al.* (2007b). Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. Science (New York, NY *318*, 1917-1920.
Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y*., et al.* (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell *4*, 381-384.
Zhou, W., and Freed, C.R. (2009). Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem cells (Dayton, Ohio) *27*, 2667-2674.
Zou, J., Maeder, M.L., Mali, P., Pruett-Miller, S.M., Thibodeau-Beganny, S., Chou, B.K., Chen, G., Ye, Z., Park, I.H., Daley, G.Q*., et al.* (2009). Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell *5*, 97-110.
Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., and Hedrick, M.H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng *7*, 211-228.