UNIVERSITY OF CALIFORNIA, SANTA CRUZ

Reprogramming Orangutan Fibroblasts to Integration-free Induced Pluripotent Stem Cells

by

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The Abstract Page

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Abstract

Bioengineering
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I have generated cell lines from fibroblasts of Sumatran orangutans that display the characteristic colony morphology and gene expression markers of pluripotent cells. The cells were reprogrammed with non-integrating Sendai virus vectors containing the transcription factors Oct4, Sox2, c-Myc, and Klf4.

The two best lines from the first reprogramming attempt are from one female, have demonstrated the ability to differentiate into cells of all three germ layers, have been verified via RT-PCR and immunofluorescence to express the pluripotency markers Oct4, TRA-1-81, and Nanog, and do not have obvious karyotypic abnormalities. However, one of them appears to retain at least one Sendai virus vector, even after dozens of passages. Differentiation assays consisted of a neural differentiation experiment (results still pending) and an undirected differentiation protocol, yielding positive immunofluorescence results for GATA4, GFAP, and α-Actin in the cell line tested.

The additional cell lines, reprogrammed later, are in the process of establishing the same features. One line is from a female and the other is from a male, and all have stained positively for TRA-1-81. They have yet to undergo differentiation assays. All lines from both species will eventually need to be injected into mice to test if they can form a teratoma. Once fully verified, these lines will be the first Orangutan iPSCs ever generated and will be applied towards research into primate biology on the molecular, cellular, and developmental level.

Acknowledgements

The work done for this project took place in the lab of Dr. David Haussler as part of the Center for Biological Science and Engineering at the University of California, Santa Cruz, with support from the Howard Hughes Medical Institute, the California Institute for Regenerative Medicine, and the National Institutes of Health. Primary fibroblast cultures came from Oliver Ryder and Marlys Houck of the San Diego Zoo Institute for Conservation Research's Frozen Zoo program in San Diego, California, and from the Coriell Biorepository in Camden, New Jersey.

My research mentors are Andrew Field M.S., Dr. Sofie Salama, and Dr. David Haussler. Andrea Reyes-Ortiz has been my lab partner throughout the project, with support from Vincent Meng. Guidance, training, and laboratory resources were provided by Mari Olsen, Kristof Tygi, and Bari Holm Nazario. This project builds off of previous work by Dr. Frank Jacobs and Jimi Rozencrantz. Microscopy was made possible by Benjamin Abrams of the UCSC Life Science Microscopy Center. Dr. Kevin Karplus helped tremendously with the editing process of this thesis. Reprogramming assistance came from Applied Stem Cell, Thermo Fischer Scientific, and the lab of Fred Gage of the the Salk Institute at the University of California, San Diego.

Contents

Abbreviations

UCSC	**U**niversity (of) **C**alifornia, **S**anta **C**ruz
CBSE	**C**enter (for) **B**iomolecular **S**cience and **E**ngineering
HHMI	**H**oward **H**ughes **M**edical **I**nstitute
IF	**I**mmuno**f**luorescence
PCR	**P**olymerase **C**hain **R**eaction
RT	**R**everse-**T**ranscription
PSC	**P**luripotent **S**tem **C**ell
ESC	**E**mbryonic **S**tem **C**ell
iPSC	**i**nduced **P**luripotent **S**tem **C**ell
MEF	**M**ouse **E**mbryonic **F**ibroblasts
FBS	**F**etal **B**ovine **S**erum
MOI	**M**ultiplicity (of) **I**nfection
CIU	**C**ell **I**nfectious **U**nits
FGF	**F**ibroblast **G**rowth **F**actor
RNA-seq	**RNA seq**uencing

Chapter 1

Pluripotent Stem Cells as a Model System for Comparing Primate Molecular Biology

For my senior thesis I have generated *induced pluripotent stem cell* (iPSC) lines from orangutan fibroblasts so that they may be used as an immortal source of orangutan tissues and a model of the early embryo. Once the lines have been fully verified to be genomically intact, cleared of reprogramming vectors, and differentiable into other cell types, they will be used as a model system in which to dynamically compare gene expression and tissue development between primate species. This work will enable expansion of previous experiments using other primate lines in the Haussler lab, as well as investigation into the molecular basis of traits that are uniquely orangutan.

The recent sequencing of all great ape genomes has revealed two facts: that the genomes of great apes have higher sequence identity than previously thought, and that the molecular basis of many phenotypic differences between hominids is not obvious from the nucleic acid sequence (King and Wilson, 1975; Locke et al., 2011). In order to study the cellular environment without harming live animals, *Center for Biomolecular Science and Engineering* (CBSE) researchers under Dr. David Haussler at the *University of California, Santa Cruz* (UCSC) have used *embryonic stem cells* (ESCs) and iPSCs as *in vitro* model systems in which to study and manipulate their cellular biology and early developmental tissues. Pluripotent stem cell lines

now exist in the lab from human, chimpanzee, rhesus macaque, mouse, and gorilla. Comparing *in vitro*-grown tissues from these animals has led to several key insights, the most recent regarding transposon regulation and its role in evolution (Jacobs et al., 2014).

Wanting to expand comparative potential of such experiments, the lab developed induced pluripotent stem cell lines from other great ape species. Embryos or gametes must be harvested from the reproductive organs of an animal in order to create a new ESC line (see Chapter 2.2), and the process for doing so is invasive and painful for the animals. To avoid harming animals, efforts were instead turned towards creating iPSCs from existing somatic primate cell lines. Creation of iPSCs involves inducing the expression of several key regulatory genes in adult cells, causing them to return to a state resembling ESCs (Takahashi and Yamanaka, 2006). Like ESCs, these cells can both divide indefinitely and become any cells in the adult body originating from the three primary germ layers (ectoderm, endoderm, and mesoderm), but they cannot form extraembryonic tissues such as the placenta or trophectoderm (Takahashi and Yamanaka, 2006). To generate these lines, the lab ordered chimpanzee, gorilla, and orangutan fibroblast samples from the Coriell Biorepository and the San Diego Zoo. Reprogramming was first performed on the chimpanzee lines, and viable chimpanzee iPSCs were produced using circular DNA vectors that maintained themselves in the nucleus but did not integrate into the genome. Soon after, gorilla iPSCs were obtained from the lab of Dr. Fred Gage at the Salk Institute in San Diego, California. Orangutans were chosen as the next target because they represent the great ape species most distantly related from humans, and have differences in their brain development, metabolism, and transposon activity that can most easily be investigated with living cells.

The task of reprogramming orangutan cells fell to me and my lab partner, Andrea Reyes-Ortiz, in the fall of 2013. After a failed attempt to reprogram orangutan fibroblasts with DNA episomes, we tried an engineered Sendai virus method and successfully generated clones from the cells of a Sumatran orangutan (as detailed in Chapter 3). These cells show the characteristic colony morphology of PSCs and have been verified to endogenously express pluripotency markers (Chapter 4). At the end of summer quarter 2014, I began the process of reprogramming another set of fibroblasts from two additional Sumatran orangutans as the main focus of my thesis work. Several clones are currently in culture and have been shown to express key pluripotency surface markers (discussed in Section 4.1.1), but still show inconsistent colony morphology and are proving difficult to expand. Once the cells are stable in culture, Andrea and I will verify their pluripotency via gene expression assays (Chapter 4) and differentiation assays. If the cells pass all quality checks, the lab plans to publish data regarding their generation, share the cells

with collaborators, and apply them towards experiments on orangutan transposon repression and cortical development.

Chapter 2

Background

2.1 Gene Expression and Cell Identity

All animals go through a single-celled bottleneck in the transition between each successive generation, and therefore all of the cells in all tissues of an adult organism are descended from that single cell and contain nearly identical DNA molecules (Kuzdzal-Fick et al., 2011). Despite this, cells of different tissues can differ radically in their morphology and behavior. This is the result of genes being up- or down-regulated in patterns specific to a cell's function, environment, and developmental lineage.

There are several different ways that a cell can regulate its gene expression. Changing the physical availability is one: in order to fit within the cell, DNA is folded, wound, and packed into superstructures called *chromatin*. The DNA winds around protein complexes called *histones*, which can in turn be modified in order to dictate gene expression. The histone-DNA complex is called a *nucleosome*, and can pack together tightly or be strung out loosely to make some sections of DNA either more accessible to or shielded from RNA polymerase based on the modifications made to the histones by regulatory proteins (Kouzarides, 2007). The DNA itself can also be modified chemically by the addition of a methyl group to cytosine or adenine bases, which can block RNA polymerase through steric hindrance. A third way DNA can be regulated is by direct, targeted binding of proteins to the DNA double helix, which can both facilitate or repress transcription. These regulators are called *transcription factors*, and many of them are unique to different tissue types as regulators of specific sets of relevant genes (Prasad et al., 2013).

Gene expression can be observed and quantified by isolating the cell's messenger RNA,

using an enzyme called *reverse transcriptase* to convert it to *complementary DNA* (or cDNA), and sequencing the resulting strands. This process of RNA sequencing, called *RNA-seq* for short, provides an array of sequences paired with the copy numbers of each sequence found. Gene expression is incredibly dynamic, so the broad patterns specific to certain tissues must be found by averaging the sequencing results of RNA-seq over many cells (Conaway, 2012).

One way of identifying different tissues and cell types is by staining for certain transcription factors or other proteins known to be unique to that tissue. *Immunofluorescence microscopy* (IF) is a common staining technique used on intact tissue samples (Suvarna et al., 2012). In this procedure, highly reactive *paraformaldehyde* (PFA) is used to open holes in the membrane and cross-link molecules inside of cells to each other and to the plate itself, fixing everything in place. Engineered antibodies are then added that very specifically bind to certain regions of the protein of interest. These antibodies are then targeted by a secondary antibody attached to a fluorophore. When light of the fluorophore's excitation wavelength is shined on the cells, the fluorophore emits a different wavelenfth of light can then be imaged directly using a fluorescence microscope (Suvarna et al., 2012). This assay allows for direct visualization of proteins inside of cells, and observation of the expected array of proteins in the correct location (such as transcription factors localizing to the nucleus or receptors on the cell surface) is considered strong evidence for cellular identity.

2.2 Stem Cells

A *stem cell* is defined as a eukaryotic cell that divides to renew itself and produce progeny of different cell types. Stem cells are essential features of the developmental landscape in all complex eukaryotes. They make it possible for the single cell formed in the fusion of a sperm and an egg to develop into the tissue and organ systems of an adult animal and for the differentiated tissues of that animal to renew themselves over time. Adult stem cells reside in what is referred to as a *stem cell niche*; a specialized environment where they divide to produce one daughter that is committed to a specific developmental state and another that may or may not be a copy of the original stem cell. An example of an adult stem cell is the hematopoietic stem cell and its niche is in the bone marrow: it divides to produce all of the blood and immune cells in the body and renew itself, but is restricted from becoming cell types like neurons or skin. By contrast an *embryonic stem cell* (ESC) can become any tissue of the fully-formed animal, including *hematopoietic stem cells*, but cannot form the cells of the outer placenta (Tuch et al.,

2006).

The different levels of developmental potential for stem cells is what is referred to as their potency. The zygote and its early daughters are totipotent: they can produce any and all cells in the developing and fully-formed animal. After a few days of successive divisions, the embryo forms what is called a *blastocyst* (Figure 2.1). The blastocyst is a sphere of cells with

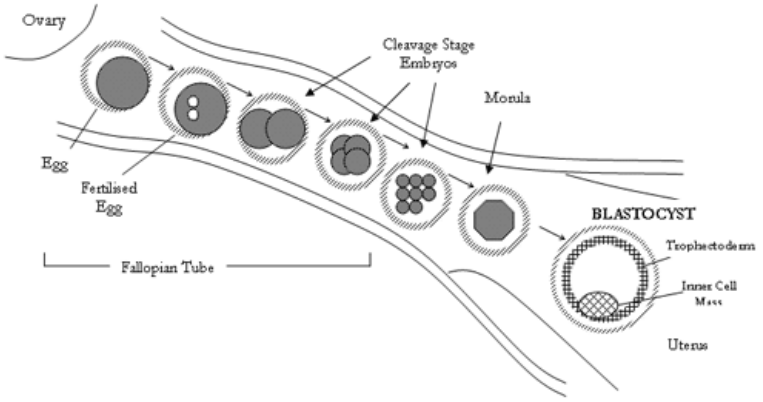

FIGURE 2.1: Diagram of early stage embryo during the first 5-6 days of release and fertilization, copied from the Boundless online textbook of Anatomy and Physiology (Boundless). All cells between fertilization and morula are totipotent. The blastocyst enters and implants in the uterus and contains embryonic stem cells in the inner cell mass (ICM). When *in vitro* fertilization is performed, an excess of embryos are grown to this stage, and the leftovers are frozen for further attempts. These leftover embryos from fertility clinics are the most common source of embryonic stem cells and are destroyed in the process of harvesting. Induced pluripotent stem cells are theoretically functionally equivalent to ESCs and require no such destruction or harvest of embryos. When viable, pluripotent stem cells can be injected into an ICM and produce a chimeric organism when brought to term. (Rippon and Bishop, 2004)

a hollow interior, save for a small cluster of cells at one side called the *inner cell mass* (ICM). The surface of the sphere is called the *trophoblast*, and it will go on to form the outer placental tissues. The cells of the ICM are embryonic stem cells, and they will form all tissues in the fully-formed animal by the time it is born (Tuch et al., 2006). These cell types have differentiated into distinct lineages: they are recently descended from *totipotent* cells, but the ICM contains *pluripotent* cells while the trophoblast could be considered *multipotent*. Hematopoietic stem cells are also multipotent; they form a variety of cell types, but are completely restricted to those types, and were produced from stem cells of a higher level or potency. *Oligopotent* cells produce a lower diversity of cell types, and *unipotent* cells only produce one type while renewing themselves (Hima Bindu, 2011).

Pluripotent cells, left to their own devices, will naturally differentiate into different cell types. In vivo, the ICM is a fleeting state of being, quickly giving rise to three different layers of cells termed the *ectoderm, mesoderm,* and *endoderm* that give rise to separate groups of

tissues (Tuch et al., 2006) (see Figure 2.2). However, when cultured in a lab, supplementing the nutrient medium with growth factors (LIF for mouse and β -FGF in primates) can forestall differentiation and cause the cells to continue dividing in their pluripotent state indefinitely. This ability to continue dividing theoretically *ad infinitum* is called *cellular immortality*, and it is significant because most other cell types divide a certain number of times before stopping and achieving a state called *senescence*. At any point during culture, certain combinations of stimuli can be used to compel stem cells towards a certain developmental lineage. These stimuli can include growth factors, mechanical stresses, and even the surface on which the cells grow (see Figure 2.2)(Tuch et al., 2006).

By definition, the daughters of pluripotent cells injected into a blastocyst will contribute to the tissues of an adult animal (Iannaccone et al., 1994). In mice, this is often done by injecting the cells of mice with a gene for one coat color into the embryo of mice coded to have a different coat color (or with the gene for GFP). This creates a visible mosaic in the coat, and the resulting animal is called a *chimera*. Though this method is time-consuming, it is necessary because no method has yet been developed to remove ESCs from an ICM without destroying it in the process.

While theoretically possible, the chimera test is not done with human cells or those of higher primates for ethical and legal reasons. The ability to form functional tissues from all three germ layers is therefore confirmed via undirected differentiation assays. The *in vitro* method used in our lab involves growing colonies in suspension without growth factors for a period of time, transferring them to a biological membrane until they "flatten out", then performing IF and RT-PCR on the cells to test for markers from all three layers. The preferred *in vivo* approach is called a *teratoma* assay, where a large number of cells is injected subcutaneously into an immunosuppressed mouse and allowed to grow for a period of months into a tumor. The tumor is excised and tested for markers from all three germ layers via IF and RT-PCR (Takahashi and Yamanaka, 2006).

2.3 Induced Pluripotent Stem Cells

In 2006 Yamanaka and Takahashi showed that acutely induced expression of just four master regulatory transcription factors would cause terminally differentiated adult cells to revert back to pluripotent state (Takahashi and Yamanaka, 2006). The resulting cell was called

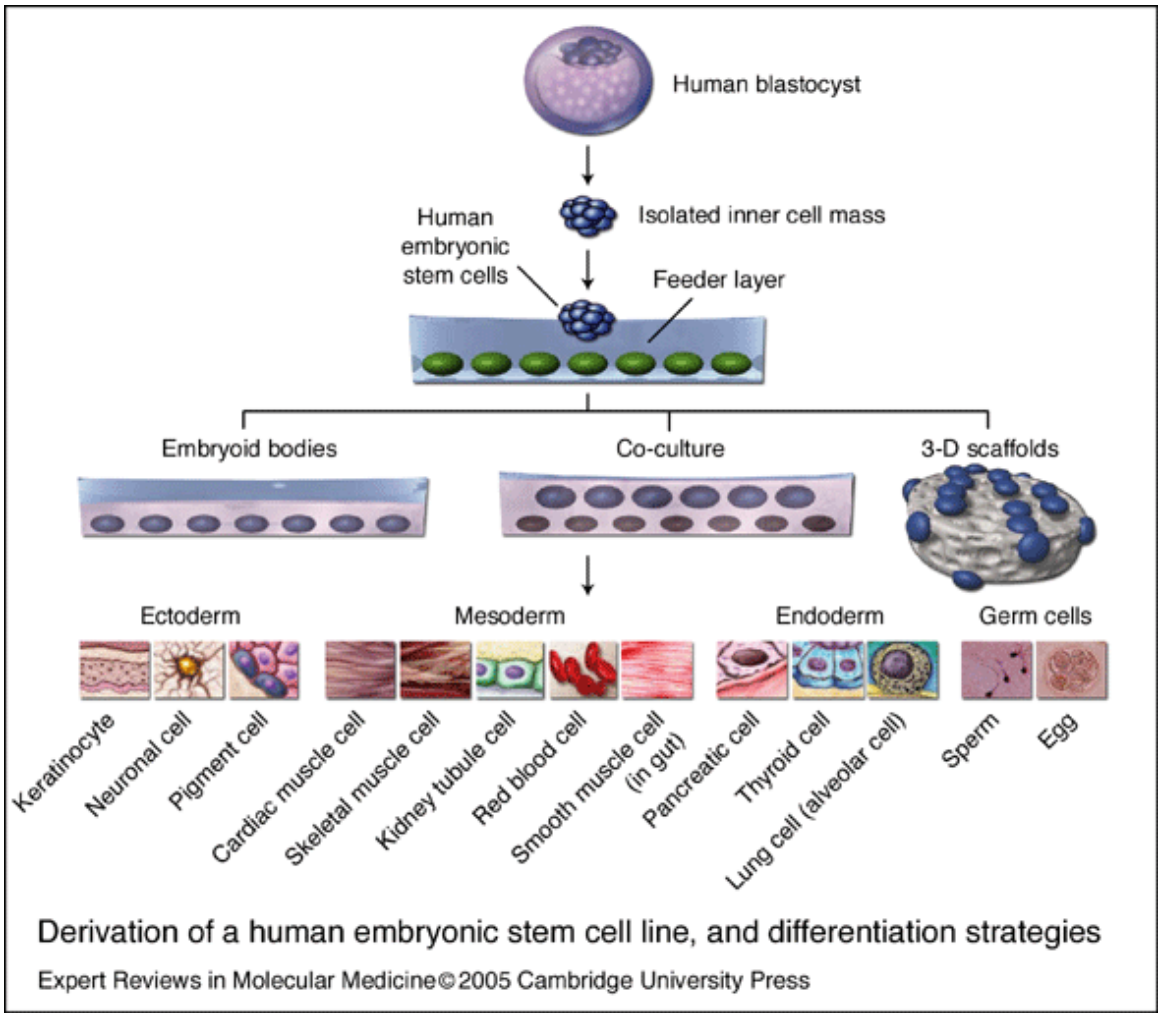

FIGURE 2.2: Schematic of embryonic stem cell isolation, culture, and differentiation, copied from (Hyslop et al., 2005). Embryonic stem cells require other cells or a biological membrane for support, and the feeder cells mentioned are often mutated, undividing mouse embryonic fibroblasts (MEFs). Embryoid bodies, co-culturing and 3D scaffolds are common strategies for encouraging and directing differentiation. Embryoid bodies are spherical, unattached colonies of cells growing in suspension, allowing for more complex interactions between cells as they differentiate. Co-culture of cells can lead to more complex models of tissues and more specialized differentiation (Hyslop et al., 2005). Three-dimentional scaffolds, made of biocompatible material, allow for organ modeling, can allow nutrients into the center of cell cultures, and can apply mechanical feedback to cells(Hyslop et al., 2005).

an *induced pluripotent stem cell* (iPSC), and in 2012 Yamanaka shared the Nobel prize in physiology and medicine for discovering and developing the technology. Previously, pluripotent cells could only be obtained by harvesting them from the ICM of a fertilized embryo, destroying the embryo in the process. Yamanaka and Takahashi enabled the creation of pluripotent cells from any cell in an adult, side-stepping the ethical concerns behind embryo destruction and the genetic incompatibility when using ESCs for stem cell therapy. Their mouse iPSCs were even able to form chimeras, proving conclusively that they had been successful (Takahashi and Yamanaka, 2006). In 2007 they and a separate, independent research group in the United States

released papers showing that they had successfully reprogrammed human fibroblasts to iPSCs as well (Takahashi et al., 2007; Yu et al., 2007).

The transcription factors used by Yamanaka and Takahashi for reprogramming somatic cells are Oct3/4, Sox2, c-Myc, and Klf4. Yu et al. used Oct4, Sox2, Nanog and Lin28. There are several different ways to induce expression of these factors in the cell. The original human and mouse iPSCs were infected with an engineered retrovirus, which inserted a customized DNA vector containing all of the reprogramming factors into the host genome. This is called an *integrative reprogramming technique*, and it is not ideal because it causes genomic disruption and potentially introduces factors that will be continuously produced and interfere with differentiation. Non-integrative techniques also exist, where transient DNA or RNA vectors are used to quickly cause expression and then degrade after successive cell divisions, as shown in Figure 2.3 (Ban et al., 2011). One useful property of iPSCs is that they are a potentially limitless

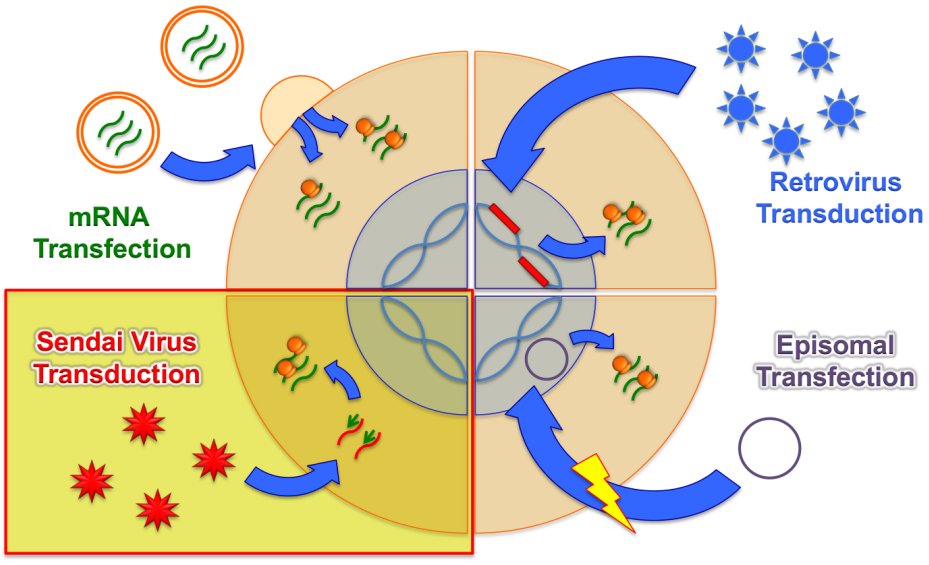

FIGURE 2.3: Schematic of four vector delivery methods. The two on the left are RNA based and never have to enter the nucleus, while the two on the right are DNA vectors that have to make their way to the nucleus with viral-based mechanisms. The only integrative method represented above is retroviral; it often leads to robust gene expression, but disrupts the genome in the process. *Sendai virus transduction* is highlighted because it is the method we chose for this project. Figure created by Andrew Field.

resource of tissue for the animal from which their original biopsy was taken. Fibroblasts (skin cells) are typically used because they are easy to obtain and grow, but many different cell types have been reprogrammed at this point.

The iPSC technology is still relatively new, and while the ideal iPSC is identical to an embryonic stem cell from the same animal, there are some important differences that have yet

to be fully addressed. One issue is that not all regulatory changes that resulted in the original differentiation of the reprogrammed tissue are removed when reverting to iPSC state, creating a statistically significant increase in the tendency to re-differentiate into the original tissue when compared to the more stochastic nature of ESC differentiation (Ji et al., 2012). The process of reprogramming is also inefficient and stressful on the cells. When reprogramming, only the best-looking colonies are perpetuated onward, meaning heavy artificial selection is required in order to obtain cells viable in culture. This does not necessarily translate into cells that still contain identical DNA to that of the donor animal, nor does it translate to the ideal pattern of epigenetic regulatory markers for a true ESC. As a result, each iPSC line is estimated to have multiple mutations in protein-coding genes, large chromosomal rearrangements are not uncommon, and gene expression patterns may not be properly regulated (Ji et al., 2012). This is a major barrier to the use of iPSCs in therapy, particularly because many of the changes turn out to be in tumor-suppressors and oncogenes. Mutations in these genes help perpetuate the cells in culture, but could result in cancer or immune rejection if transplanted to a living environment (Ji et al., 2012; Tuch et al., 2006).

2.4 Mammalian Cell Culture

The culture of live mammalian cells, like that of other organisms, largely takes place in a petri dish filled with nutrient-rich medium. The medium is liquid, and cells may be cultured in suspension or flat on the bottom of the dish. Monolayer adherent cells may be plated on top of a biological matrix like gelatin, or a more intricate and expensive material of purified membrane material. The cultures are maintained in an incubator that controls temperature, humidity, and gas concentration similar to the natural levels of a living body. Some cells, like pluripotent stem cells, may even require what is called a *feeder layer* of other cells present to provide support. Transferring cells from one culture vessel to another is called a *passage*, and is often required periodically in order to maintain an optimal density or colony size. Because of the absence of a circulatory system to continuously supply nutrients and carry away metabolic by-products, medium has to be changed out periodically. Each cell type has a different set of optimal conditions and medium components, and modifying these parameters is one of the main ways to direct stem cells to differentiate toward a specific cell type or lineage. Protocols significantly changed for the design of this project are recreated in detail in Appendix

B, but many routine protocols in this research are adapted from pages on the Life Technologies (Thermo Fischer Scientific Inc., 2015a) or the WiCell (WiCell Research Institute, 2015) websites.

2.5 Hominid Evolution

Humans (*Homo sapiens*), chimpanzees (*Pan troglodytes* & *paniscus*), gorillas (*Gorilla gorilla* & *beringei*), and orangutans (*Pongo pygmaeus* & *abelii*) belong to the primate family hominidae, more colloquially known as the great apes. Humans split from *Pan* about 5 million years ago (mya), *Gorilla* about 7 mya, and Pongo 14 mya; gibbons, or so called lesser apes, split from the line about 18 Mya (Figure 2.4) (Locke et al., 2011). All species more related to humans than the chimpanzees have gone extinct, though the argument could be made that Neandertals survived by breeding into the human germline. When it comes to living animals, the great apes are the only other comparison point when it comes to examining recent evolution (Locke et al., 2011).

Before the advent of DNA technology, anthropologists and paleontologists comparing the skeletons of humans with those of the great apes thought that the common ancestor of the two was much more distant. When DNA analysis by Mary-Claire King and A. C. Wilson revealed in 1975that the genomes were extremely similar, they suspected that human evolution had accelerated on a more subtle level than simply mutations in the genes for brain size and skeletal structure. They hypothesized that small differences in the regulation of genes could lead to different levels of gene expression in certain tissues at certain times, causing drastic changes in gross phenotypes (King and Wilson, 1975).

One possible example of this is the rapid expansion of the human neocortex. The human brain is more than twice the size of that of the chimpanzee, but it appears there is not a correspondingly obvious change in the genetic sequence of relevant genes, suggesting a more subtle mechanism. For example, during mammalian fetal development, there is a period of time where the cerebral cortex, an important structure in higher cognition, is constructed from the inside-out. Neural stem cells called *radial glia* form around the center of the developing brain, send projections to the outermost layer, and divide to form *neural progenitors* (Noctor et al., 2001). The progenitors then radiate outwards along the spindles and settle into their final location before differentiating into mature neurons. The number of radial glia, the angle at which they radiate outwards, the radii of their expansion, and the time over which they continue to divide may all be governed by a transient gene expression pattern (as discussed

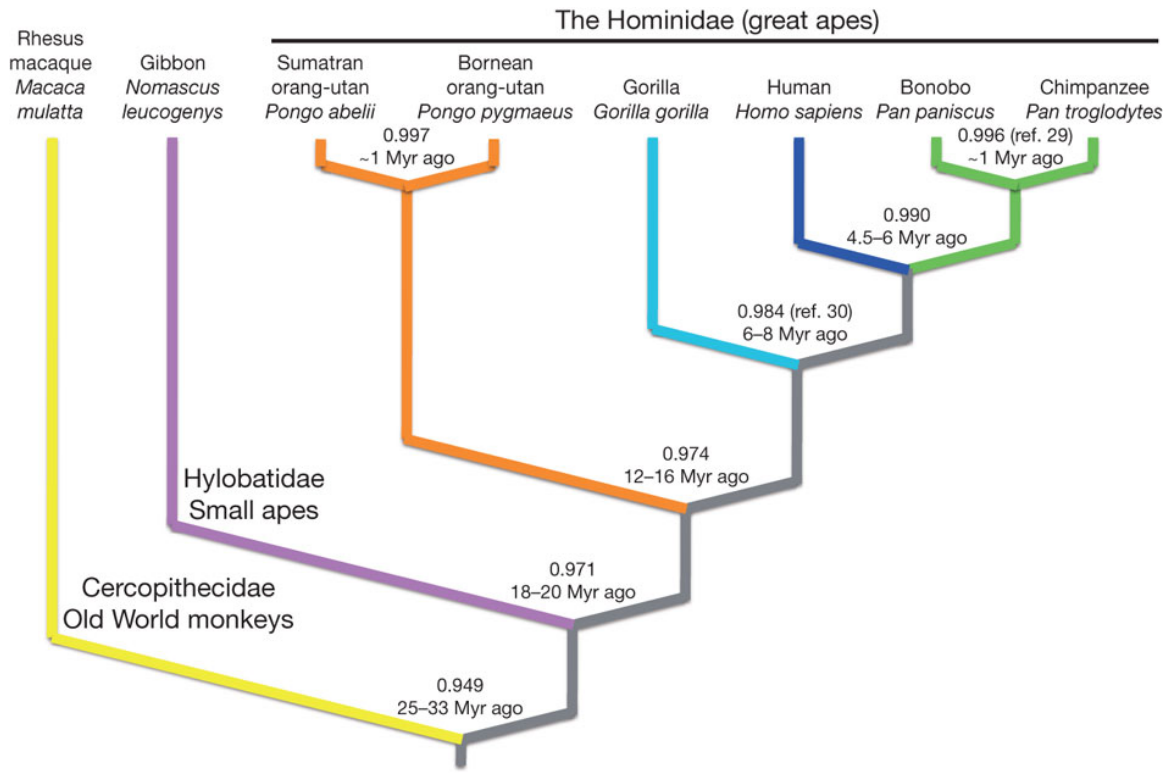

FIGURE 2.4: Schematic phylogenetic tree of recent primate evolution, copied from Locke et al., 2011. Distances and times are based off of genome sequence variation (with the decimal values at each branch being the seqence similarity between its constituent species), revealing relatively recent divergences in the orangutan and chimpanzee lines. From this data, orangutan's distance from our own line is clearly visible. iPSCs were previously generated for chimpanzee and gorilla, and either ESCs or iPSCs are available from human and rhesus, but not for bonobo, gibbon, or orangutan.

in Section 2.1). An idea called the *radial unit hypothesis* states that changes in any of these variables would lead to a differently sized or shaped brain in the fully-formed animal (Noctor et al., 2001). Methods currently exist for differentiating pluripotent stem cells to this stage in development. They even form radial structures called *neural rosettes*, mirroring the formation of the early neurotube, and so examining gene expression at certain time points can effectively simulate this early developmental stage (Shi et al., 2012).

The genes that regulate these expression patterns may be able to tolerate mutations more readily than those coding for more basal traits. A slightly differently shaped brain could still be functional, whereas a brain that has mutations in genes coding for cytoskeletal proteins could be seriously deformed, or not form at all. Regulatory genes, by definition, control the expression of other genes, so any changes in them can have far-reaching effects such as an increase in brain size and complexity over time. By comparing gene expression across species, it is hoped that the molecular cause of human brain expansion can be better understood. This is one of the main avenues of investigation in the Haussler lab, and something we hope to apply our stem

cells to studying.

Another area of study is the regulation of transposons within the primate genome. The Haussler lab has focused on this topic recently, using a combination of molecular and computational approaches to study transcription factors that repress these rogue genetic elements (Jacobs et al., 2014). Transposons are relevant to evolutionary divergence in two distinct, but related, ways. One is that the transposable elements are, by their very nature, a major driver of genomic change. They splice themselves in and out of the genome, causing disruptions in nucleotide sequences and even sometimes duplicating the sequences surrounding their insertion sites. Active and defunct elements make up large stretches of the genome, leading to an excess of code that can recombine, mutate, and be re-purposed over time. Too much mutagenesis is generally evolutionary disadvantageous, and a major finding of Jacobs and Greenburg was showing that KZNFs had, multiple times in the past, mutated to recognize and repress these transposon sequences after they had mutated out of de-repression, leading them to coin the interaction an "arms race" taking place within the genome itself (Jacobs et al., 2014). KZNFs do not just repress the specific sequence to which they bind, however. A less potent repressive effect extends to nearby genes, potentially leading to larger and more complex downstream effects in gene regulation depending on what genes are affected (Jacobs et al., 2014).

It has been shown that *Alu elements*, a class of retrotransposons particularly active in primate genomes, are comparatively quiescent in the orangutan genome, but the mechanism for that quiescence not yet known (Walker et al., 2012). Alus are present in large numbers in the human genome, have been shown to cause human disease via genome disruption and homologous recombination at their insertion sites (Deininger and Batzer, 1999). During the summer of 2014, Andrew Field and Kasey Feng in the Haussler lab performed RNA sequencing in order to build a gene expression profile of the iPSCs Andrea and I generated. That data is being analyzed by a team of bioinformaticians including Dr. Benedict Paten and Ian Fiddles in order to identify ZNFs that are unique to the lineage and highly expressed in these cells. Progress is slow because of problems aligning sequence with the current orangutan genome assembly, but the effort will hopefully reveal candidates for investigation into the mechanism of orangutan Alu repression. Elucidating the mechanism of their quiescence in the orangutan lineage could lead to revelations on the contributions of Alus to evolutionary differences between the two lineages and perhaps lead to a strategy for mitigating their contribution to genome disruption during cell culture and iPSC reprogramming.

Chapter 3

Reprogramming Orangutan Fibroblasts

3.1 Sendai Virus Transducibility

The Cytotune-iPS 2.0 Sendai Reprogramming kit from Life Technologies is a set of three engineered viruses, each containing an RNA vector designed to induce high expression of a unique combination of the Yamanaka factors (Thermo Fischer Scientific Inc., 2015b). In human cells, the three viruses used in conjunction have been shown to have a success rate of 0.49% successful reprogramming chance per cell, and the Yamanaka factors are so highly conserved between mammals that the kit has successfully been used to reprogram cells from the distantly related drill (a monkey closely related to baboons) and the critically endangered white rhinoceros (Ben-Nun et al., 2011). Viruses do not always infect hosts in the same way, so it was important to confirm that Sendai virus would infect orangutan cells without causing them to undergo programmed cell death before using the reprogramming kit. To this end, Life Technologies also sells the Cytotune-EmGFP Fluorescence Reporter, a single Sendai virus containing the gene for green fluorescent protein (GFP). Andrea and I used the reporter on all five San Diego Zoo orangutan fibroblast lines in our posession, and 48 hours after administration of the virus all cultures were analyzed by IF (Figure 3.1). All of them contained a population of cells that were positive for both GFP and Sendai virus coat protein, and little to no cell death was observed in the cultures. Having confirmed that the virus would infect the cells, we proceeded to ensure they were free of mutations.

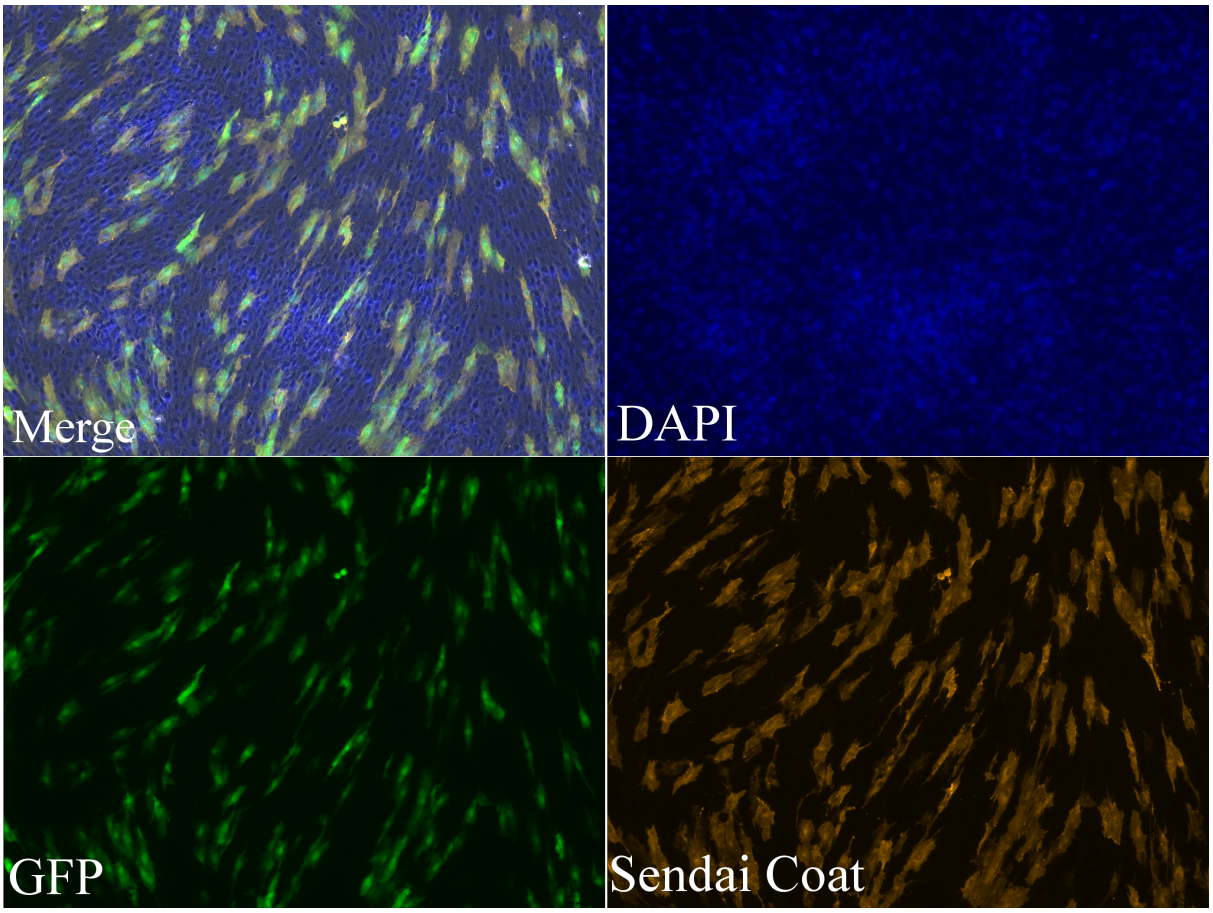

FIGURE 3.1: Fluorescence microscopy image of fibroblasts stained for GFP and Sendai Virus coat protein 48 hours after transduction with Cytotune-EmGFP Fluorescence Reporter. These particular cells are 9018-3265 "Karen" fibroblasts and were the most confluent, but all five lines in this assay also presented positive results for both stains. Cell death appeared to be negligible, though no formal counts were done. Image was taken under microscope at 100× magnification.

3.2 Cell Line Selection and Karyotyping

Because each reprogramming kit contains enough virus to transduce only three lines at a time, Andrea and I had to narrow down which lines to focus on for each experiment. Only the San Diego Zoo lines were tested for the firt reprogramming attempt, and we chose the three that replicated most quickly during the GFP test. Two of these lines were Sumatran orangutans (*Pongo abelii*) and one was Bornean (*Pongo pygmaeus*). The Sumatran lines chosen were 11045-4593 and 9018-3265, or "Josephine" and "Karen," respectively. The Bornean orangutan line was called 13692-6363, or "Chelsea". Cell lines often have a systematic number designation, but since these cells came from captive animals in a zoo, they were often called colloquially by the names of their source animals.

To assess the cells for potential gross genomic rearrangements and damage to chromosomes, we use G-band karyotyping services provided by *Cell Line Genetics* (www.clgenetics.com).

A common mutation that can arise during reprogramming, one shared with some cancer phenotypes, is large chromosomal aberrations such as duplications or fusions (Laurent et al., 2011). Such mutations are often lethal to the cell, but on rare occasions can greatly increase its fecundity in culture instead. Cells with abnormal chromosomes are far from ideal for collecting data meant to be representative for normal tissues. While it is a relatively low-resolution approach to detecting mutations, it is standard procedure to have the chromosomes examined through karyotyping before and after reprogramming to ensure that they have not been damaged by the stressful process. In addition, it is important to repeat the procedure periodically during subsequent culture because the heavy artificial selection that culturing places on the cells can also encourage chromosomal damage. In April, samples of all three lines above were sent to CLG just prior to initiating the reprogramming protocol, but Andrea and I did not receive the results until after the reprogramming had begun. We terminated the wells containing cells from Karen. In the second transduction, I made sure all lines were karyotyped beforehand. The results of both tests are summarized in Table 3.1.

ID No. / Name	Source	Species	Sex	Passage No.	Date Tested	Normal?
PR00054	Coriell	Sumatran	Male	14	8/8/14	Yes
PR01110	Coriell	Sumatran	Female	11	8/8/14	Yes
10853-4479 / Indah	SD Zoo	Sumatran	Female	10	8/8/14	No
4993-6474 / Janey	SD Zoo	Bornean	Female	9	8/8/14	No
9018-3265 / Karen	SD Zoo	Sumatran	Female	8	4/15/14	No
11045-4593 / Josephine	SD Zoo	Sumatran	Female	10	4/15/14	Yes
13692-6363 / Chelsea	SD Zoo	Bornean	Female	10	4/15/14	Yes

TABLE 3.1: Summary of karyotype results as reported by CLG. Unfortunately, almost half of the orangutan lines tested had some kind of aberration. However there were still representatives of both species and both sexes among the lines that tested normal. Reprogramming was attempted for all four of those lines. Abberations included a chromosomal duplication in Karen, extra genetic material of unknown origin on Indah's chromosome 17, and several assorted additions and deletions on chromosomes of cells in Janey's culture.

The severity of aberrations varied between the different lines, but in order to reduce the chance of wasting the very expensive Sendai Virus kits I chose to consider only the lines that returned completely normal. This meant that only four of our lines were suitable candidates for reprogramming. In April, we attempted reprogramming with "Josephine," "Karen," and "Chelsea," only succeeding with the first of the three. Chelsea's cultures never yielded colonies. In September I chose to attempt reprogramming with PR00054 and PR01110, and to retry it with 113692-6363 "Chelsea." At the time, our lab records indicated that PR01110 and PR00054

were Bornean orangutan samples and while it would only leave us with one Sumatran specimen to work with, I reasoned that having multiple orangutans and representatives of both sexes could give us more confidence in experimental results. On March 10th, 2015, we found out that the PR01110 and PR00054 were actually Sumatran orangutans.

3.3 Reprogramming

A detailed protocol for the reprogramming process can be found in Appendix B.1. The protocol begins with thawing the cell lines to be reprogrammed several days before actual viral infection. I thawed out cell stocks and passaged each plate when the cells covered approximately 50% to 80% of the plate's surface as determined by examination under a microscope at 100× magnification. While an ideal experiment would have an equal number of cells for each line, the different lines had different growth rates. This made coordination difficult, with 9018-3265 "Karen" growing fastest at a rate almost twice as fast as 113692-6363 "Chelsea," the slowest. PR00054 was the fastest by far and PR01110 grew at an intermediate rate. The protocol called for between 2×10^5 and 3×10^5 cells per well on the day of transduction (Thermo Fischer Scientific Inc., 2015b), and the counts stayed mainly within that range during the first time. Both 113692-6363 "Chelsea" and PR01110 were within that range, but PR00054 had more than twice the maximum at 6.08×10^5 cells per well. The final cell counts one week after transduction varied wildly as well, and during the first run it appeared that the Chelsea cells suffered some kind of population crash, possibly due to cytotoxicity or some error in their handling. The differences in cell counts are summarized in Figures 3.2 and 3.3.

The Sendai kit has three separate viral vectors that are added together, called KOS, hc-Myc, and hKlf4 at a certain *Multiplicity of Infection* (MOI), or ratio of active or infectious virus particles to cells. Some details of their construction and composition can be found in the technical documents for the kit (Thermo Fischer Scientific Inc., 2015b). Essentially the KOS vector contains RNA for Klf4, Oct3/4, and Sox2 while the other two were separate vectors for c-Myc and another Klf4. The additional Klf4 vector serves to provide an extra dose of the virus' RNA polymerase, which is needed to compensate for the polycistronic KOS vector' extra genes. Additional Klf4 also increases reprogramming efficiency, according to the product's technical resources, but its MOI often has to be optimized for each cell line (Thermo Fischer Scientific Inc.,

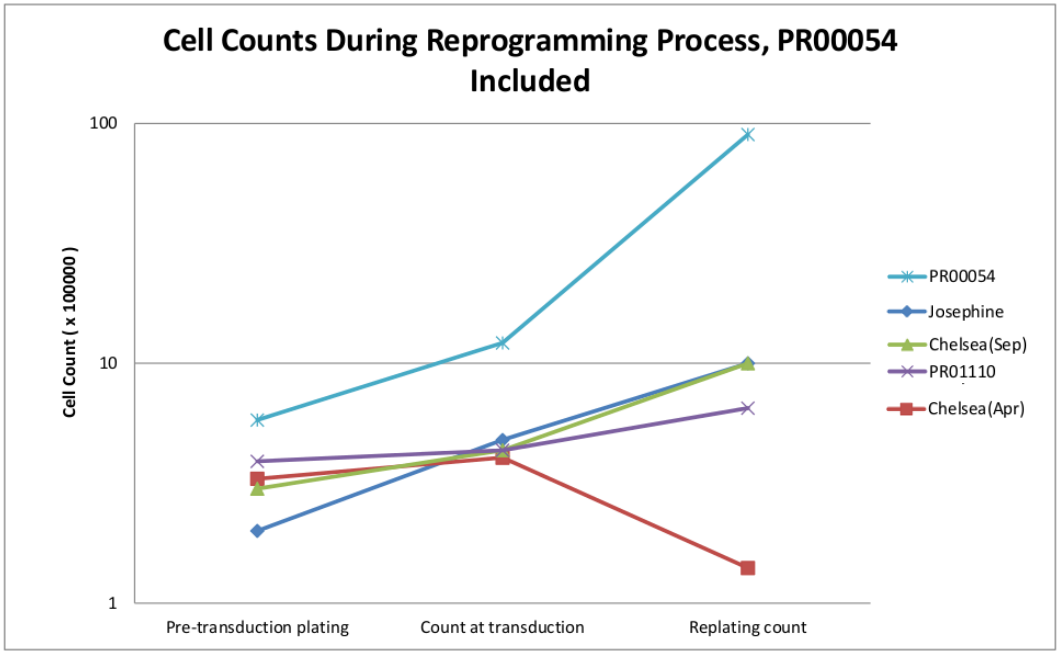

FIGURE 3.2: Graph of cell counts as they were taken before, during, and after transduction with Sendai Virus. This graph serves mainly to illustrate the large difference in cell count between PR00054 and the rest of the lines, as the distinctions between them are dwarfed in comparison. I initially seeded that line at a higher density because it had seemed to grow relatively slowly during the culture for karyotyping, but it took off more than I had imagined it would.

2015b). According to the protocol, the best MOI for KOS and hc-Myc is 5 virus particles per cell (Thermo Fischer Scientific Inc., 2015b). In April, Andrea and I tried both MOI 6 and 3 and had success only with the former, and so I chose to use only that MOI in September.

The other design choice lay in how to re-plate the cells one week after transduction. While the official protocol (Thermo Fischer Scientific Inc., 2015b) calls for plating them onto 10 cm cell-culture dishes, I chose to instead use 6-well cell cutlure dishes. An entire 6-well dish has approximately the same amount of effective growth area as a 10 cm dish, but is more modular and therefore allows for more flexibility and greater usage of space because cells of multiple lines can be self-contained in different wells of the same dish. This was useful because of the very large discrepancy in final cell counts between the three lines.

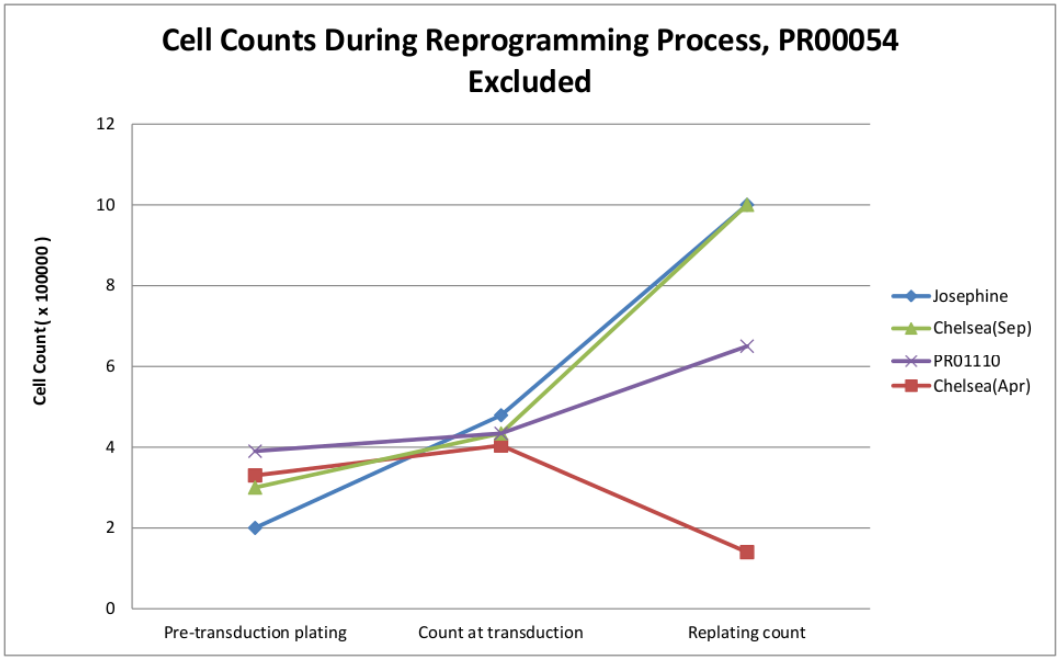

FIGURE 3.3: Graph of cell counts as they were taken before, during, and after transduction with Sendai Virus, excluding PR00054. This graph illustrates the population crash that Chelsea observed in April, for which I do not have an explanation but may have led to no iPSC clones being generated from those cells during that run. It also shows the relatively slow growth rate of PR01110, about half of that of Chelsea or Josephine. That line grew very slowly in culture as fibroblasts, too, and could be a result of either natural variation or some kind of growth-modifying phenotype shared by some lines and not others. As of March 4th, 2015, both PR01110 and PR00054 have only yielded 1 clone in the September transduction and no Chelsea clones remain.

3.3.1 April Reprogramming

This round used cells from Chelsea, Karen, and Josephine before Karen had to be removed from the experiment for karyotypical abnormalities (Table 3.1). For the re-plating step (one week after the transduction with Sendai virus), we had a relatively even spread of cell densities, adjusted for cell count when one line was more numerous than another. When colonies began to emerge in April, Andrea and I transferred them to 12-well plates and passaged one colony at a time until their morphology improved. Each 12-well plate was numbered in the order that it came into use, and the subsequent naming of each clone was a combination of the primary fibroblast designation, the 12-well plate it was first passaged to, and the coordinates of the

well into which it was placed (with the three rows labeled A-C and columns 1-4). As they were continually passaged, the cell culture vessel in which they were contained grew as the number of viable colonies they displayed increased. Only Josephine produced clones during that transduction, and four clones made it to the post-transduction karyotyping test: 3C1, 2A3, 2A4, and 1B4. After verifying their pluripotency (Chapter 4), 3C1 and 2A3 were chosen as the best clones to continue forward with and the rest were placed in liquid nitrogen for storage. Currently, as of April 6th, 2015, I am in process of thawing and re-expanding 3C1 and 2A3 for further characterization.

3.3.2 September Reprogramming

This round used cells from Chelsea, PR01110, and PR00054. Ultimately, because it had the most success in the April transduction, I decided to plate most of the cells at the middle density recommended (1.5×10^5 cells / 6-well plate) with a subset at 3.0×10^5 and another subset at 7.5×10^4. Though we plated everything on top of MEF feeder cells in April, for the run in September I also had one 6-well plate coated in Matrigel and 2×10^6 cells for each, with the hopes that some clones could be generated in feeder-free conditions. When it became apparent that I did not prepare enough plates to handle the number of cells that had grown, I plated the rest of what was left of PR00054 and Chelsea at a relatively high density. By the time colonies emerged, Andrea had returned after being away for the summer and assisted me in transferring new clones to 12-well plates of MEFs. That time, the high-density plates got so confluent with fibroblasts that it became difficult to remove only the iPSC colonies, leading me to use a 5-minute incubation with 2mg/mL of collogenase to break up the MEF layer before performing the first passage. As of April 6th, 2015 work was still ongoing with one clone of each PR00054 and PR01110, designated PR00054-1B1 and PR01110-5A1. On March 13th, 2015, the PR01110 stem cells were confirmed to be karyotypically normal by Cell Line Genetics.

Chapter 4

Verification of Pluripotent Marker Expression Via Immunofluorescence and RT-PCR

4.1 Immunofluorescence

4.1.1 Live Staining for TRA-1-81

In order to reduce the chance of wasting resources on mutant or partially reprogrammed cells, Andrea and I tested live cells for the presence of a pluripotent cell surface protein, TRA-1-81. Stegment manufactures a monocolonal antibody covalently attached to a 488 nm fluorophore designed to stain living cells in culture, the protocol for which can be found on their website (Stegment Inc., 2015). Before passaging beyond the 6-well-plate stage, Andrea and I performed the live cell stain on the cells while still in culture. Colonies that visibly expressed TRA-1-81 through fluorescence were selected for further passaging. This assay was important because early-stage or partially differentiated iPSC colonies can be difficult to distinguish from fully differentiated colonies through colony morphology alone. Some of the colonies were heterogeneous for TRA-1-81 staining, but had to be passaged in their entirety anyway with the hope that the negative cells were not iPSCs and would eventually senesce. The colonies that did not yield positive results were discarded.

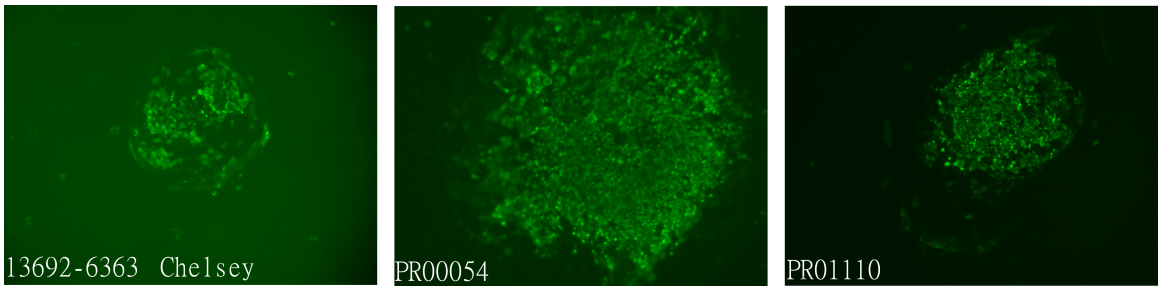

FIGURE 4.1: Representative pictures of the three orangutan cell lines reprogrammed in September stained with anti-TRA-1-81 antibody, showing positive cell surface staining as compared to the background. The leftmost picture is of a heterogeneous differentiating colony; only a subset of cells are positive and others are dark, in marked contrast to the bright and uniform colonies of the other two specimens. Image was taken under microscope at 100× magnification.

4.1.2 Fixed Staining for Defined Factors

We performed IF staining periodically in order verify the expression of pluripotency factors. Once each clone was stable in culture, we set aside a subset of cells during passaging and grew them on a 24-well cell culture plate. Once they were easily visible under the microscope, we stained them according to a similar protocol to that outlined in Appendix B.2, with the only difference being that they remained within the plate for imaging and were placed underneath coverslips following staining. Staining for Yamanaka factors such as Sox2 or Oct3/4 is important because they are essential for pluripotency, but those factors were introduced in the vectors and are therefore not sufficient to prove endogenous expression. Nanog, however, is an endogenous pluripotency marker that is not included in the Yamanaka factors, so its expression was used to confirm reprogramming as well. Staining for Sendai virus coat protein was also performed to verify that the vector has cleared the cells. An example is shown in Figures 4.2 and 4.3.

While powerful, IF is not definitive enough to draw solid conclusions without molecular biology experiments to back them up. For example, expired or contaminated antibodies could interfere with antibody-antigen interactions, and expired paraformaldahyde could improperly fix the cells to the plate. Another factor worth mentioning is that though the genes being tested for are fairly conserved across species, these antibodies are generated using human antigens. Coding differences in the antigen site could lead to less binding, while duplication or fusion could lead to false positives. We chose RT-PCR to provide independent verification of marker expression through a completely different mechanism.

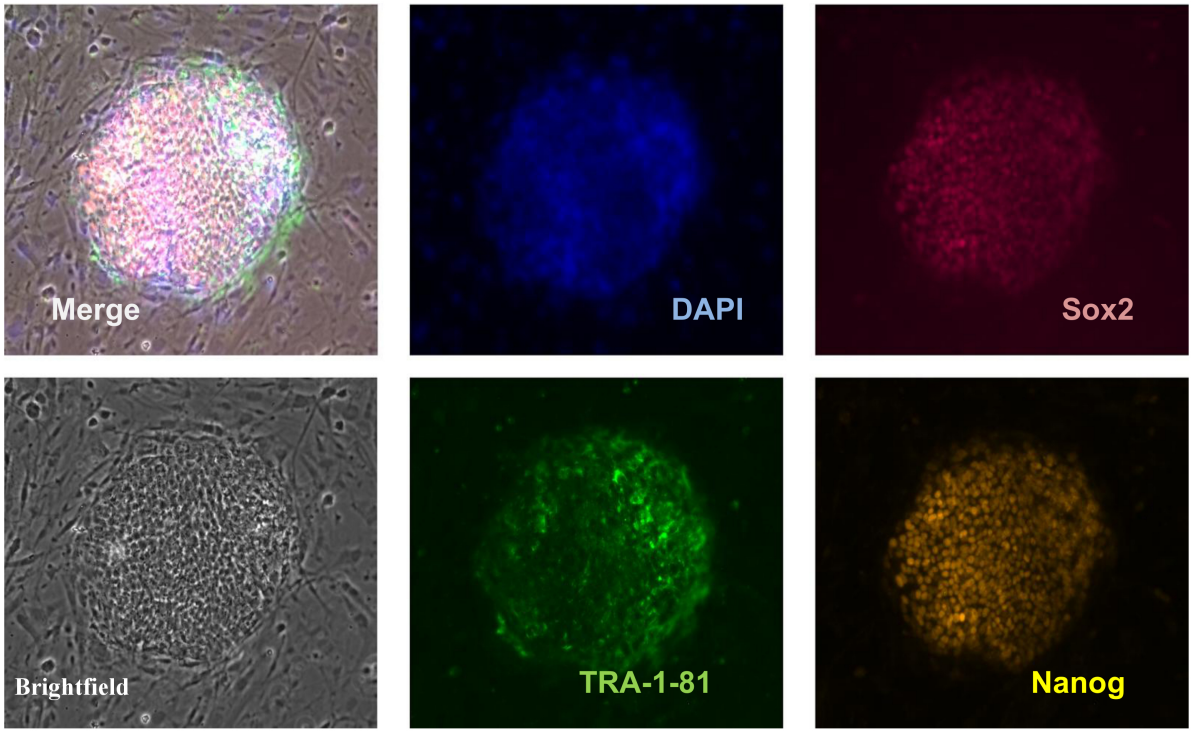

FIGURE 4.2: Fixed Stain of Josephine-3C1 iPSCs for pluripotency factors. The bottom left image shows a brightfield image of an iPSC colony on feeder MEFs, displaying a consistent density with defined borders and much smaller cell size than observed in the surrounding fibroblasts. DAPI stain, though slightly off-focus here, stains the nuclei of all cells in the field of view. Sox2 co-stains in the nucleus as expected for a pluripotency transcription factor, but its presence alone is not enough to establish pluripotency because it is one of the Yamanaka factors transduced with the Sendai virus. TRA-1-81 and Nanog, however, are endogenous markers of pluripotency, and they, too, stain where expected. TRA-1-81 stains the cell surfaces, leading to a globular appearance, while Nanog stains the nuclei and looks more like Sox2 or DAPI. There is some background staining observed in the MEFs, but it is at a much lower intensity than that corresponding to the iPSC colony. Image was taken under microscope at 100× magnification.

4.2 RT-PCR

Direct tests for the presence of mRNA are some of the most powerful lines of evidence available for discerning the properties of generated stem cells, allowing us to verify gene expression and test for the continued presence of the RNA Sendai virus genome. We use two kits from Qiagen in tandem to analyze RNA: the RNeasy Mini kit (QIAGEN, 2015b) to extract it and the OneStep RT-PCR kit (QIAGEN, 2015a) to reverse-transcribe and amplify RNA sequences of interest. The protocol I used to extract the RNA is found in Appendix B.4, while the RT-PCR itself was performed according to the OneStep kit's protocol except that all volumes were halved in order to conserve reagent. RT-PCR uses RNA isolated from each fibroblast line just before reprogramming, fibroblasts 1 week after transduction, each stem cell line, and RNA from human ESCs as a positive pluripotent primate control (Figures 4.4 and 4.5). The primers we

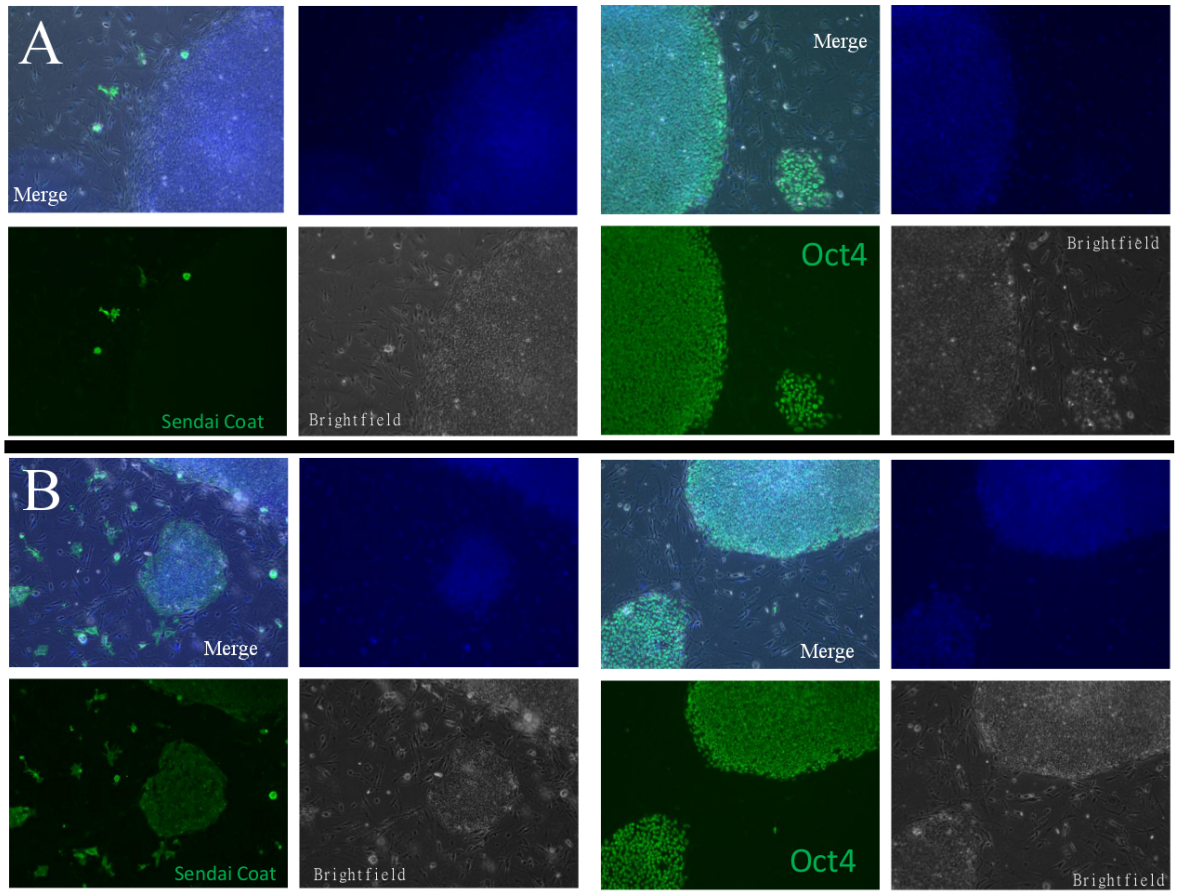

FIGURE 4.3: Panel A: Josephine 3C1 clone expresses Oct4 but not Sendai virus coat protein. Panel B: Josephine-2A3 expresses both. This test was run after the ones shown in Figures 4.2, 4.4 and 4.5. It shows evidence of recalcitrant Sendai virus reproduction in the 2A3 line long after it had already cleared in 3C1. Background staining does appear in the 3C1 Sendai image, but only in cells that appear to be lysed and therefore the staining could be an example of GFP-wavelength autofluorescence; the colony does not show staining. Image was taken under microscope at 100× magnification.

used include those for human Oct3/4 and Nanog, as well as a positive control using GAPDH (an enzyme necessary for glycolysis) and a negative control using water instead of primers. The primers were not checked against the Orangutan genome at the time, but they have since been verified to match via BLAT on the UCSC genome browser (Kent, 2002; Kent et al., 2002). We recently ordered new primers for each Sendai virus vector to make sure that the vectors have actually cleared from the cells. The Sendai primers (whose sequence is found in the technical documents for the kit (Thermo Fischer Scientific Inc., 2015b)) span the viral genome and the factors they encode, and the reaction run using them can be found in Figure 4.6.

Together, these reactions provide valuable evidence for the quality of the generated cells and the tools used to analyze them. If results are negative for pluripotency factors, it potentially indicates either that the cells lack the required gene expression profile or that the gene

sequence of interest has diverged significantly where the primers are supposed to bind. Presence of Sendai virus sequence indicates that the vectors have failed to clear the cell, as is the case with the Josephine 1B4, 2A4, and 2A3 clones. The Cytotune manual has several methods for encouraging recalcitrant vectors to clear, including heavy selection and culturing at elevated temperatures (Thermo Fischer Scientific Inc., 2015b).

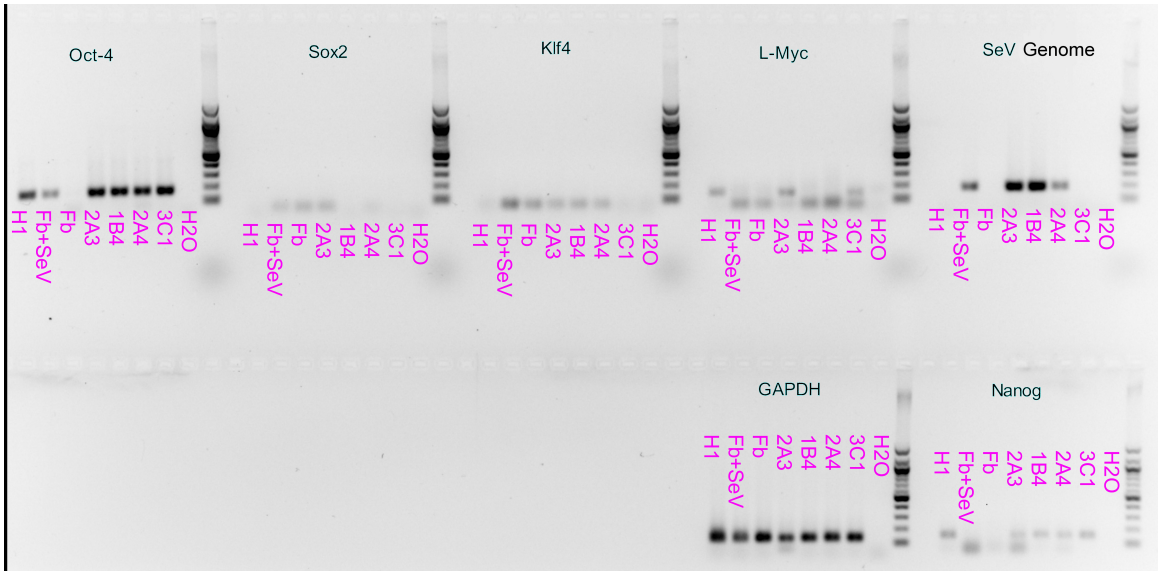

FIGURE 4.4: Agarose gel of RT-PCR products: 25 μL reactions, 1 ng of RNA per reaction, run for 35 cycles. Lanes are clustered by primer pair and labeled with shorthand names for the RNA source. H20 is a water negative control; 3C1, 2A4, 1B4, and 2A3 are four different iPSC clones from Josephine's fibroblasts; Fb is from pre-transduction fibroblasts, while Fb+SeV is RNA from 1 week following transduction; H1 is human ESC positive control. Oct4, Sox2, Klf4, and L-Myc are the factors transduced into the cells, and Nanog is an endogenous pluripotency transcription factor. GAPDH is a gene used in glycolysis, and serves as a positive control. The results of this test are mixed. Oct4, GAPDH, and Nanog are almost exactly as expected. Positive bands of expected size appear in all lines for GAPDH. Oct4 appears in the Fibroblasts after Sendai transduction, the iPSC lines and in H1. Nanog has bands of the correct size in the iPSC lines and H1, with ambiguous bands in the lines of Fb+SeV and 2A3. Sox2, Klf4, and L-Myc had ambiguous results, with absent bands in the H1 control in the former two and multiple bands in the latter. These were human primers, so a genomic difference in the loci between orangutan and human could have led to that result. A pipeting error could also explain it. The other significant result out of this test is the presence of Sendai virus in three of the lines even though it should have cleared the cells at this time (about passage 15 after transduction).

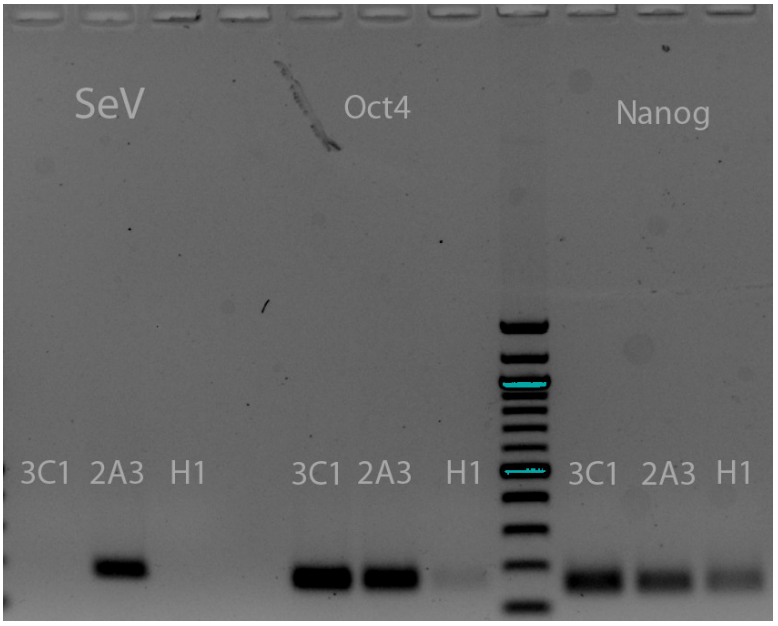

FIGURE 4.5: Agarose gel of RT-PCR products: 25 μ L reactions, 1 ng of RNA per reaction, run for 30 cycles. A test for Sendai virus genome, Oct4 and Nanog expression in the Josephine-3C1 and Josephine-2A3 clones about 10 passages later, compared to H1 human ESCs. Sendai virus only appears in 2A3. Nanog and Oct4 appear in all three, but some volume was lost in pipeting the H1 fraction so it did not turn out as well in the gel. This helped confirm the recalcitrant SeV vector remaining in the 2A3 clone.

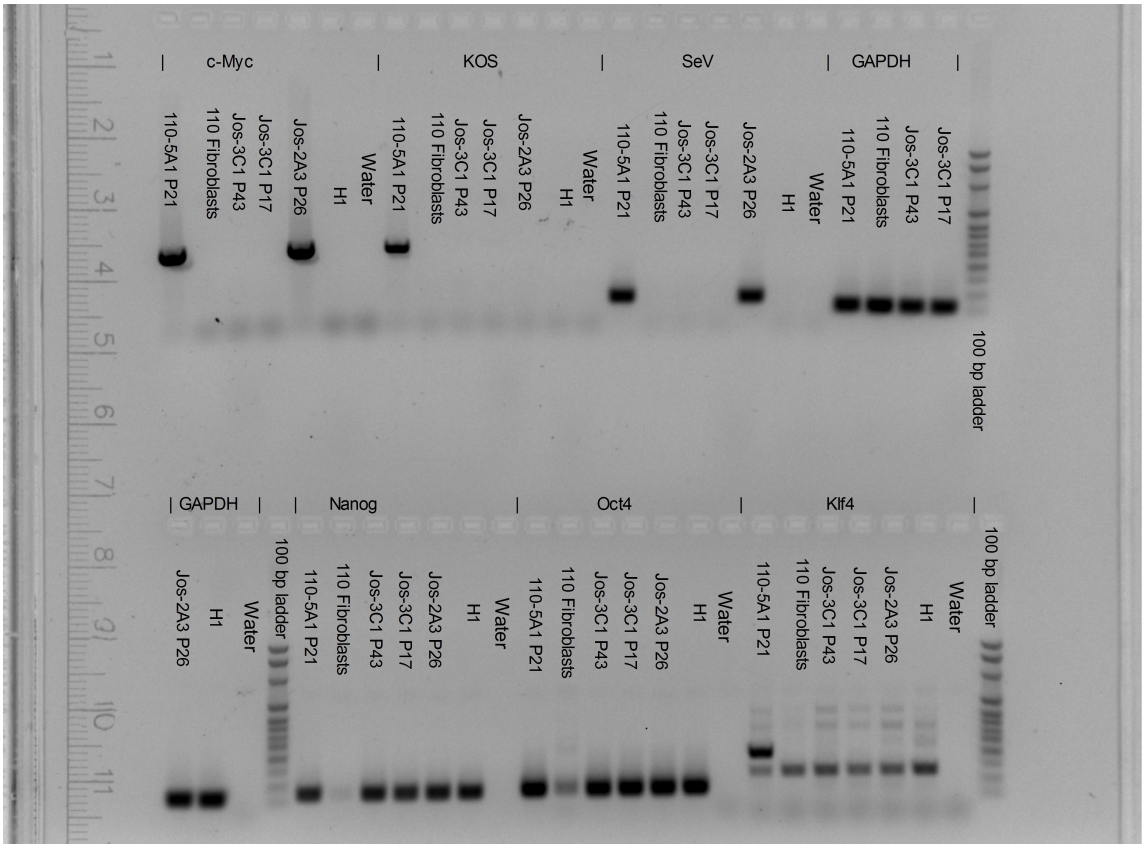

FIGURE 4.6: Agarose gel of RT-PCR products: 25 μ L reactions, 1 ng of RNA per reaction, run for 30 cycles. This test was designed to verify pluripotency and viral clearance in successful iPSC lines. Primer names are at the top, while each lane is labeled with its origin RNA source. GAPDH is a positive control, Nanog and Oct4 are for the primate genes, and the rest of the primers were designed to target Sendai virus. SeV targets directly to Sendai Virus genome. KOS, c-Myc, and Klf4 target the gene sequence with one primer and Sendai genome sequence with the other. This reaction suggests persistent retention of the c-Myc vector in Josephine 2A3 and retention of all three vectors in PR0110-5A1. H1 serves as positive control for human ES cells. The Klf4 vector appears the most puzzling, with various products in all cell lines including that of the H1 cells even though there should not be any vector. The only correctly sized products for the Klf4 vector appears in 110-5A1, suggesting that the other bands may be side products.

Chapter 5

Differentiation Assay

By definition, pluripotent cells must have the ability to spontaneously differentiate into any tissue type from the three primary germ layers. As discussed in Section 2.2, there are assays for this property that can be done *in vitro* as well as *in vivo*. The tests I did for the Josephine Sumatran orangutan line are described in this chapter, and the assays will be repeated when the current group of cells are stable enough for experimentation.

5.1 Undirected Differentiation

5.1.1 *In vitro*

In order to test the spontaneous differentiation properties of the cells *in vitro*, I modified an old protocol from the notebook of a former lab technician, named Jimi Rozencrantz, which she developed while working with Dr. Frank Jacobs. A detailed protocol can be found in Appendix B.2. The protocol involved culturing the cells suspended in a high concentration of fetal bovine serum. After two weeks of growing them in suspension, they were transferred to microscope slide cover slips coated in biological membrane, where they were allowed to flatten and spread out for easier imaging and further differentiation. They were then fixed with paraformaldehyde and I stained for six different proteins using various combinations of 8 monocolonal antibodies. The factors stained for and the germ layer they represent can be found in Table 5.1.

While RT-PCR still needs to be run in order to corroborate the results, the Josephine 3C1

Factor	AFP	Nestin	GFAP	Tuj1	GATA4	α-Actin
Layer	endoderm	meso/ectoderm	ectoderm	ectoderm	endoderm	mesoderm

TABLE 5.1: Summary of the markers stained in order to test for differentiation potential, spanning all three germ layers. Not shown: α-actin was represented by three different antibodies.

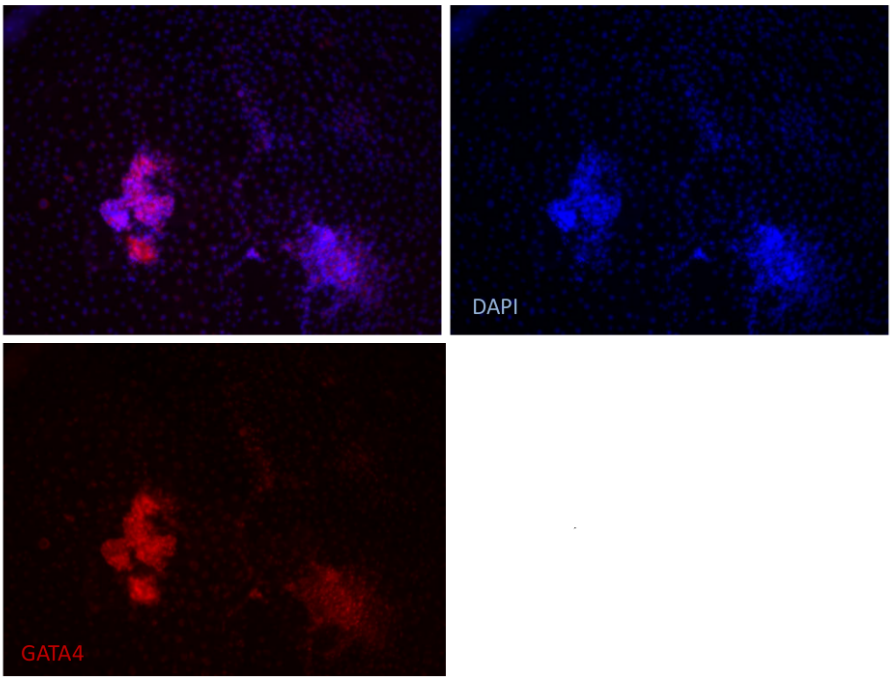

FIGURE 5.1: Fluorescence microscopy image of immunostained 3C1 iPSCs following differentiation. GATA4 is a transcription factor found in cells of the primitive endoderm. In the bottom-left corner, it appears to co-stain nuclei with DAPI, a nuclear stain shown in the upper-left corner. This suggests that this transcription factor is present and that cells have differentiated into endodermal tissues. Image was taken under microscope at 100× magnification.

cells stained positively for factors in all three germ layers (Figures 5.1, 5.2, and 5.3).

This test was performed on Josephine 2A3 in parallel, but unfortunately I ran out of several critical antibodies and was only able to confirm expression of GATA4 in those cells. This protocol, or a similar one, should be performed on any line for which we wish to publish results, including Josephine 2A3 and any new clones from the September transduction.

5.1.2 *In vivo*: Teratoma Assay

(we are still figuring out if the teratoma assay is going to happen while I am working here and how the logistics will work out on that, but a section on it will go here soon regardless)

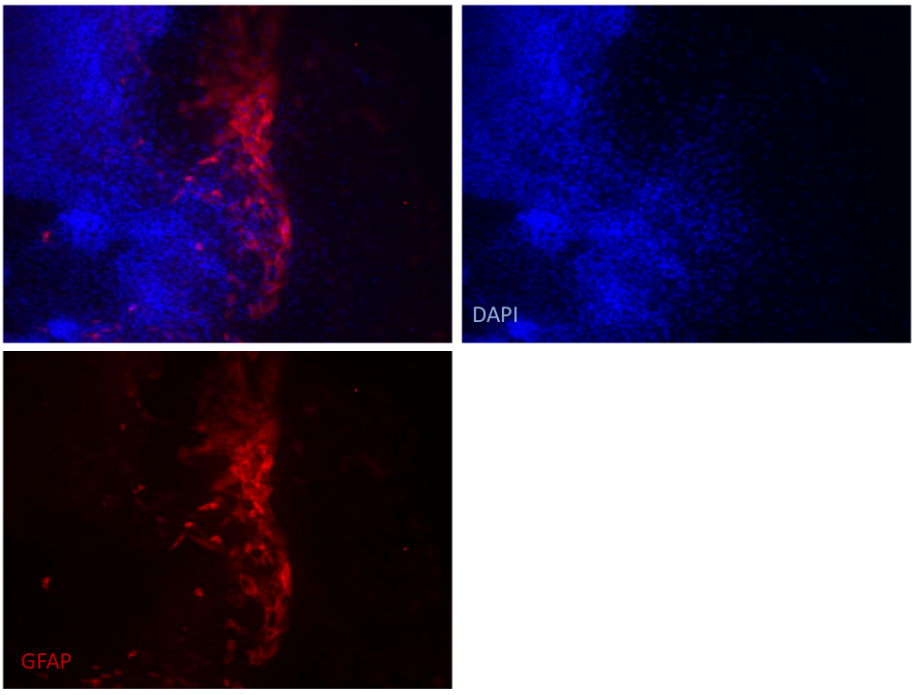

FIGURE 5.2: Fluorescence microscopy image of immunostained 3C1 iPSCs following differentiation. GFAP is a filament protein found in the central nervous system. In the lower-left, the cells appear to have nets of positive staining in their cytoplasm, appearing around the DAPI nuclear markers when merged with that image. Image was taken under microscope at 100× magnification.

5.2 Neural Differentiation Assay

Methods for differentiating human pluripotent stem cells into neurons of the cerebral cortex were previously developed in 2008 (Eiraku et al., 2008) and have since been adapted to several primates in the Haussler lab. The lab has working protocols for mouse, rhesus, and chimpanzee. I decided to try the human cell protocol in Appendix B.3 on the 2A3 and 3C1 Sumatran orangutan iPSC to achieve multiple goals at once. I wanted to verify their capacity to differentiate into other tissues, test to see if the same protocols would work between chimpanzee and orangutan, and generate RNA expression profiles of orangutan neural development. To summarize, I transferred whole colonies of iPSCs to a low-adhesion plate to encourage them to grow as embryoid bodies in suspension. I then replaced stem cell medium containing fibroblast growth factor with EB neural differentiation media containing other factors designed to encourage the growth of neuroepithelial cells (for media recipes and the full list of factors, see Appendix A). After 2.5 weeks, I switched to Neural Basal/N2 with cyclopamine for 8 days to maintain the developing neurons, then withheld cyclopamine for nine more days before harvesting the resulting EBs for RT-PCR and IF. I also harvested a few EBs every week in order to

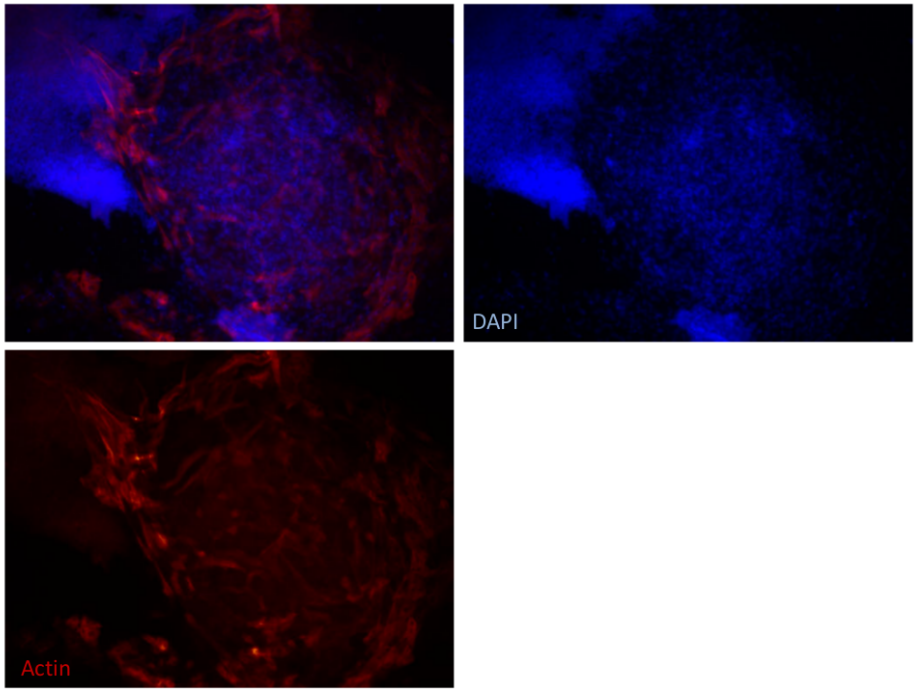

FIGURE 5.3: Fluorescence microscopy image of immunostained 3C1 iPSCs following differentiation, with colors inverted and shifted to gray scale for better visualization on paper. α-Actin is a classic marker of smooth muscle cells. It is filaments of this protein that give them strength and the ability to contract. Positive filamentous staining appears in this assay and appears in the lower-left corner, surrounding the DAPI stained nuclei in the upper-left merged image. Image was taken under microscope at 100$\times$ magnification.

perform multiple rounds of RNA-seq and develop an expression profile for the neural development process from start to finish. By the end of the experiment, long projections (reminiscent of axons) could be seen between cells on the surviving EBs, but no easily visible neural rosettes had formed. I still have yet to perform PCR and IF on the EBs in order to verify the presence of neurons. When I do, I will use primers and antibodies to target the neocortical development markers Ctip2, Sox1, and Tbr2.

Chapter 6

Future Work

6.1 Vector Clearance

All three Sendai Virus vectors remain in the PR01110-5A1 clone, and the C-Myc vector remains in Josephine-2A3 (Figure 4.6). Currently, PR01110 is undergoing heavy selection in an attempt to remove the Klf4 vector (at least) from the cells. This is because the KOS and Myc vectors contain a temperature-sensitive mutation in their basic viral machinery that will inhibit their replication in 38-39° C, but the Klf4 vector does not. Once 2A3 is thawed, we will attempt to remove the vector by culturing at these temperatures for 5 days as specified in the Cytotune technical documents (Thermo Fischer Scientific Inc., 2015b)).

The "heavy selection" that PR01110-5A1 is undergoing involves passaging individual colonies in two parts. One half of the colony is perpetuated onward in a 12-well cull-culture plate, while the other half is plated onto a 24-well plate and is stained for Sendai coat protein several days later. If any clones stain negatively, the corresponding living half of the original colony will be expanded until this result is verified by PCR. Once the virus is cleared from all lines, we will be free to perform differentiation experiments on them.

6.2 Differentiation Assays

By early Spring 2015, I plan performing an undirected differentiation assay with the PR01110 and PR00054 Cells and staining for a similar array of factors. When that is completed, I will use RT-PCR to test RNA from that experiment and the previous differentiation assay

experiments for markers in all three germ layers, such as muscle actin, GFAP, and GATA4.

In parallel I want to do a directed differentiation assay into cardiomyocytes for all lines, though I do not have a protocol planned out for doing so as of March 19, 2015. Successful differentiation will be confirmed by morphology, RT-PCR, and autonomous beating of cells in culture.

6.3 Further Characterization of New Lines

PR01110 (also known as 110-5A1) is currently undergoing karyotype analysis at Cell Line Genetics. Staining for pluripotency factors and Sendai Virus coat protein are currently underway. RT-PCR analysis will have to be re-run to confirm if the Oct4 observed in pre-transfection fibroblasts is a real signal or an artifact of human error or contamination (Figure 4.6). PR00054 still needs all aforementioned tests, but Andrea and I plan on executing them within the next two weeks. As mentioned in Section 6.2, they will undergo differentiation assay before the year is up.

6.4 Thawing Josephine Stocks

To free up more time during the September reprogramming, all of the Josephine lines were frozen down. Both 3C1 and 2A3 will need to be thawed soon in order to continue analysis, and to verify that the stocks are viable.

6.5 Teratoma Assay

The teratoma assay will be handled almost entirely by the lab technician, Kristof Tygi. I will grow a large volume of cells cells and give them to him to be injected into an immunodeficient mouse. After several months, the mouse will be sacrificed and the tumor sectioned in a cryostat, fixed with paraformaldahyde, stained for germ layer markers and examined with fluorescence microscopy.

Chapter 7

Bibliography

Hiroshi Ban, Naoki Nishishita, Noemi Fusaki, Toshiaki Tabata, Koichi Saeki, Masayuki Shikamura, Nozomi Takada, Makoto Inoue, Mamoru Hasegawa, Shin Kawamata, et al. Efficient generation of transgene-free human induced pluripotent stem cells (ipscs) by temperature-sensitive sendai virus vectors. *Proceedings of the National Academy of Sciences*, 108(34): 14234–14239, 2011.

Inbar Friedrich Ben-Nun, Susanne C Montague, Marlys L Houck, Ha T Tran, Ibon Garitaonandia, Trevor R Leonardo, Yu-Chieh Wang, Suellen J Charter, Louise C Laurent, Oliver A Ryder, et al. Induced pluripotent stem cells from highly endangered species. *Nature methods*, 8(10):829–831, 2011.

Boundless. Blastocyst formation, November . URL `https://www.boundless.com/physiology/textbooks/boundless-anatomy-and-physiology-textbook/human-development-and-pregnancy-28/first-week-of-development-261/blastocyst-formation-1287-5986/`.

Joan W Conaway. Introduction to theme chromatin, epigenetics, and transcription. *Annual review of biochemistry*, 81:61–64, 2012.

Prescott L. Deininger and Mark A. Batzer. Alu repeats and human disease. *Molecular Genetics and Metabolism*, 67(3):183 – 193, 1999. ISSN 1096-7192. doi: http://dx.doi.org/10.1006/mgme.1999.2864. URL `http://www.sciencedirect.com/science/article/pii/S1096719299928640`.

Mototsugu Eiraku, Kiichi Watanabe, Mami Matsuo-Takasaki, Masako Kawada, Shigenobu Yonemura, Michiru Matsumura, Takafumi Wataya, Ayaka Nishiyama, Keiko Muguruma, and

Yoshiki Sasai. Self-organized formation of polarized cortical tissues from {ESCs} and its active manipulation by extrinsicsignals. *Cell Stem Cell*, 3(5):519 – 532, 2008. ISSN 1934-5909. doi: http://dx.doi.org/10.1016/j.stem.2008.09.002. URL `http://www.sciencedirect.com/science/article/pii/S1934590908004554`.

A Hima Bindu. Potency of various types of stem cells and their transplantation. *Journal of Stem Cell Research & Therapy*, 2011. URL `http://omicsonline.org/2157-7633/2157-7633-1-115.php`.

Louise A. Hyslop, Lyle Armstrong, Miodrag Stojkovic, and Majlinda Lako. Human embryonic stem cells: biology and clinical implications. *Expert Reviews in Molecular Medicine*, 7: 1–21, 2005. ISSN 1462-3994. doi: 10.1017/S1462399405009804. URL `http://journals.cambridge.org/article_S1462399405009804`.

Philip M. Iannaccone, Greg U. Taborn, Ray L. Garton, Matthew D. Caplice, and David R. Brenin. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. *Developmental Biology*, 163(1):288 – 292, 1994. ISSN 0012-1606. doi: http://dx.doi.org/10.1006/dbio.1994.1146. URL `http://www.sciencedirect.com/science/article/pii/S0012160684711468`.

Frank MJ Jacobs, David Greenberg, Ngan Nguyen, Maximilian Haeussler, Adam D Ewing, Sol Katzman, Benedict Paten, Sofie R Salama, and David Haussler. An evolutionary arms race between krab zinc-finger genes znf91/93 and sva/l1 retrotransposons. *Nature*, 2014. doi: doi:10.1038/nature13760.

Junfeng Ji, Siemon H Ng, Vivek Sharma, Dante Neculai, Samer Hussein, Michelle Sam, Quang Trinh, George M Church, John D Mcpherson, Andras Nagy, et al. Elevated coding mutation rate during the reprogramming of human somatic cells into induced pluripotent stem cells. *Stem Cells*, 30(3):435–440, 2012.

W. James Kent. BLATThe BLAST-Like Alignment Tool. *Genome Research*, 12(4):656–664, April 2002. doi: 10.1101/gr.229202. URL `http://genome.cshlp.org/content/12/4/656.abstract`.

W. James Kent, Charles W. Sugnet, Terrence S. Furey, Krishna M. Roskin, Tom H. Pringle, Alan M. Zahler, and and David Haussler. The Human Genome Browser at UCSC. *Genome Research*, 12(6):996–1006, June 2002. doi: 10.1101/gr.229102. URL `http://genome.cshlp.org/content/12/6/996.abstract`.

Mary-Claire King and AC Wilson. Evolution at two levels in humans and chimpanzees. *Essential Readings in Evolutionary Biology*, 188(4184):301, 1975.

Tony Kouzarides. Chromatin modifications and their function. *Cell*, 128(4):693–705, 2007.

Jennie J Kuzdzal-Fick, Sara A Fox, Joan E Strassmann, and David C Queller. High relatedness is necessary and sufficient to maintain multicellularity in dictyostelium. *Science*, 334(6062): 1548–1551, 2011.

Louise C. Laurent, Igor Ulitsky, Ileana Slavin, Ha Tran, Andrew Schork, Robert Morey, Candace Lynch, Julie V. Harness, Sunray Lee, Maria J. Barrero, Sherman Ku, Marina Martynova, Ruslan Semechkin, Vasiliy Galat, Joel Gottesfeld, Juan Carlos Izpisua Belmonte, Chuck Murry, Hans S. Keirstead, Hyun-Sook Park, Uli Schmidt, Andrew L. Laslett, Franz-Josef Muller, Caroline M. Nievergelt, Ron Shamir, and Jeanne F. Loring. Dynamic changes in the copy number of pluripotency and cell proliferation genes in human {ESCs} and ipscs during reprogramming and time in culture. *Cell Stem Cell*, 8(1):106 – 118, 2011. ISSN 1934-5909. doi: http://dx.doi.org/10.1016/j.stem.2010.12.003. URL `http://www.sciencedirect.com/science/article/pii/S1934590910006983`.

Devin P Locke, LaDeana W Hillier, Wesley C Warren, Kim C Worley, Lynne V Nazareth, Donna M Muzny, Shiaw-Pyng Yang, Zhengyuan Wang, Asif T Chinwalla, Pat Minx, et al. Comparative and demographic analysis of orang-utan genomes. *Nature*, 469(7331):529–533, 2011.

Stephen C Noctor, Alexander C Flint, Tamily A Weissman, Ryan S Dammerman, and Arnold R Kriegstein. Neurons derived from radial glial cells establish radial units in neocortex. *Nature*, 409(6821):714–720, 2001.

Ajay Prasad, Suchitra Suresh Kumar, Christophe Dessimoz, Vincent Jaquet, Stefan Bleuler, Oliver Laule, Tomas Hruz, Wilhelm Gruissem, and Philip Zimmermann. Global regulatory architecture of human, mouse and rat tissue transcriptomes. *BMC genomics*, 14(1):716, 2013.

QIAGEN. QIAGEN OneStep RT-PCR kit, February 2015a. URL `http://www.qiagen.com/us/products/catalog/assay-technologies/end-point-pcr-and-rt-pcr-reagents/qiagen-onestep-rt-pcr-kit/#resources`.

QIAGEN. RNeasy mini kit - QIAGEN, February 2015b. URL `http://www.qiagen.com/us/products/catalog/assay-technologies/end-point-pcr-and-rt-pcr-reagents/rneasy-mini-kit`.

H. J. Rippon and A. E. Bishop. Embryonic stem cells. *Cell Proliferation*, 37(1):23–34, 2004. ISSN 1365-2184. doi: 10.1111/j.1365-2184.2004.00298.x. URL http://dx.doi.org/10.1111/j.1365-2184.2004.00298.x.

Yichen Shi, Peter Kirwan, and Frederick J Livesey. Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nature protocols*, 7(10):1836–1846, 2012.

Stegment Inc. Stemgent - TRA-1-81 antibody (DyLight 488), mouse anti-human, February 2015. URL https://www.stemgent.com/products/135#.

Kim S Suvarna, S Kim Suvarna, Christopher Layton, and John D Bancroft. *Bancroft's Theory and Practice of Histological Techniques, Expert Consult: Online and Print, 7: Bancroft's Theory and Practice of Histological Techniques*. Elsevier Health Sciences, 2012.

Kazutoshi Takahashi and Shinya Yamanaka. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4):663–676, 2006.

Kazutoshi Takahashi, Koji Tanabe, Mari Ohnuki, Megumi Narita, Tomoko Ichisaka, Kiichiro Tomoda, and Shinya Yamanaka. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131(5):861 – 872, 2007. ISSN 0092-8674. doi: http://dx.doi.org/10.1016/j.cell.2007.11.019. URL http://www.sciencedirect.com/science/article/pii/S0092867407014717.

Thermo Fischer Scientific Inc. Cell culture protocols | life technologies, February 2015a. URL http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols.html.

Thermo Fischer Scientific Inc. CytoTune-iPS 2.0 sendai reprogramming kit - life technologies, February 2015b. URL https://www.lifetechnologies.com/order/catalog/product/A16517.

Bernard E Tuch et al. Stem cells: A clinical update. *Australian family physician*, 35(9):719, 2006.

Jerilyn A Walker, Miriam K Konkel, Brygg Ullmer, Christopher P Monceaux, Oliver A Ryder, Robert Hubley, Arian FA Smit, and Mark A Batzer. Orangutan alu quiescence reveals possible source element: support for ancient backseat drivers. *Mob DNA*, 3(8), 2012. URL http://www.biomedcentral.com/content/pdf/1759-8753-3-8.pdf.

WiCell Research Institute. Stem cell protocols - WiCell, February 2015. URL `http://www.wicell.org/home/support/stem-cell-protocols/stem-cell-protocols.cmsx`.

Junying Yu, Maxim A. Vodyanik, Kim Smuga-Otto, Jessica Antosiewicz-Bourget, Jennifer L. Frane, Shulan Tian, Jeff Nie, Gudrun A. Jonsdottir, Victor Ruotti, Ron Stewart, Igor I. Slukvin, and James A. Thomson. Induced pluripotent stem cell lines derived from human somatic cells. *Science*, 318(5858):1917–1920, 2007. doi: 10.1126/science.1151526. URL `http://www.sciencemag.org/content/318/5858/1917.abstract`.

Appendix A

Media Recipes

All cell culture medium is mixed from previously made aliquots and sterile filtered prior to storage at $4°C$.

"KSR" Stem Cell Culture Medium

Note: in PSC culture, FGF is supplemented in order to suppress differentiation. The concentration is optimized for each cell line, with the most common during this project at 150 ng/mL but ranging from 100ng/mL to 300ng/mL. Colloquially, these concentrations are referred to as KSR 15, 10, and 30 respectively, because each involves adding 15, 10, or 30 μL of FGF stock (10μg/mL) to every 10 mL of media.

20% Gibco ® KnockOut®Serum Replacement

1% 100× Gibco ® GlutaMAX®

1% 100× Gibco ® MEM Non-Essential Amino Acids

1% 100× Gibco ® Penicillin / Streptomycin

0.18% 2-Mercaptoethanol

72.81% Gibco ® KnockOut® DMEM/F12 Culture Medium

Primate Fibroblast Media

10% Gibco ®Qualified Fetal Bovine Serum

1% Gibco ® 100× Penicillin / Streptomycin

89% Gibco ® MEM-Alpha®

Mouse Embryonic Fibroblast Medium

10% Gibco ®Qualified Fetal Bovine Serum

1% Gibco ® 100× Penicillin / Streptomycin

89% Gibco ® DMEM + GlutaMAX®

Undirected Differentiation Medium

20% Gibco ® Qualified Fetal Bovine Serum

1% Gibco ® 100× Penicillin / Streptomycin

79% Gibco ® DMEM + GlutaMAX®

EB Neural Differentiation Medium

20% Gibco ® KnockOut®Serum Replacement

1% 100× Gibco ® GlutaMAX®

1% 100× Gibco ® MEM Non-Essential Amino Acids

1% 100× Gibco ® Penicillin / Streptomycin

1% 100× Gibco ® Sodium Pyruvate

0.18% 2-Mercaptoethanol

71.81% DMEM/F12 Culture Medium

When aliquoted, supplement with 1 microliter of 1000x DKK-1, Noggin, SB431542 and cyclopamine per milliliter of media for standard lab stocks. Final Concentration should be 0.5 ng/mL of Noggin and DKK-1, 1μM cyclopanine and 10μM SB431542.

Neurobasal / N2 Media

1% N2 Supplement

1% Gibco ® 100× Penicillin / Streptomycin

1% Gibco ®100× GlutaMAX®

97% Gibco ® Neurobasal®Medium

Appendix B

Protocols

B.1 Reprogramming Three Fibroblast Lines With the Cytotune^{TM}-iPS 2.0 Sendai Reprogramming Kit

Adapted from the Cytotune^{TM}-iPS 2.0 Sendai Reprogramming Kit user manual (Thermo Fischer Scientific Inc., 2015b).

Day 1 Thawed 1 frozen vial each of 13692-6363 "Chelsea", PR01110, and PR00054 fibroblast stocks onto 6 cm diameter cell culture dish.

Day 2 Passage PR01110 and 13692-6363 "Chelsey" up to 10 cm diameter cell culture dish.

Day 3 Passage PR00054 up to 10 cm cell culture dish.

Day 4 Lifted all three lines with trypsin and counted the cells by taking a small aliquot to a Bio-Rad TC-20 cell counter. Passaged each to three wells of a 6 well cell culture plate; two of each for viral transduction, the third to be sacrificed for counting.

Day 5 Viral transduction

1. Warmed a separate 1 mL aliquot of fibroblast medium for each well (6 total).
2. Harvested 1 well of each for counting by lifting with TrypLE^{TM} and counting in Bio-Rad TC-20 cell counter. This yielded a concentration, and the number of cells per well was obtained with a simple volume multiplication.

3. The volume of viral solution for each well was found by the following equation: Volume of virus (μL / well) = $\frac{MOI(CIU/cell)\ (number\ of\ cells}{titer\ of\ virus\ (CIU/\mu L)}$
4. One at a time, I removed each virus tube from storage and held for 5 seconds in 37°C water bath before briefly pulsing it in a tabletop centrifuge and placing on ice.
5. Added calculated volume of virus to each aliquot of fibroblast medium and pipetted up and down to mix.
6. Aspirated existing medium from each well and replaced with viral-infused medium.
7. Placed cells in 37°C incubator for 24 hours.

Day 6, 8, 10 Replaced fibroblast medium.

Day 11 Prepared 14 × 6 well MEF feeder plates and 1 × 24 well plate for immunofluorescence.

Day 12 Diluted cells to separate out clones, so each reprogrammed cell would from its own independent colony

1. Harvested all wells with TrypLE^{TM}, obtained cell count with Bio-Rad TC-20 cell counter.
2. Set aside small aliquots of cell suspension for RNA isolation and immunofluorescence.
3. Plated cells at low density still in fibroblast medium.

Day 13 Changed to KSR medium with FGF.

Day 14 - Onward Changed medium every day and monitored for colonies. When colonies were found, passaged them to 12 well cell culture plates and expanded them from there.

B.2 *In vitro* Undirected Differentiation

August 8 In medium lacking FGF, gently lifted iPSC colonies with cell scraper, keeping them intact, and transferred to a 6 cm diameter low adhesion cell culture plate.

August 9 Replaced half of media with Undirected Differentiation Medium (Appendix A).

August 10 Replaced all media with Undirected Differentiation Medium

August 12-20 Changed media every two days, checked on embryoid bodies at that time but tried not to disturb them otherwise.

August 22 Harvested half of EB's in TrypLE for RNA isolation and analysis. The other half were plated to wells of a 24 well cell culture plate. Wells had round microscope cover slips in the bottom coated in either Matrigel or porcine gelatin; 11 wells of each material.

August 24 Replaced medium.

August 26 Bodies had flattened out somewhat by this time. I performed a standard 24-well plate antibody staining for the markers stated in Chapter 5.1. Staining protocol as follows:

1. Aspirate media, rinse once with phosphate buffered saline (PBS).
2. In fume hood, fill wells with 0.5 mL of 4% paraformaldehyde (PFA) and let sit at room temperature for 10 minutes.
3. Aspirate PFA to appropriate hazardous waste container. Replace with same volume of PBS and rock gently for 3 minutes.
4. Aspirate again to hazardous waste. Rinse twice more with PBS for 3 minutes each time, aspirate to standard liquid waste.
5. Add blocking solution (3% Bovine Serum Albumin, 0.1% Triton detergent, 96.9% PBS) to wells, incubate at 4°C for 4 hours.
6. Prepare antibody mix in blockin solution. After 4 hour period, replace blocking solution in wells with antibody mix and leave at 4°C overnight.
7. Aspirate antibody mix, rinse 3 times with PBS + Tween detergent, gently rocking for 3 minutes each time.
8. Prepare and add secondary antibody mix, incubate 1 hour room temperature or 4 hours at 4°C, shielding plate from light.
9. Rinse 3 times with PBS + Tween again
10. Remove the cover slips from the wells with tweezers and inverted them onto drops of SlowFade Gold antifade mountant with DAPI (material used to both stain the nuclei of cells and extend the lifespan of the fluorophores) resting on microscope slides. After allowing them to dry for several hours, I seal the outside of the cover slip with clear nail polish. Store at 4°C until they can be imaged.

B.3 Neural Differentiation

Day 0 Replace the media on one plate of pluripotent cells with EB Neural Differentiation Media (rinsing once with phosphate buffered saline to remove residual FGF) and lift whole colonies with a cell lifter, being very careful to keep them as intact as possible. Transfer to low adhesion plate and place in 37°C incubator at 5% carbon dioxide.

Day 1 Replace culture medium with more EB Neural Differentiation Media:

1. Gently swirl the medium such that the EBs cluster in the center of the plate.
2. With a 5 mL serological pipette, slowly aspirate most of the media so that the EBs settle on the floor of the plate, repositioning the pipette as needed to avoid aspirating any EBs.
3. With a new 5 mL serological pippette, slowly add the same amount of media as withdrawn in the previous step drop-wise, taking care not to directly strike any EBs with liquid or add turbulence to the culture.
4. If harvesting EBs for RNA, withdraw them with a 1 mL micropipette, thoroughly triturate in 1 mL Trizol and store in -80°C until ready to extract.
5. Replace plate into 37°C incubator at 5% carbon dioxide.

Days 3 - 17 Replace media with fresh EB Neural Differentiation media according to previous step every other day.

Days 19 - 25 Replace media with fresh Neural Basal/N2 Media + 1x cyclopamine every other day.

Days 27 - 35 Replace media with fresh Neural Basal/N2 Media without factors every other day.

Day 36 Harvest all EBs. Save most on Trizol for RNA. For any that will be used for IF:

1. Widen the tip of a 200 microliter pipette tip using a razor blade.
2. Use widened tip to transfer EBs to 1.5 mL microcentrifuge tube.
3. Allow EBs to settle to bottom of tube, then aspirate as much media as possible.
4. Flush once with phosphate buffered saline (PBS) to rinse, then allow the EBs to settle to the bottom. Aspirate as much as possible while leaving EBs in the tube.

5. In a fume hood, add 1 mL of 4% para-formaldehyde (in PBS) solution and incubate at room temperature for 10 minutes.
6. Rinse and aspirate three times with 1 mL PBS, placing aspirated liquid in appropriate hazardous waste container.
7. With another widened pipette tip, transfer EBs to 1 mL sucrose solution and store at 4°C overnight until they sink to the bottom.
8. The next day, place tissue freezing medium into a cryo-mold and transfer the EBs to it.
9. With an intact 200 microliter pipette tip, remove as much sucrose solution as possible (the EBs should be too big and the tissue freezing medium too viscous to aspirate).
10. Mark the location of the EBs, then store at -80°C until they can be sectioned in a cryostat.

B.4 RNA Prep and Analysis

Each RNA sample from adherent cells was obtained from a single 10 cm tissue-culture plate, as described by the first part of the protocol below.

Harvesting RNA From Adherent iPSCs on 10 cm MEF Feeder Plates

1. In a biological safety cabinet, aspirate media from the plate and replace with at least 5 mL fresh, pre-warmed media containing 2mg/mL of 200 unit/mg Gibco Collagenase Type 4.
2. Place plate in 37°C incubator for 5-15 minutes, checking under 100 × magnification every 5 minutes.
3. Once the fibroblast matrix has begun to break up, and any iPSC colonies are beginning to curl up at the edges, take plate back to the biological safety cabinet.
4. Aspirate media and rinse with an equal volume of the same fresh, pre-warmed media lacking collagenase. Gently swirl plate before aspirating media again.
5. Gently tap the plate on the cabinet's working surface 7-10 times to further break up the cellular matrix.
6. Pipette 10 mL of fresh, pre-warmed media onto the cells.

7. As quickly as possible, use a cell lifter to remove as many cells as possible from the plate surface, scraping across the entire surface in steadily decreasing circles from the outer edge in towards the center of the plate.
8. Once cells are in suspension, use a 10 mL serological pipette to asiprate the media from the plate. Place the tip of the pipette flat against the surface of the plate and pipette the media and cells back onto the plate, gently rocking the pipette in a circular motion to allow for a small opening through which the cells will travel. Repeat this trituration 5-7 times to further reduce colony size and break up MEF matrix.
9. Transfer media and cells to a 15 mL conical tube. Place in an upright position and the cells collect at the bottom of the tube for 5 minutes.
10. Aspirate as much media as possible without disturbing the cell pellet.
11. With a 1 mL micropipette, triturate cells in 1 mL Trizol and transfer to a labeled 1.5 mL microcentrifuge tube. Transfer tube to -80∘C for storage.

Isolating RNA preps with QIAGEN RNeasy cleanup kit

1. Remove tube from storage and place in fume hood. Allow to thaw at room temperature for approximately 5 minutes.
2. Add 0.2 mL Chloroform. Invert $\geq$ 10 times, then incubate at room temperature for 2 minutes.
3. Centrifuge at 12,000 $\times$ g for 15 minutes at 4°C.
4. With a 200 μL micropippette, carefully transfer clear phase to fresh 1.5 mL tube containing 0.5 mL isopropanol. Do not disturb or aspirate phenol (pink) layer. Tubes can be spun again for 2 minutes at a time if phases begin to mix.
5. Discard remaining phenol tube. Invert isopropanol tube $\geq$ 10 times and then incubate for 10 minutes at room temperature.
6. Centrifuge at 12,000 $\times$ g for 10 minutes at 4°C.
7. Pour off supernatant, then add 1 mL 75% ethanol chilled below -50∘C. Vortex or invert and flick tube several times.
8. Centrifuge at 7,500 $\times$ g for 5 minutes at 4°C.
9. Aspirate ethanol, taking care not to disturb pellet. Open tubes and allow ethanol to evaporate, 5-10 minutes.

10. Resuspend pellet in 90 μL of 50∘C nuclease-free water. Add 10 μL of RQ1 Dnase buffer or 10 $\times$ NEB2 buffer.
11. Add 10 units of SUPERase In RNase Inhibitor (by Life Technologies) and 3.5 units of RQ1 RNase-Free DNase (by Promega).
12. Mix gently, incubate 30 minutes at 37°C
13. Add 350 μL QIAGEN RLT buffer, mix well.
14. Add 250 μL 96-100% ethanol and mix well by pipetting. Do not spin down after adding ethanol.
15. Immediately transfer mix to RNeasy Mini spin column placed in a 2 mL collection tube. Close lid tightly, centrifuge 15 seconds at $\geq$ 8,000 $\times$ g.
16. Discard flow-through, taking care not to allow the column tip to contact the flow-through when returning to the same collection tube.
17. Wash with 500 μL QIAGEN RPE buffer. Close lid tightly and centrifuge for 15 seconds at $\geq$ 8,000 $\times$ g. Discard flow-through and return to same collection tube.
18. Wash again with 500 μL QIAGEN RPE buffer. Close lid tightly and centrifuge for 2 minutes at $\geq$ 8,000 $\times$ g.
19. Discard collection tube and transfer the column to a fresh collection tube. Centrifuge 1 minute at $\geq$ 8,000 $\times$ g to remove any residual RPE buffer.
20. Discard collection tube and transfer the column to a 1.5 mL microcentrifuge tube. Add 30 μL of 50∘C nuclease-free water directly to the resin in the column. Incubate for 1 minute.
21. Spin 1 minute at $\geq$ 8,000 $\times$ g to elute RNA.
22. Measure yield on nanodrop spectrometer according to manufacturer's specifications.
23. Record yield, write concentration on outside of tube.
24. Make a 10 ng/μL dilution of the stock eluted RNA, at least 10 μL in volume. This is what will be used for RNA-seq and RT-PCR experiments.