In Vitro Models of Cellular Dedifferentiation for Regenerative Medicine

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ACADEMIC ABSTRACT

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Stem cells have the ability to self-renew and to differentiate into a variety of cell types. Stem cells can be found naturally in the body, can be derived from the inner cell mass of blastocysts, or can be made by dedifferentiation of adult cells. Regenerative medicine aims to utilize the potential of stem cells to treat disease and injury. The ability to create stem cell lines from a patient’s own tissues allows for transplantation without immunosuppressive therapy as well as patient-specific disease modeling and drug testing. The objective of this study was to use cellular dedifferentiation to create in vitro cell lines with which to study regenerative medicine. First, we used siRNA targeted against myogenin to induce the dedifferentiation of murine C2C12 myotubes into myoblasts. Timelapse photography, immunofluorescence, and western blot analysis support successful dedifferentiation into myoblasts. However, the inability to separate the myotubes and myoblasts prior to siRNA treatment confounded the results. This system has the potential to be used to study mechanisms behind muscle cell regeneration and wound healing, but a better method for separating out the myoblasts needs to be developed before this will be achievable. Second, we used a doxycycline-inducible lentiviral vector encoding the transcription factors Oct4, Sox2, cMyc, and Klf4 to create a line of naïve-like porcine induced pluripotent stem cells (iPSCs). This reprogramming vector was verified first in murine cells, the system in which it was developed. Successful production of both murine and porcine iPSC lines was achieved. Both showed alkaline phosphatase activity, immunofluorescence for pluripotency marker (Oct4, Sox2, and Nanog) expression, PCR for upregulation of endogenous pluripotency factors (Oct4, Sox2, cMyc, Klf4, and Nanog), and the ability to form embryoid bodies that expressed markers of all three germ layers. Additionally, we were able to create secondary porcine iPSC lines by exposing cellular outgrowths from embryoid bodies to doxycycline to initiate more efficient production of porcine iPSCs. The secondary porcine iPSCs were similar to the primary porcine iPSCs in their morphology, behavior, alkaline phosphatase expression, and Nanog expression with immunofluorescence. The porcine iPSCs were dependent on doxycycline to maintain pluripotency, indicating that they are not fully reprogrammed. Despite this dependence on doxycycline, this system can be used in the future to study the process of reprogramming, to develop directed differentiation protocols, and to model diseases.
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Stem cells have the ability to self-renew and to differentiate into a variety of cell types. Stem cells can be found naturally in the body, can be derived from the inner cell mass of blastocysts (the stage of development just prior to implantation), or can be made by dedifferentiating, or reprogramming, adult cells into stem cells. Regenerative medicine aims to utilize the potential of stem cells to treat disease and injury. The ability to create stem cell lines from a patient’s own tissues allows for transplantation without immunosuppressive therapy as well as patient-specific disease modeling and drug testing. The objective of this study was to use cellular dedifferentiation to create cell lines in the laboratory with which to study regenerative medicine.

First, we knocked down the expression of myogenin, a key factor in muscle cell development, to induce the dedifferentiation of mouse myotubes (adult muscle cells) into myoblasts (progenitor cells). Various methods of analysis supported successful dedifferentiation into myoblasts, but the inability to completely separate myotubes and myoblasts prior to myogenin knockdown confounded the results. With better separation of the cells, this system has the potential to be used to study mechanisms behind muscle cell regeneration and wound healing.

Second, we used a viral vector encoding reprogramming factors to create both mouse and pig induced pluripotent stem cells (iPSCs) from skin cells. Pluripotent cells have the ability to differentiate into any cell type in the body, except for the placenta. Multiple pluripotency assays indicated that both the mouse and pig iPSCs were truly pluripotent. Additionally, we were able to differentiate the iPSCs into adult cells, then reprogram those back into “secondary” iPSCs. The production of secondary iPSCs is much more efficient compared to the initial creation of the primary iPSCs, which increases the usefulness of these cells for future experiments.

Unfortunately, the porcine iPSCs were dependent on the reprogramming vector to maintain pluripotency. This indicates that these cells are not fully reprogrammed. Despite this, the system can still be used in the future to study the process of reprogramming, to develop cellular differentiation protocols, and to model diseases.
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TABLE OF CONTENTS

Academic Abstract...........................................................................................................ii
General Audience Abstract..........................................................................................iii
Acknowledgements.....................................................................................................iv
Table of contents..........................................................................................................v
List of figures................................................................................................................vii
List of tables..................................................................................................................viii
Abbreviations...............................................................................................................ix

Chapter 1: Literature review........................................................................................1
  1.1 Introduction to regenerative medicine.................................................................2
  1.2 Factor-based reprogramming................................................................................4
  1.3 Characterizing pluripotency....................................................................................5
  1.4 Porcine stem cells..................................................................................................6
  1.5 The future of regenerative medicine.....................................................................7

Chapter 2: Induction of de-differentiation in C2C12 cells using siRNA targeted at myogenin......................................................................................................................9
  2.1 Introduction...........................................................................................................10
  2.2 Methods and materials........................................................................................11
    2.2.1 Cell culture....................................................................................................11
      2.2.1.1 Starting cell culture from frozen cells.......................................................11
      2.2.1.2 Subculture of adherent cells.................................................................11
      2.2.1.3 Counting cells.......................................................................................12
      2.2.1.4 Freezing cells.......................................................................................12
      2.2.1.5 C2C12 cells.........................................................................................12
    2.2.2 Myotube formation..........................................................................................12
    2.2.3 Myotube separation.........................................................................................12
      2.2.3.1 Cytosine-beta-d-arabinofuranoside / Ara-c............................................12
      2.2.3.2 Mesh filters.........................................................................................12
    2.2.4 SiRNA knockdown of myogenin.....................................................................13
    2.2.5 Western blot..................................................................................................13
      2.2.5.1 Protein extraction and quantification......................................................13
      2.2.5.2 Electrophoresis.....................................................................................13
      2.2.5.3 Electroblotting....................................................................................14
      2.2.5.4 Detection.............................................................................................14
    2.2.6 Immunofluorescence.......................................................................................14
  2.3 Results...................................................................................................................15
2.4 Discussion ........................................................................................................... 20
2.5 Conclusions ......................................................................................................... 20

Chapter 3: Generation and characterization of naïve-like porcine induced pluripotent stem cells ................................................................................................................. 22

3.1 Introduction ........................................................................................................... 23
3.2 Methods and materials ........................................................................................ 25
  3.2.1 Virus preparation .............................................................................................. 25
    3.2.1.1 Plasmid growth .......................................................................................... 25
    3.2.1.2 Approximate check for desired plasmids by PCR ........................................ 26
    3.2.1.3 Plasmid extraction ...................................................................................... 26
    3.2.1.4 Precise check of plasmids by sequencing ....................................................... 27
    3.2.1.5 Production of viral particles ....................................................................... 27
  3.2.2 Cell culture ....................................................................................................... 30
    3.2.2.1 Embryonic stem cell or induced pluripotent stem cell culture......................... 30
  3.2.3 Titration of virus ............................................................................................... 30
  3.2.4 iPSC production ............................................................................................... 32
  3.2.5 Establishment of porcine fibroblast lines .......................................................... 32
  3.2.6 Immunofluorescence ....................................................................................... 33
  3.2.7 Alkaline phosphatase test ............................................................................... 33
  3.2.8 Polymerase chain reaction ............................................................................. 34
  3.2.9 Embryoid body formation .............................................................................. 34
  3.2.10 Secondary piPSC formation ......................................................................... 35

3.3 Results .................................................................................................................... 35
  3.3.1 Establishment of lentiviral reprogramming vector ............................................. 35
    3.3.1.1 Approximate check for plasmids by PCR ...................................................... 35
    3.3.1.2 Precise check of plasmids by sequencing ...................................................... 36
    3.3.1.3 Virus titration ............................................................................................ 37
  3.3.2 iPSC production ............................................................................................... 38
    3.3.2.1 Establishment of porcine fibroblast lines ....................................................... 38
    3.3.2.2 Reprogramming ......................................................................................... 38
  3.3.3 Characterization of mouse induced pluripotent stem cells ................................. 40
  3.3.4 Characterization of porcine induced pluripotent stem cells ............................... 44
  3.3.5 Production and characterization of secondary porcine induced pluripotent stem cells ...................................................................................................................... 50

3.4 Discussion .............................................................................................................. 54
3.5 Conclusions ............................................................................................................ 56

References .................................................................................................................. 57
Appendix ....................................................................................................................... 66
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>C2C12 differentiation western blots.</td>
</tr>
<tr>
<td>2.2</td>
<td>Myotubes after transfection with fluorescein-tagged siRNA.</td>
</tr>
<tr>
<td>2.3</td>
<td>Western blot for myogenin knockdown by siRNA.</td>
</tr>
<tr>
<td>2.4</td>
<td>Time-lapse photography of myotube dedifferentiation after transfection with active siRNA.</td>
</tr>
<tr>
<td>2.5</td>
<td>C2C12 Immunofluorescence.</td>
</tr>
<tr>
<td>2.6</td>
<td>Mesh-separated myotubes.</td>
</tr>
<tr>
<td>3.1</td>
<td>Arrangement of reprogramming factors in 4F2A vector.</td>
</tr>
<tr>
<td>3.2</td>
<td>Set-up for approximate check for plasmids in bacteria by PCR.</td>
</tr>
<tr>
<td>3.3</td>
<td>Ideal concentration of 293T cells for transfection.</td>
</tr>
<tr>
<td>3.4</td>
<td>Plate design for virus titration.</td>
</tr>
<tr>
<td>3.5</td>
<td>PCR confirmation of presence of plasmids in bacteria prior to extraction.</td>
</tr>
<tr>
<td>3.6</td>
<td>Schematic of reprogramming plasmids with sequencing primers.</td>
</tr>
<tr>
<td>3.7</td>
<td>Generation of porcine fibroblast cells.</td>
</tr>
<tr>
<td>3.8</td>
<td>Reprogramming of MEFs to miPSCs.</td>
</tr>
<tr>
<td>3.9</td>
<td>Reprogramming of PEFs to piPSCs.</td>
</tr>
<tr>
<td>3.10</td>
<td>Expanded iPSCs.</td>
</tr>
<tr>
<td>3.11</td>
<td>Immunofluorescence for characterization of pluripotency of miPSCs.</td>
</tr>
<tr>
<td>3.12</td>
<td>Alkaline phosphatase activity of mouse cells.</td>
</tr>
<tr>
<td>3.13</td>
<td>PCR for characterization of pluripotency in mouse cells.</td>
</tr>
<tr>
<td>3.14</td>
<td>miPSC embryoid bodies.</td>
</tr>
<tr>
<td>3.15</td>
<td>Immunofluorescence for characterization of pluripotency of piPSCs.</td>
</tr>
<tr>
<td>3.16</td>
<td>Alkaline phosphatase activity of piPSCs.</td>
</tr>
<tr>
<td>3.17</td>
<td>piPSC embryoid bodies.</td>
</tr>
<tr>
<td>3.18</td>
<td>PCR for germ layer markers in pig cells.</td>
</tr>
<tr>
<td>3.19</td>
<td>PCR for endogenous expression of pluripotency factors in pig cells.</td>
</tr>
<tr>
<td>3.20</td>
<td>PCR for exogenous expression of pluripotency factors in pig cells.</td>
</tr>
<tr>
<td>3.21</td>
<td>Timeline for production of secondary piPSCS.</td>
</tr>
<tr>
<td>3.22</td>
<td>Production of secondary piPSCs.</td>
</tr>
<tr>
<td>3.23</td>
<td>Doxycycline removal from secondary piPSCs.</td>
</tr>
<tr>
<td>3.24</td>
<td>Alkaline phosphatase activity of secondary piPSCs.</td>
</tr>
<tr>
<td>3.25</td>
<td>Immunofluorescence for Nanog and Cd44 for secondary piPSCs.</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 3.1  Components for transfection of 293T cells.
Table 3.2  Guide for estimation of pig fetus gestational age by crown-to-rump length.
Table 3.3  Viral titration calculations.
Table 3.4  Pig fetuses used to establish porcine fibroblast lines.
ABBREVIATIONS

4F2A = doxycycline-inducible polycistronic lentiviral vector
AraC = Cytosine-β-D-Arabinofuranoside
bp = base pairs
DM = differentiation media
DMSO = dimethyl sulphoxide
EB = embryoid bodies
ESC = embryonic stem cell
FBS = fetal bovine serum
FGF = fibroblast growth factor
GFP = green fluorescent protein
GM = growth media
iPSC = induced pluripotent stem cell
IR-MEF = irradiated mouse embryonic fibroblasts
LIF = leukemia inhibitory factor
MEF = mouse embryonic fibroblast
mEB = mouse embryoid body
mESC = mouse embryonic stem cell
MHC = myosin heavy chain
miPSC = mouse induced pluripotent stem cell
MOI = multiplicity of infection
PBS = phosphate buffered saline
PCR = polymerase chain reaction
pEB = porcine embryoid body
PEF = porcine embryonic fibroblast
piPSC = porcine induced pluripotent stem cell
rtTA = reverse tetracycline transactivator
siRNA = small interfering RNA
CHAPTER 1:

LITERATURE REVIEW
1.1 Introduction to regenerative medicine

Regenerative medicine aims to utilize the potential of stem cells to treat disease and injury. Stem cells have the ability to self-renew through asymmetric cell division and to differentiate into all or many cell types of the body [1-3]. This second characteristic depends on the degree of potency of the stem cell. Totipotent cells can give rise to any cell type in the body, including extra-embryonic issues such as the placenta [4]. Pluripotent cells can give rise to any cell type of the three germ layers mesoderm, endoderm, and ectoderm, but cannot give rise to extra-embryonic tissues [2,5,6]. Multipotent cells can only give rise to cell types within their lineage [6].

There are three main types of stem cells. The first are adult stem cells. These cells are unipotent or multipotent and are extracted from various locations in the body. They have a restricted differentiation potential and are typically used by the body to replace old or damaged cells. Adult stem cells were first discovered in the 1960’s with the practice of bone marrow transplants. Since then these cells have been isolated from nearly every part of the body [6,7]. The second type of stem cells are embryonic stem cells (ESCs). These are pluripotent cells isolated from the inner cell mass of a blastocyst [5]. This was first done in the mouse by Martin Evans and Matthew Kaufman in 1983 [8]. James Thomson’s lab was the first to make human ESCs from human blastocysts in 1998 [9]. The last type of stem cells are induced pluripotent stem cells (iPSCs). These are made by de-differentiating fully specialized adult cells into unspecialized pluripotent cells. This was first done in 2006 by Kazutoshi Takahashi and Shinya Yamanaka [10] and has since been done by many groups with varied methods of reprogramming. While ESCs and iPSCs are both pluripotent, ESCs are considered the more truly pluripotent of the two. Most iPSCs have DNA methylation and RNA expression profiles that retain some similarities to their differentiated cells of origin compared to expression profiles in ESCs [11,12]. Furthermore, iPSCs commonly have a more reduced and variable potential for cell types into which they can be differentiated [13]. Based on these characteristics, it is thought that iPSCs retain an “epigenetic memory” of their cell type of origin. This can be somewhat attenuated through passaging and treatment with chromatin-modifying drugs [14], but improvement of iPSC production technologies should provide better reprogramming at the epigenetic level.

Cellular reprogramming is used to generate pluripotent stem cells by three techniques. The first, and original, technique is nuclear transfer. Nuclear transfer refers to the transplantation of a nucleus from one cell into an enucleated oocyte. Factors, which are still not completely understood, within the cytoplasm of the enucleated oocyte reprograms the transplanted nucleus to a pluripotent state [5]. This method is useful for studying the mechanisms of reprogramming and early development. However, it doesn’t yield clinically relevant numbers of stem cells, is associated with ethical concerns surrounding the destruction of an embryo, and is technically challenging [5,15,16]. Robert Briggs and Thomas King developed the technique in 1952 using frog eggs [17]. They then transplanted nuclei of embryos at multiple stages of development.
In 1962, John Gurdon was the first to successfully reprogram adult cells using nuclear transfer [20]. These publications demonstrated the important concept that genetic material is not lost or permanently silenced during development, but is only temporarily inactivated by reversible epigenetic changes. This concept was ultimately verified with the production of the first mammalian clone, Dolly the sheep, in 1997 by Wilmut et al. [21]. Subsequently clones have been made in the cow, goat, and pig [22-24]. The second method is cell fusion. Cell fusion involves fusing two or more separate cell types to form a single cell. When a pluripotent cell is fused with an adult cell, the pluripotent cell can reprogram the adult cell nuclei into a pluripotent state [5]. This was first done by Masako Tada et al. in 2001 using embryonic stem cells [25].

This technique is useful for studying the molecular mechanisms of reprogramming. For example, cell fusion was used to elucidate tumor-suppressor proteins in the late 1960s [26,27]. However, unless cells with haploid nuclei are used, then the fused cell cannot replicate and will not yield clinically useful cells [5]. The last reprogramming method is factor-based reprogramming. Factor-based reprogramming induces dedifferentiation of adult cells into pluripotent cells through the overexpression of certain transcription factors. Fibroblasts are often the adult cell of choice because they are highly available and can be easily maintained in vitro [16]. The cells must undergo several rounds of division before reprogramming occurs. This method has the greatest capacity for scale-up for therapeutic use. Additionally, this technique can be used to make autologous cells that eliminate the risk of immunorejection following transplantation [5,16,28].

Factor-based reprogramming was first done by Yamanaka and Takahashi in 2006 using the transcription factors Oct4, Sox2, Klf4, and cMyc [10]. These four factors play important roles during early embryo development [29]. Oct4 and Sox2 were later shown to be the only essential reprogramming factors [30-32]. Klf4 and cMyc can be left out or can be replaced by Lin28 and Nanog [9,33]. Nuclear transfer and cell fusion take 24-48 hours to reprogram cells, while factor-based reprogramming takes 10-30 days [28,34,35]. Cell fusion can achieve a reprogramming efficiency of up to 70%, while nuclear transfer and factor-based reprogramming are around 1% [5,28,36]. While factor-based reprogramming is currently the slowest and least efficient method of cellular reprogramming, it is still a new technology that has immense potential and is the subject of considerable research.

An alternative to the reprogramming of adult cells into pluripotent stem cells is the dedifferentiation of mature cells into proliferating precursors that can expand and re-differentiate. This can occur in certain fish, amphibians and reptiles where tissue and organ regeneration is much more extensive than in mammals [165,166]. Dedifferentiation does not naturally occur in mammals, suggesting that it was lost or suppressed during evolution [167]. Emulating dedifferentiation could serve as a regenerative therapy in mammals. Currently the generation of therapeutically useful numbers of pluripotent stem cells is very time, labor, and monetarily extensive. Inducing de-differentiation could serve as a substitute or adjunct to more traditional stem cell therapy.

1.2 Factor-based reprogramming
The theory behind why the transcription factors Oct4, Sox2, Klf4, and cMyc can initiate reprogramming is that they facilitate opening of the chromatin to allow for changes in gene expression [28,65-67]. From there factor-based reprogramming is hypothesized to occur in a step-wise fashion. First, somatic markers are down-regulated and cells undergo morphological changes similar to those that occur during the epithelial-to-mesenchymal transition of oncogenesis [2,67-70]. It is thought that cMyc in particular plays an early role to promote cellular proliferation and to switch the cell towards an energy metabolism more typical of quickly dividing cells like cancer cells and stem cells [40,41]. Second, early-stage pluripotency markers such as alkaline phosphatase and SSEA1 are activated. Finally, late-stage pluripotency markers such as Nanog, Sox2, and Oct4 are activated [2,67-70].

There are three general methods used for factor-based reprogramming. The original technique utilizes retroviruses. The first iPSCs were made using four separate constitutively active retroviral vectors that encoded the four transcription factors for reprogramming [10]. Retroviruses have an RNA genome that is converted to double-stranded DNA by reverse transcriptase. The DNA is then permanently integrated into the host genome and transcribed by the host’s transcription machinery. Transgenes can be inserted into the viral RNA genome and will thus be expressed after integration of the DNA into the host’s genome [34]. The second method uses lentiviruses. Lentiviruses are more efficient than retroviruses and can infect a greater number of cell types, including non-dividing or slowly dividing cells [34,37,38]. Both retroviral and lentiviral vectors permanently integrate into the host cell genome. The encoded transgenes should be silenced at the end of the reprogramming process, once endogenous genes expression has been up-regulated. However, if the transgenes are not silenced then maintenance of the pluripotent phenotype is dependent on expression from the exogenous factors encoded by the transgenes. The iPSCs are therefore only partially reprogrammed and have decreased differentiation potential and increased likelihood of tumor formation [39-41]. To combat this, inducible vectors were created to use instead of constitutively active vectors. With the inducible system, the exogenous transgenes are turned on only in the presence of an activator and without the activator the cells must maintain their pluripotency with endogenous factors [28,37]. The efficiency of viral-mediated reprogramming can also be increased by using a secondary reprogramming system. An inducible vector is used to make the primary iPSCs, which are then differentiated into somatic cells. These somatic cells can then be reprogrammed back into secondary iPSCs simply by adding the activator, since every somatic cell present has the integrated viral vector [37,42,43]. Retroviral and lentiviral vectors are established methods for factor-based reprogramming, making them useful research tools. However, viral-mediated transgene integration into the host genome comes with a risk of insertional mutagenesis that makes iPSCs derived from this method undesirable for clinical application. Additionally the inserted transgenes could be activated after transplantation, resulting in oncogenesis in the patient [34]. This has led to the development of the third method for factor-based reprogramming: integration-free vectors. There are three main types. The first are vectors that never integrate into the host genome. These include adenoviral vectors [44-46], Sendai virus
[34,47], minicircle DNA [48], plasmids [49,50], and episomal vectors [51-53]. The second type integrate into the genome, but can be removed later. These vectors have loxP sites [54,55] or PiggyBac transposons [56,57] around the transgenes, which allows for the exogenous transgenes to be removed once reprogramming has been achieved. The last type of integration-free vectors are a broad group of mechanisms that do not involve nucleic acids. These include purified recombinant proteins [58,59], whole-cell extracts from ESCs [60], and mRNAs [61-64]. Integration-free vectors lower the risk of oncogenecity, but currently the reprogramming efficiency is very low, usually less than 0.003% [44,45].

To date, the mechanisms and timeline of the changes behind transcription factor based reprogramming are still not completely understood. This lack of knowledge results in inefficient factor-based protocols that are reprogramming through a stochastic process in which all cells in the dish are equally susceptible, but only a random few undergo the necessary changes to become reprogrammed [66]. As a consequence, factor-based reprogramming is only 0.01-1% efficient and take approximately two weeks to produce iPSC colonies [6,28]. However, once scientists better understand the underlying mechanisms they can vastly improve the efficiency of iPSC production by developing protocols that reprogram through a more deterministic process.

1.3 Characterizing pluripotency

An unofficial set of requirements have been established for claiming pluripotency in an iPS cell line. First, up-regulation of expression of pluripotency markers such as Oct4, Sox2, Klf4, cMyc and Nanog must be shown through immunofluorescence and polymerase chain reaction (PCR) [161,162]. Second, the cells should be strongly positive for alkaline phosphatase activity, indicating an increased telomere length and telomerase activity which is a feature of pluripotent cells [71,161,162]. Third, following transfer to a non-adherent plate without leukemia inhibitory factor (LIF) the cells should be able to form embryoid bodies that contain cell types from all three of the germ layers endoderm, ectoderm, and mesoderm. Fourth, when injected into severe combined immune deficiency (SCID) mice, the cells should form teratomas. The final, and most stringent, indicator of pluripotency is germline transmission. This is achieved through injection of pluripotent cells into a developing blastocyst, resulting in contribution of the pluripotent cells in the formation of a live chimeric animal, including all three primary germ layers and the germline [161,162].

Pluripotent cells can further be characterized as naïve or primed based on their growth factor requirements to maintain pluripotency as well as their developmental properties. Naïve cells are more primitive and thus considered more truly pluripotent. They are dependent on the growth factor LIF and bone morphogenic protein (BMP) [73], express the cell surface antigen SSEA1 [74], and don’t implement X chromosome inactivation [51]. Colonies have a raised 3-dimensional morphology, show rapid proliferation, can be passaged as single cells, have greater viability following cryopreservation, and more efficiently form germline chimeras [75-77]. Mouse ESCs and iPSCs are primarily naïve [15,73,80]. Primed or epiblast stem cells are slightly
more differentiated than naïve cells. They are dependent on fibroblast growth factor (FGF) and Activin [78,79], express cell surface antigens SSEA3, SSEA4, Tra-1-60, and Tra-1-81 [74], and will perform X chromosome inactivation [15,51]. They have a flattened morphology, divide more slowly, cannot be passaged as single cells without significant cell death, are difficult to cryopreserve, are prone to spontaneous differentiation, and are difficult to form chimeras from [78-82]. Human ESCs and iPSCs are primarily primed/epiblast [15,78,80].

1.4 Porcine stem cells

As established in the Nuremberg code of 1947, animal studies must be performed before clinical trials can be conducted in humans [83]. The majority of past research with pluripotent stem cells has been done with mouse models. Mice are a commonly used research tool because they have short gestation and maturation times, are inexpensive to maintain, and there are a plethora of mutant strains available [3,84]. However, many mouse models fail to recapitulate human diseases [84] and most of the clinical trials that fail in humans had previously shown success in rodent models [85]. These failures are likely due to the smaller organ anatomy and physiology, shorter lifespans, and differences in immune cells in mice [86]. Acknowledgement of the disadvantages of using mouse models for human disease has led to more effort being directed towards developing better large animal models, particularly the pig. Apart from non-human primates, pigs have the most morphological, physiological, anatomical, and immunological similarities to humans [3,15,84,87-89]. A longer lifespan than rodents allows for conduction of long-term studies to better follow disease progression, treatment outcomes, and potential side effects [63,64,89,90]. Similar organ size and overall body size allows for the direct use of techniques and equipment from human medicine. This provides a more accurate representation of how the routes of administration, dosages, surgical techniques, side effects, and costs of treatment will translate to humans [3,84]. Pigs have a history of being used as animal models in development of therapies for human diseases [92,93] and as sources of products such as heart valves and insulin for transplants into humans [94,95]. They are especially good models for regenerative medicine therapies for spinal cord and cardiovascular injuries because researchers can consistently induce injuries in pigs that closely mimic what occurs in humans [84]. Additionally, the majority of porcine ESCs and iPSCs have a morphology and gene expression profile more similar to primed human pluripotent cells than naive mice cells [96-98]. The above reasons make pigs a good model for humans [16,87,92-98].

To date, ESCs meeting all pluripotency requirements, including production of viable chimeras, have only been established for mice [99] and rats [100,101]. Partial ESCs that only meet some of the requirements for pluripotency have been generated from the cells of many species, including humans and pigs [9,97,102-120]. Porcine ESCs are unable to form teratomas or contribute to germline transmission. In general these cells are similar to human ESCs in that they are feeder-dependent, refractory to LIF, express alkaline phosphatase, and express classic stem cell markers such as Oct4, Sox2, Nanog, SSEA4, and TRA-1-60/81 [92,119,121]. However, pig ESCs often have difficulty maintaining their pluripotency and self-renewal capabilities over prolonged
passaging [95,97,98]. It is not entirely understood why true ESCs have not been successfully made from pig blastocysts but it is likely due to species-specific morphological and developmental differences that have yet to be elucidated.

Compared to ESCs, more progress has been made with porcine iPSCs. The first piPSCs were developed by three separate groups in 2009 [122-124]. They reprogrammed porcine fibroblasts using Oct4, Sox2, Klf4, and cMyc mouse or human genes in either lentiviral or retroviral vectors [122-124]. Since 2009 many groups have successfully made piPSCs using a variety of reprogramming methods [51,98,119,122-141]. Pig iPSCs have been shown to form teratomas following injection into SCID mice as well as embryoid bodies with cell types from all three germ layers [51,123,124,126,129]. Germline transmission, however, has been unsuccessful [127,128,136]. Only one paper has reported production of chimeric offspring and even then the efficiency was only 5.9% and none of the chimeric piglets survived more than three days [128]. Morphologically, like porcine ESCs, most piPSC colonies also resemble primed human ESCs and iPSCs [119,122-124, 126-130, 132-141]. However, naïve-like piPSCs that are dependent on LIF can be produced by adding the small molecule inhibitors mitogen-activated protein kinase (MEK, PD0325901) and glycogen synthase kinase-3 beta (GSK-3, CHIR99021) to the culture media [51,98,120,125,131]. Additionally, all published papers report issues maintaining the pluripotent state with passaging. Firstly, the piPSCs have a high rate of spontaneous differentiation. Secondly, the exogenous transgenes are not being silenced and the endogenous transgenes are not being up-regulated [51,98,119,122-141]. Incomplete transition to a pluripotent epigenetic pattern during reprogramming is one of the most likely reasons behind issues with poor germline transmission, maintaining pluripotency, and failing to silence exogenous transgenes [1,15,45,80,90,98,121]. To date, piPSCS have been differentiated into neural cells [142,143], cardiomyocytes [129,144], hepatocytes [145], retinal rod cells [146], and endothelial cells [147]. However, once the issues surrounding maintenance of pluripotency have been resolved, it is likely that piPSCs will be differentiated into many more cell types and can be used as better models for human diseases.

1.5 The future of regenerative medicine

At present, the use of stem cells in the clinic is limited to procedures such as bone marrow transplants and skin grafts [3]. There are still significant issues with in vitro derived pluripotent stem cells that must be addressed before they can be used clinically. First, current production methods are too inefficient, expensive, and time consuming to be practical for large scale use [2,45,84,150]. Second, naïve stem cells are more clinically useful than primed/epiblast stem cells, but unfortunately the primed cells are the predominant type derived from all mammals except rodents [15,51,74,75,80]. The technology to make naïve stem cells from humans and large animal models is important for translation into the clinic. Third, there are safety concerns regarding the potential of pluripotent stem cells to become oncogenic [3,44,151,152]. Since these cells can differentiate into any cell type, it is possible that they may give rise to cancerous cells. This concern is further supported by evidence showing genomic instability over prolonged
passaging and following the use of integrated reprogramming vectors [45]. Using non-integrating vectors and differentiating the stem cells into a specific cell type before transplantation decreases the risk of carcinogenicity [44,151]. Lastly, there are no standard practices for the production and use of pluripotent stem cells. In addition to the variety of methodologies available for reprogramming, optimal culture conditions have not been established for non-rodent species [121]. The lack of established differentiation protocols, in particular, is a major barrier to bringing stem cells to the clinic [2,84,149,150]. This is further complicated in porcine iPSCs because the exogenous transgenes that aren’t silenced during reprogramming will subsequently interfere with differentiation [122,152]. Following transplantation for disease therapy, many of the transplanted cells are not being able to functionally integrate into the host [3,44,84,149,151]. There has also been some evidence of immune rejection, even with autologous cell transplants [141,153]. Therefore, at the present time, the high degree of variability in derived pluripotent cells makes it very difficult to use these cells in the clinic.

Once these challenges have been overcome, pluripotent stem cells have enormous potential to revolutionize medicine. Firstly, they have the ability to generate any cell type in the body, apart from the placenta [1-3,5,6,16]. This is important because most adult cells in the body have a very limited capacity for self-renewal and once they are damaged they are replaced by fibrous tissue and lost forever. This is why cardiac infarctions and spinal cord injuries have been such popular areas of interest in regards to stem cell therapies, because both of these injuries result in permanent damage and there is currently no cure [154-156]. A particularly useful feature of cellular reprogramming is that it can be used to create patient-specific cells for transplantation. Patients who receive cell, tissue, or organ transplants from imperfect matches must take immunosuppressive drugs for the remainder of their lives. These drugs can have serious side effects in addition to being costly. The use of iPSCs circumvents this because they are made from the patient’s own cells and are thus genetically identical and should not be rejected. Generation of replacement cells with iPSCs instead of ESCs also avoids the ethical concerns surrounding destruction of human embryos [6,44,149-151]. There is also potential for iPSCs to be used to treat genetic diseases. Gene targeting and homologous recombination can be used to repair the disease-causing mutations in cells taken from the patient. By making iPSCs, the cells can be manipulated without the risk of undergoing senescence. Those cells can then be grown up in vitro and injected into the patient [6,44,149,150,157,158]. Hannah et al. 2007 demonstrated this by curing sickle cell anemia in a mouse model using factor-based reprogramming and gene targeting [159]. Finally, pluripotent stem cells have immense use in disease modeling and drug testing. The study of many degenerative diseases such as Alzheimer’s and Parkinson’s is limited by the inability to grow the cell types in vitro for extended periods of time. Also, the use of a patient’s own cells allows for screening of drug candidates that will be most effective in that particular patient [6,44,149-151]. For example, drug testing in neurons differentiated from iPSCs made from amyotrophic lateral sclerosis (ALS) patients revealed a drug used to treat epilepsy as a promising candidate for ALS treatment [160]. Regenerative medicine is a quickly growing field with great potential to contribute to the advancement of veterinary and human medicine.
CHAPTER 2:

INDUCTION OF DE-DIFFERENTIATION IN C2C12 CELLS USING SIRNA TARGETED AT MYOGENIN
2.1 Introduction

Regenerative medicine aims to utilize the potential of stem cells to treat disease and injury. Stem cells have the ability to self-renew through asymmetric cell division and to differentiate into all or many cell types of the body [1-3]. Adult stem cells are unipotent or multipotent, meaning they can only give rise to cell types within their lineage [6]. They have a restricted differentiation potential and are typically used by the body to replace old or damaged cells. Adult stem cells were first discovered in the 1960’s with the practice of bone marrow transplants. Since then these cells have been isolated from nearly every part of the body [6,7]. Satellite cells are the adult stem cells of skeletal muscle. They comprise approximately 3-6% of all nuclei in a muscle fiber, but once activated they rapidly divide and subsequently differentiate into multi-nucleated muscle fibers [168].

An alternative to the recruitment of stem cell populations in the body for regeneration of tissues, is the dedifferentiation of mature cells into proliferating precursors that expand and re-differentiate. This occurs in certain fish, amphibians and reptiles where tissue and organ regeneration is much more extensive than in mammals [165]. An illustration of this is the ability of newts to regrow their tails [166]. This de-differentiation process does not occur in most mammalian tissues, suggesting that the mechanism was lost or suppressed during evolution [167]. Emulating dedifferentiation could serve as a regenerative therapy in mammals. Currently the generation of therapeutically useful numbers of stem cells is very time, labor, and monetarily extensive. Inducing de-differentiation could serve as a substitute or adjunct to more traditional stem cell therapy.

Dedifferentiation of muscle cells is a key aspect of regrowth of appendages in lower vertebrates. Fortuitously, the differentiation process of myogenesis has been well characterized. Together, this supports the use of muscle cells as a good model for studying dedifferentiation and regeneration. There are naturally-occurring muscle adult stem cells, or myoblasts, within the musculature. After injury, myoblasts are signaled to expand, differentiate, and fuse to form myotubes [171,172]. This process is regulated by the basic-helix-loop-helix transcription factors Myogenin, MyoD, Myf6, and Myf5. Myogenin and Myf6 are expressed during differentiation into myotubes, with myogenin being absolutely essential [173,174]. In fact mice born without myogenin gene expression die from complications associated with skeletal muscle deficiency [175]. Dedifferentiation of myotubes into myoblasts has been achieved through exposure to chemicals [166,176], overexpression of certain genes [177-178], and knockdown of certain genes [165].

Mastroyiannopoulos et al. 2012 have shown that siRNA-mediated knockdown of myogenin results in de-differentiation of C2C12 myotubes into myoblasts. Myogenin is a regulatory factor for terminal differentiation in myogenesis, and is thus a good target [168]. siRNA technology is used to temporarily knock down specific mRNA expression. Since the goal is to redeifferentiate the myoblasts into myotubes, knockdown of myogenin mRNA is desired over permanent gene
expression knockout. C2C12 cells are a subclone, established by Blau et al 1985 [169], of the mouse myoblast cell line originally established by Yaffe and Saxel 1977 [170]. The C2C12 subtype is able to rapidly differentiate into contractile myotubes. Our goal was to repeat and verify the work of Mastroyiannopoulos et al. 2012 then to isolate the dedifferentiated myoblasts, expand them in vitro, then redifferentiate them into myotubes in order to illustrate their true potential as a regenerative therapy.

This system can be used to study mechanisms behind muscle cell regeneration and wound healing. There is also potential for this technology to be used in the treatment of diseases such as muscular dystrophy. Muscular dystrophy is an inherited disorder of progressive muscle wasting and weakness. Severe forms of the disease can lead to cardiac or respiratory failure and death. It is usually caused by gene mutations, making it a difficult disease to treat [165,167,168].

2.2 Materials and methods

2.2.1. Cell culture:

2.2.1.1 Starting cell culture from frozen cells

In a cell culture hood, 9 ml of pre-warmed standard growth media (GM) was put in a 15 ml conical tube. GM consisted of 90% DMEM High Glucose with L-glutamine (HyClone #SH30022.01) + 10% Standard Fetal Bovine Serum (FBS; HyClone #SH30088.03) + 50 µg/ml Gentamycin Sulfate (Lonza #17-5182). The vial of frozen cells was put in a 37°C water bath until contents had just melted. Cells were diluted in the tube containing 9 ml of GM then the tubes were centrifuged at 120g for 5 minutes. During centrifugation, culture dishes were prepared in the hood by labeling the dish and adding an appropriate amount of GM. After centrifugation, the supernatant was removed and discarded. The pellet was re-suspended in growth media and added drop-wise to the culture dishes. Murine cells were cultured in the incubator at 37°C and 5% CO₂ and porcine cells were cultured at 38.5°C and 5% CO₂.

2.2.1.2 Subculture of adherent cells

Culture dishes were placed in cell culture hood. Media was removed from dishes and dishes were washed with Dulbecco’s Phosphate Buffered Saline (PBS; HyClone #SH3002802). Pre-warmed 0.25% trypsin (Corning Cellgro #25-053-C1) was added, 1 ml per 25cm² of surface area of dish. The dish was rotated to cover the entire surface with trypsin and was then placed in the incubator for five minutes or until cells detached. An equivalent volume of GM was added to the dish to inactivate the trypsin. Cell suspension was collected and placed in a conical tube. Cells counted at this stage (see “Counting cells” section below). The conical tube was centrifuged at 120g for five minutes to pellet the cells. The supernatant was removed and discarded. The cell pellet was re-suspended in appropriate volume of pre-warmed GM and was transferred to new dishes or frozen for storage (see “Freezing cells” section below).
2.2.1.3 Counting cells

Both chambers of a hemocytometer were filled with 10 µl of cell suspension and were viewed under an inverted phase contrast microscope using 20X magnification. The number of cells was then counted. In order to increase accuracy, more than 100 cells were counted, with an equal number of squares counted on each side. The smaller squares inside of the large middle square were counted. Only the cells in the middle and that overlapped on two sides of each square were counted. If the number of cells counted in both chambers differed by more than 10% then the sample was reloaded and recounted. The following equation was used to calculate the concentration of cells:

\[
\text{Equation 2: } \frac{\text{cell count}}{\text{squares counted}} \times 10^4 = \text{cells/ml x sample volume = total # cells}
\]

2.2.1.4 Freezing cells

Cells were detached from the dish using 0.25% trypsin (see “subculture” protocol) and were counted on the hemacytometer (see “counting cells” protocol). Cells were frozen at 1x10⁶ cells per ml per vial. Freezing medium consisted of a 1:9 ratio of Dimethyl Sulphoxide (DMSO; Sigma #D2650) to growth media. After centrifugation, the cells were wiped down with 70% ethanol and transferred into the hood. The supernatant was removed and the pellet was resuspended in freezing medium. Cryovials were placed at 4°C for one hour and were then transferred to a -80°C freezer overnight. The next day the vials were put in a liquid nitrogen tank for long-term storage at vapor phase.

2.2.1.5 C2C12 cells

Ordered from ATCC (CRL-1772, lot#59398548).

Maintenance media = DMEM High Glucose + 1 µl/ml gentamycin + 10% fetal bovine serum.

2.2.2. Myotube formation:

Once C2C12 cells are at >80% confluency, media was switched to differentiation media (DMEM High Glucose + 1 µl/ml gentamycin + 2% horse serum).

2.2.3. Myotube separation

2.2.3.1 Cytosine-Beta-D-Arabinofuranoside / Ara-C

The day following transition to differentiation media, 4µg Ara-C per 1 ml culture media was added to halt proliferation of myoblasts. Concentration of Ara-C later increased to 8 ug in attempts to halt division of myocytes

2.2.3.2. Mesh filters
Differentiation media (DM) was removed and the cells were washed with PBS. PBS removed and replaced with 0.05% Trypsin (1 ml per 25 cm$^2$ of surface area of dish). Cells allowed to incubate at 37 °C with trypsin until cell adhesions disrupted. Equal amount of DM was added to trypsin and all contents transferred to a 15 ml conical tube. Cells allowed to settle to bottom of tube by gravity for 10 minutes. Supernatant removed and cells resuspended with fresh DM. Suspension passed sequentially through a 100 µm (Fisher Scientific #22363549) then 40 µm mesh filters (Fisher Scientific #22363547). Myotubes washed off filters into culture dishes using DM.

### 2.2.4. siRNA knockdown of myogenin:

Resuspended lyophilized siRNA duplex (Invitrogen) in 330 µl of RNAase-free water to create a 9 pmol/µl solution. 9 µl, or 81 pmol, RNAi duplex was diluted in 250 µl DMEM in a 15-mL conical tube for each 35-mm culture dish to be used for transfection. 5 µl of Lipofectamine was diluted in 250 µl DMEM in a 15-mL conical tube for each 35-mm culture dish to be used for transfection. RNAi duplex and Lipofectamine were gently combined and incubated for 20 min at room temperature. Media was aspirated from culture dishes to be transfected, and replaced with 2.5 ml plain DMEM. 500 µl of RNAi-Lipofectamine mix was added per dish. Cells were incubated at 37°C at 5% CO$_2$ for 8 hours, then the media was aspirated and replaced with differentiation media.

### 2.2.5. Western blot:

#### 2.2.5.1 Protein extraction and quantification

Placed PBS and lysis buffer (440mg NaCl + 61 mg Tris + 2.5 ml of 10% stock of Triton-X-100 + 250 mg Deoxycholate + deionized water to achieve 50 ml total volume) on ice for at least 15 minutes. Trypsinize cells (see 2.2.2 Subculture of adherent cells). Removed supernatant with 5ml cold DPBS. Centrifuged 5 minutes at 120 g to wash cells. Combined 1 ml cold lysis buffer with 5 ul Protease Inhibitor (174 mg PMSF + 10 ml 100% ethanol). Removed supernatant and resuspended pellet in equivalent volume of lysis buffer + protease inhibitor. Incubated for 30 minutes on ice, pipetting to mix contents every 10 minutes. Stored at -70°C.

The amount of protein was quantified using a BCA Protein Assay Kit (ThermoFisher Scientific #23225).

#### 2.2.5.2 Electrophoresis

Diluted samples 1:1 with Laemmlı Buffer in 1.5 ml microfuge tubes and boiled them for 5 minutes. 10-30 µg/ml of each sample as well as 2 ul of a ladder were loaded into a 4-20% gel (Bio-RAD #456-1094) and ran at 125 Volts in a Bio-Rad Mini-Protean 3 electrophoresis module (Bio-Rad #179-3930) using 1x working solution (100 ml 10x stock Running Buffer + 900 ml)
deionized water) from a 10x stock of Running Buffer (30.3 g Trizma base + 144 g Glycine + 10 g SDS in 1 L deionized water).

2.2.5.3. Electroblotting

Once electrophoresis is complete, gel was transferred to 50 ml of 1x working solution (100 ml 10x stock Blotting Buffer + 800 ml deionized water + 100 ml methanol) of 10x stock of Blotting Buffer (30.3 g Trizma base + 144 g Glycine in 1 L of deionized water), which was placed on a rotamixer for 30 minutes to wash the gel. PVDF membrane (Immobilon-F, Millipore) was presoaked in methanol for 1 minute then in 1x Blotting Buffer for 2-30 min. Mini Trans-Blot Cell (Bio-RAD #170-3935) placed in ice bath on top of a stir plate. Transfer “sandwich” assembled, in the following order: (1) filter paper (Bio-rad #1703932) (2) PVDF membrane (3) gel (4) filter paper (5) sponge. Transferred “sandwich” inserted into Mini Trans-Blot Cell, which was filled with 1x Blotting Buffer and run at 100 volts for 60 minutes, making sure not to exceed 350 mA.

2.2.5.4. Detection

Placed membrane in 25 ml 0.5x Odyssey Blocking Buffer (Li-Cor #927-40000) for 1 hour at room temperature or overnight at 4°C on rotamixer. Diluted primary antibody in 10 ml of 1x Blocking Buffer + 100 µl 10% Tween-20 stock (5 ml Tween-20 + 45 ml deionized water). Incubated membrane in primary antibody for 1 hour at room temperature on a rotamixer. Washed membrane 4 times for 5 minutes per wash at room temperature in 50 ml PBST (5 ml of 10x PBS stock + 45 ml deionized water + 250 µl of 10% Tween-20 solution) on a rotamixer. 10x PBS stock = 85 g NaCl + 9.94 g Na₂HPO₄ + 4.14 g NaH₂PO₄·H₂O, pH adjusted to 7.4. Diluted secondary antibody in 10 ml of 1x Blocking Buffer + 100 µl 10% Tween-20 stock. Incubated membrane in second antibody for 30-60 min at room temperature on a rotamixer, covered to keep from light. Washed membrane 4 times for 5 minutes per wash at room temperature in 50 ml PBST on a rotamixer. Rinsed membrane once with 50 ml of 1x PBS (100 ml 10x PBS stock + 900 ml deionized water) for 5 min. Odyssey Infrared Imaging system used to read membrane.

Primary antibodies used were Myogenin (Santa Cruz #576, 1:200 dilution), Myosin Heavy Chain (Santa Cruz #20641, 1:200 dilution), Rb (Santa Cruz #102, 1:200 dilution), and beta-Actin (Santa Cruz #10731, 1:400 dilution).

Secondary antibodies used were Goat anti-Rabbit (Odyssey #926-32221, 1:5000 dilution) and Goat anti-Mouse (Odyssey #926-32210, 1:5000 dilution).

2.2.6. Immunofluorescence:

Media on the cell culture was removed and the cells were washed one time with ice-cold PBS (HyClone #SH3002802). The cells were then fixed with 4% paraformaldehyde (Wako #163-20145) for 20 minutes. The paraformaldehyde was aspirated off and properly disposed of. The
cells were washed two times, for five minutes each, with PBS. 0.2% Triton-X-100 (ICN Biomedicals #807423) was put on the cells for 20 minutes to permeabilize the cellular membranes. Western Blocking Buffer (Odyssey #927-40000) was put on the cells for one hour. The primary antibody was added to the blocking buffer and was incubated overnight at 4°C on a rotamixer. All primary antibodies were diluted to achieve a final concentration of 0.01 ug primary antibody per 100 ul of diluted blocking buffer. The next day the cells were washed three times, for 10 minutes each, with PBS. The secondary antibody was added to Western Blocking Buffer diluted 1:1 with PBS and was incubated with the cells for one hour at room temperature. All secondary antibodies were diluted to achieve a final concentration of 0.5 ug secondary antibody per 100 ul of diluted blocking buffer. Cells were kept covered during this incubation because fluorescent secondary antibodies are light sensitive. After an hour the cells were washed three times, for 10 minutes each, with PBS. DAPI (Vectashield Mounting Medium with Dapi #H-1200) was added to visualize the nuclei and the cells were viewed under an inverted phase contrast microscope with fluorescence.

Primary antibodies used were Myogenin (Santa Cruz #576, 1:200 dilution), Pax7 (Santa Cruz #7748, 1:100 dilution), Ki67 (Santa Cruz #7846, 1:100 dilution).

Secondary antibodies used were Donkey anti-Goat IgG (ThermoFisher #A11058, 1:400 dilution) and Goat anti-Rabbit IgG (ThermoFisher #A11008, 1:400 dilution).

2.3 Results

Using the differentiation protocols described in the methods section, C2C12 cells were successfully differentiated into myotubes. The myotubes were elongated, spindle-shaped, multinucleated cells that would spontaneously contract. Formation took approximately 4-6 days.

Western blots were performed to assess the levels of myogenin and myosin heavy chain (MHC) before and after formation of myotubes from C2C12 myoblasts. Both of these are highly expressed in myotubes and are poorly expressed in myoblasts, and therefore the levels of both were expected to rise as the myoblasts differentiated into myotubes. MHC expression increased approximately 20-fold and myogenin expression increased approximately 4.5-fold.
To verify that the siRNA was able to penetrate into the C2C12 cells, the same amount of a fluorescein-tagged siRNA was transfected into a batch of C2C12 cells. Fluorescein can be clearly visualized within the cells, indicating successful uptake of the siRNA.

**Figure 2.1. C2C12 differentiation western blots.** Done to compare (a) Myosin Heavy Chain (MHC) and (b) Myogenin levels in day 1 undifferentiated myoblasts vs. day 7 differentiated myotubes. One replicate was performed. Histogram quantitatively shows MHC and Myogenin expression changes. Results concur with literature.
A western blot was performed to quantitatively assess siRNA-mediated knockdown of myogenin. Compared to the fluorescent siRNA control and the no siRNA control there was a 60% and 58%, respectively, knockdown of myogenin.

**Figure 2.2. Myotubes after transfection with fluorescein-tagged siRNA.** Done to qualitatively assess cellular uptake of siRNA.

**Figure 2.3. Western blot to for myogenin knockdown by siRNA.** Active siRNA is siRNA that is directed against myogenin mRNA and is expected to decrease translation into functional proteins. Fluorescein siRNA is a control to assess how well the siRNA is getting into cells and if there are negative consequences on cell health. It should have no impact on myogenin expression. Cells in “DM” group were kept in differentiation media and allowed to continue myotube formation. Histogram quantitatively shows myogenin expression changes.
Images were taken for the first three days following treatment of myotubes with siRNA against myogenin. After day three the cells were too confluent and would require passaging. Myoblasts were arrested before siRNA transfection using Cytosine-β-D-Arabinofuranoside (AraC). From day zero to day three, there were morphological changes consistent with the dedifferentiation of myotubes to myoblasts. The number of distinct elongated, multinucleated myotubes decreased and the number of smaller, round, mononuclear myoblast-like cells increased. Additionally, many of the myoblast-like cells were aggregated together in rows with a similar shape of myotubes.

Immunofluorescence was performed to assess the expression of markers for myotubes (Myogenin) and myoblasts (Pax7, Ki67) in C2C12 cells three days after treatment with myogenin-targeting siRNA. Positive expression of myogenin in figure 2.5a shows that there are definitely myotubes still present. However, there are many dapi-positive nuclei that do not express myogenin, suggesting that they are not myotubes. Pax7 expression was very low, but there was a significant amount of Ki67 expression, which further supporting the hypothesized presence of myoblasts.

Figure 2.4. Time-lapse photography of myotube dedifferentiation after transfection with active siRNA. Images taken at same time each day, but not in same area of dish.
AraC treatment did not eliminate all of the myoblasts. In an attempt to better separate the myoblasts from the myotubes, the cells were trypsinized and passed through a 100 µm mesh filter followed by a 40 µm mesh filter and then washed off the 40 µm filter into a culture dish. The 100 µm filter was used to filter out larger pieces of debris and allowed both myotubes and myoblasts to pass through. The pores in the 40 µm filter were small enough for myoblasts to pass through, but not myotubes. The majority of the cells that were washed off the 40 µm filter were larger, multinucleated cells consistent with myotubes. However, there was significant loss of cells during the filtration process and the myotubes that made it back into culture had altered morphology and behavior. These cells did not have the typical elongated appearance of a myotube and did not spontaneously contract as they did prior to trypsinization and mesh separation.

Figure 2.5. C2C12 immunofluorescence. (a) Myogenin. This is a marker of myotubes. (b) Pax7. This is a marker of myoblasts. (c) Ki67. This is another marker of myoblasts.

Figure 2.6. Mesh-separated myotubes. Image was taken 24 hours after separation and re-plating.
2.4 Discussion

C2C12 cells were successfully differentiated into myotubes. This has been well documented with this cell line, but was a necessary step in our experiment before dedifferentiation could be attempted. The presence of fluorescein within the myotubes in conjunction with a 60% knockdown of myogenin on western blot indicated that the siRNA was getting into cells and having the desired effect. siRNA typically does not achieve 100% knockdown. While closer to 80% knockdown was the goal, 60% was adequate to proceed with the experiment.

The cellular changes following siRNA administration were consistent with changes expected from dedifferentiation of myotubes into myoblasts. There was a decrease in the number of spindle-shaped multinucleate myotubes with a concurrent increase in the number of round mononucleate myoblast-like cells, indicating that the myotubes were turning into myoblasts. Additionally, some of the myoblast-like cells were arranged in rows that approximated the shape of myotubes, which was suggestive that they had formed from myotubes. Immunofluorescence provided additional support for dedifferentiation. While expression of myogenin indicated that there were myotubes still present, only the larger multinucleate spindle-shaped cells expressed it. The smaller, round, mononuclear cells were myogenin negative. Additionally, there was fairly widespread expression of Ki67, a marker of myoblasts.

While it is apparent that the number of myoblasts increased and the number of myotubes decreased after the introduction of myogenin-targeting siRNA, it is unclear whether that was due to dedifferentiation of the myotubes or contamination of myoblasts that survived AraC treatment. After the differentiation protocol to form the myotubes and the AraC treatment to kill the more actively dividing myoblasts, there were still myoblasts present. The amount of AraC was increased, but it was not possible to eliminate all of the myoblasts and the amount of cell death rose with every increase. We also tried filtering out the myoblasts by passing the differentiated C2C12 cells through a series of mesh filters. Again, this did not fully eliminate the myoblasts and it was also harmful to the myotubes. Many were lost during the filtration process and the few that re-plated had an altered morphology. While it does appear that dedifferentiation is occurring, repopulation by myoblasts that survived AraC treatment cannot be ruled out.

2.5 Conclusions

The work performed here shows that myotubes can be generated from C2C12 myoblasts, that myogenin can be knocked down in C2C12 myotubes with siRNA, and that myogenin knockdown appears to induce dedifferentiation of C2C12 myotubes into myoblasts. Unfortunately, since it was not possible to eliminate all of the myoblasts during myotube formation, we were unable to definitively differentiate dedifferentiation of myotubes from proliferation of myoblasts that escaped AraC treatment. Therefore, a better method for separating myoblasts from myotubes is needed in order to continue to use this system as a model to study regenerative medicine. Once this is achieved, though, this system could be used to study and
potentially develop therapeutics for diseases such as muscular dystrophy. The idea of using siRNA to knock down lineage-determining genes could also be expanded to other cells types. This could be especially useful for tissues, such as cardiac and neural tissues, that are commonly injured but do not have a lot of innate regenerative capacity.
CHAPTER 3:

GENERATION OF NAÏVE-LIKE PORCINE INDUCED PLURIPOTENT STEM CELLS
3.1 Introduction

Induced pluripotent stem cells (iPSCs) are cells that have the capacity to both self-renew and to differentiate into any cell type of the body, aside from extra-embryonic tissues [2,5,6]. iPSCs can be made by nuclear transfer, cell fusion, or factor-based reprogramming [5]. Factor-based reprogramming induces de-differentiation of somatic cells to a pluripotent state through the overexpression of certain transcription factors, most commonly Oct4, Sox2, Klf4, and cMyc [10,16,28]. This technique avoids the destruction of embryos, has the greatest potential for scale-up production, and can be used to make autologous cells that eliminate the risk of immunorejection following transplantation [5,16,28]. The majority of factor-based reprogramming has been done in the mouse. However, pigs have the most morphological, physiological, anatomical, immunological, and lifespan similarities to humans, making them better biomedical models than mice [3,15,84,87-89]. The first piPSCs were made in 2009 [122-124], but all derived cell lines to date have issues with high rates of spontaneous differentiation, exogenous transgenes that are not silenced, largely unsuccessful germline transmission, and a propensity for primed over naïve cells [51,98,119,122-141]. While factor-based reprogramming is currently a stochastic process that is only 0.01-1% efficient and takes approximately two weeks to produce iPSC colonies [6,28], it is a new technology that has immense potential and is the subject of considerable research.

Characterization of pluripotent stem cells is usually done through immunofluorescence, PCR, alkaline phosphatase activity, embryoid body formation, teratoma formation, and chimera formation. Immunofluorescence and PCR are used to confirm expression of pluripotency-associated genes such as Oct4, Sox2, Klf4, cMyc, and Nanog [161,162]. Alkaline phosphatase is an indicator of increased telomerase activity and cell immortality [71,161,162]. Embryoid bodies are formed following transfer to a non-adherent plate without LIF, where they differentiate into cell types of all three germ layers. PCR can then be used to confirm this differentiation. Teratomas form in SCID mice following injection. Chimeric animals demonstrate the ability of the iPSCs to contribute to germline transmission. It is the most stringent and most difficult test of pluripotency [161,162]. In this study we characterized pluripotency through immunofluorescence, PCR, alkaline phosphatase activity, and embryoid body formation.

Stem cells can further be characterized as naïve or primed based on their growth factor requirements to maintain pluripotency as well as their developmental properties. Naïve cells are more primitive and thus considered more truly pluripotent [73-77]. Primed cells divide more slowly, cannot be passaged as single cells without significant cell death, are difficult to cryopreserve, are prone to spontaneous differentiation, and are difficult to form chimeras from [78-82]. Naïve cells are dependent on the growth factor leukemia inhibitory factor (LIF) [73], express the cell surface antigen SSEA1 [74], have a raised morphology, can be passaged as single cells, and more efficiently form germline chimeras [75-77]. Mouse ESCs and iPSCs are primarily naïve [15,73,80]. Primed or epiblast stem cells are slightly more differentiated than naïve cells. They are dependent on fibroblast growth factor (FGF) [78,79], express cell surface
antigens SSEA3, SSEA4, Tra-1-60, and Tra-1-81 [74], have a flattened morphology, cannot be passed as single cells without significant cell death, and are difficult to form chimeras from [78-82]. Human ESCs and iPSCs are primarily primed/epiblast [15,78,80]. In addition, the majority of porcine iPSCs have more closed resembled primed human iPSCs [119,122-124, 126-130, 132-141]. To evaluate whether the cells derived in our lab were naïve or primed, we looked at morphology, ability to passage as single cells, reliance on LIF vs. FGF, and immunofluorescence for cell surface markers SSEA1, Tra-1-60, and Tra-1-81.

In this study, we used a doxycycline-inducible polycistronic lentiviral vector (4F2A) encoding mouse pluripotency transcription factors Oct4, Sox2, Klf4, and cMyc to reprogram both mouse and porcine fibroblasts into iPSCs. The four factors are separated on the vector by 2A sequences, which allow ribosome skipping during translation and result in four separate peptides from a single transcript (Figure 3.1). A companion vector encoding a constitutively expressed reverse tetracycline transactivator (rtTA) must be co-transfected with the 4F2A vector. This is a TetOn set-up, in which the protein produced from the rtTA vector only binds to the TetO region of the 4F2A transcript in the presence of doxycycline [34,37]. The inducible system allows for identification of iPSCs that are not dependent on the exogenous transgenes to maintain their pluripotency. These vectors have been shown to effectively reprogram mouse and human fibroblasts into both primary and secondary induced pluripotent stem cells by Carey et al. 2009 [37]. However, this system has not yet been shown to reprogram cells of other species. The pig was our species of interest, but reprogramming was also done in the mouse as a control.

**Figure 3.1: Arrangement of reprogramming factors in 4F2A vector.** The four transcription factors Oct4, Sox2, Klf4, and cMyc serve as the reprogramming factors that facilitate the transition of somatic adult cells into induced pluripotent stem cells. The 2A sequences are derived from agents in the foot-and-mouth disease family. During translation, the ribosome skips over the 2A sequence. This allows for production of four separate peptides from a single vector [34,37]. The use of a single vector instead of four separate vectors, one for each reprogramming factor, greatly increases the efficiency of reprogramming. This image was taken from Carey et al. 2009 [37].

This work is important because derivation of truly pluripotent pig iPSCs has not been achieved yet. The pig has potential to be an important animal model for stem cells therapy, but current technology limits its usefulness. However, even if truly pluripotent piPSCs are not achieved with
this system, this study will still result in a piPSC line made using a reprogramming vector previously only shown to work in mice and human cells. Additionally, it should allow for production of secondary iPSCs. These are made by differentiating iPSCs into somatic cells through embryoid body formation, then adding doxycycline to reprogram them again into iPSCs. Since the cells already contain the integrated lentiviral vector, they only require addition of doxycycline to reprogram. This results in a much faster and efficient system, usually decreasing the time for reprogramming to full colonies in half while also initiating reprogramming in nearly all of the cells, in comparison to the <1% efficiency that you achieve on average with traditional factor-based reprogramming [5,28,36, 37,42,43]. The secondary iPSC system is not commonly used with pig cells and could increase the usefulness of this cell type.

3.2 Methods and materials

3.2.1 Virus preparation

3.2.1.1 Plasmid growth

The rtTA (Plasmid 20342: FUW-M2rtTA) and 4F2A (Plasmid 20321: TetO-FUW-OSKM) plasmids were ordered from Addgene and arrived packaged in Stab13 bacteria in stab culture format. Bacterial growth media was made by combining 4 ml of Luria-Bertani (LB) broth with 0.1 mg/ml Ampicillin in a 14 ml polypropylene round bottom tube (BD Falcon #352059) for each sample to be grown up. The LB broth was made by adding 2.5g LB powder (Amresco #J106-1KG) to 100ml deionized (DI) water. Sample set-up was as follows:

- Tube 1: rtTA
- Tube 2: 4F2A
- Tube 3: control (environmental bacteria)

A sample of the rtTA bacteria was taken from the LB-agarose gel stab culture and transferred to its designated tube. This was repeated for the other two samples. All three tubes were placed in a New Brunswick Scientific I2400 Incubator Shaker set at 37°C for 5-6 hours.

After 5-6 hours the expanded bacteria was transferred to LB-agarose plates. Prior to transferring bacteria, the plates were put in a ThermoScientific Forma Series II Water Jacket CO2 incubator set at 37°C to warm up for 30 minutes. Ten µl of rtTA bacteria was transferred to a plate and streaked in progressive dilutions with a flame-sterilized loop. Plate streaking was repeated for 4F2A. For a control, 100 µl of control LB+bacteria was diluted in 900 µl LB, and then 10 µl of that was plated and streaked. All three plates were placed in the Incubator Shaker at 37°C until decent sized, non-touching colonies appeared. From each plate, three isolated colonies were picked and put into separate 14 ml polypropylene round bottom tubes containing 4 ml LB broth + 0.1 mg/ml Ampicillin. Tubes were placed in the Incubator Shaker at 37°C for 5-6 hours, then stored at -4°C. Placement in -4°C is suitable for short-term storage of up to a week.
3.2.1.2 Approximate check for desired plasmids by PCR

Polymerase chain reaction (PCR) was used to check for the presence of the desired plasmids by using a forward and reverse primer to amplify a fragment of the plasmid. The primers were designed to be complimentary to approximately 20 base pairs (bp) in the plasmid DNA sequence, and should have therefore only resulted in a PCR product if the correct plasmid was present. For rtTA, primers KW08 and MC10 were used, which resulted in a fragment of 1,408 bp (Figure 3.2a). For 4F2A, primers KW14 and MC13 were used, which resulted in a fragment of 2,200 bp (Figure 3.2b). For the controls, the plasmid was left out of the reaction. The protocol for PCR is detailed in a section below. For the electrophoresis, a 0.7% gel was chosen. A combination of Lambda DNA – Hind III digest ladder (New England Biolabs #N30125) and φX174 DNA – Hae III digest ladder (New England Biolabs #N30265) were chosen since the DNA fragments from rtTA and 4F2A were not within the range of just one of these ladders.

For the setup, the plasmids are present in the bacteria, PCR should amplify the fragment between the two primers. This does not provide information on the integrity of the plasmid, but is used to confirm the presence of the plasmid prior to extraction. (a) Amplification of the segment of the rtTA plasmid between primers MC10 and KW08 should produce a 1408 base pair product. (b) Amplification of the segment of the 4F2A plasmid between primers KW14 and MC13 should produce a 2200 base pair fragment. (c) Primer sequences.

3.2.1.3 Plasmid extraction

From the samples stored at 4°C, 10 µl of bacteria from rtTA and 4F2A were transferred into sterile 500 ml glass flasks containing 100 ml LB broth + 50 µg/ml Ampicillin and were put in 37°C Incubator Shaker overnight. After 36 hours, the plasmids were extracted using a 5Prime PerfectPrep Endofree Maxi Kit (#2300120). The amount of DNA was measured using a nanodrop spectrophotometer. Bacteria samples for all three replicates of rtTA and 4F2A were frozen at -80°C for long-term storage by combining 500 µl of bacteria with 500 µl of 50% glycerol in ThermoScientific Nalgene System 100 cryovials (#5000-1020).
3.2.1.4 Precise check of plasmids by sequencing

Sequencing primers were designed with the following specifications: approximately 20 bp in length, distance between primers approximately 500 bp, no self-dimerization or hair pin formation by more than 3 bp, 50-60% GC content, and a melting temperature between 55°C and 65°C. The website [http://www.basic.northwestern.edu/biotools/oligocalc.html](http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to design the primers. Primers were ordered from Eurofins MWG Operon.

When primers arrived they were resuspended in the amount of DNase, RNase-free H₂O indicated on the data sheet to achieve a stock solution of 100 µM/µl. Working solutions of 10µM/µl were made by diluting the stock solution 1:9 with DNase, RNase-free H₂O.

Samples were submitted to the Virginia Bioinformatics Institute (VBI) at Virginia Tech for sequencing. The SeqMan Pro program was used to compare VBI sequencing results with the sequence provided by AddGene.

3.2.1.5 Production of viral particles

Protocol was adapted from Hotta et al. 2009 [163].

Viral packaging plasmids (Tat, Rev, PSPAX2, and PMD2.G) were grown up and extracted. Viral packing plasmids were a gift from Dr. Colin Bishop. Frozen plasmid-containing bacterial stocks were thawed and 10 µl each of each of the packaging plasmids were put in separate flasks containing 100 ml LB broth + 0.1 mg/ml Ampicillin. The flasks were cultured in the Incubator Shaker at 37°C overnight and the bacterial stocks were put back in -80°C storage. The next day the plasmids were extracted using the 5Prime PerfectPrep Endofree Maxi Kit (#2300120).

293T cells were used as hosts for viral particle production. In order to ensure these cells were growing properly, they were put in culture at least one week before transfection (see “Starting cell culture” protocol below). The cells were split every 2-3 days with a split ratio of 1:4 (see “Subculturing” protocol below). Cells were never allowed to reach confluency, since that would have reduced their transfection efficiency.

The day before transfection, 7.5x10⁶ 293T cells were seeded into a 10-cm dish with 10 ml of growth media (GM) so that the cell concentration would look like that in Figure 3.3 for transfection the following day. The next day the viral plasmids were transfected into the 293T cells. For each dish to be used for vector production, two 15-ml conical tubes containing 1.5 ml of Opti-MEM I media (Gibco #31985-070) each were prepared. Sixty µl of Lipofectamine 2000 (Invitrogen #11668-019) was added to one of the tubes containing 1.5 ml of Opti-MEM I, and was incubated for five minutes at room temperature. Plasmids were added to the other tube containing Opti-MEM I (Table 3.1). Glass pipettes were not used because they can absorb plasmid DNA. After the five minute incubation, the Opti-MEM I + Lipofectamine 2000 solution was added to the tube containing Opti-MEM I and plasmids. This combination was mixed gently
and allowed to incubate for 20 minutes at room temperature. During incubation, the media on the 293T cells was removed and replaced with 10 ml of pre-warmed GM without gentamycin. After incubation the plasmids + Lipofectamine was added dropwise to each dish. The dishes were gently rocked back and forth to mix and were then put into an incubator set at 37°C and 5% CO² for 8-16 hours. From this point forward the 293T cells started producing live lentivirus and Biosafety Contaminant Level II guidelines with enhanced Level II handling procedures were strictly followed.

![Image](image.png)

**Figure 3.3: Ideal concentration of 293T cells for transfection.** Production of viral particles is optimized when viral packaging plasmids are introduced to 293T cells at this concentration. This image was taken from Hotta et al. 2009 [191].

<table>
<thead>
<tr>
<th>Component</th>
<th>Tube 1 – Plasmid vectors</th>
<th>Tube 2 – Lipofectamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opti-MEM I</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Lipofectamine 2000</td>
<td></td>
<td>60 µl</td>
</tr>
<tr>
<td>Transfer vector (gene of interest)</td>
<td>15 µg</td>
<td>-</td>
</tr>
<tr>
<td>Gag-Pol-Rev expression plasmid</td>
<td>10 µg</td>
<td>-</td>
</tr>
<tr>
<td>(PSPAX2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tat expression plasmid</td>
<td>10 µg</td>
<td>-</td>
</tr>
<tr>
<td>Rev expression plasmid</td>
<td>10 µg</td>
<td>-</td>
</tr>
<tr>
<td>VSV-G expression plasmid</td>
<td>5 µg</td>
<td>-</td>
</tr>
<tr>
<td>(pMD2.G)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.1: Components for transfection of 293T cells.** Gag-Pol-Rev, Tat, Rev, and VSV-G are packaging proteins that combine to form a functional lentivirus. The transfer vectors are the 4F2A and rtTA plasmids. Lipofectamine 2000 facilitates the movement of plasmids into cells. Opti-MEM I is a reduced serum medium that improves the efficiency of transfection procedures. These amounts are for use in a 10-cm plate. This table was taken from Hotta et al. 2009 [191].
After 8-16 hours, the old media was discarded and 25 ml of pre-warmed GM + gentamycin was added to each 10cm cell culture dish. The discarded media was put into a disposable plastic bottle containing 10% bleach. Virus-containing media was always handled with disposable, plastic pipettes. Virus-containing media was harvested at 48 and 72 hours post transfection by transferring the media into sterile 50 ml conical tubes and was stored at 4°C. Virus half-life at 4°C is a little over a week and the half-life is greatly reduced at higher temperatures since viruses are temperature sensitive. The virus-containing media was centrifuged at 3000 rpm for 15 minutes at 4°C to pellet any cell debris. The supernatant was aspirated and filtered through a 0.45 µm syringe filter (Millex HP #HSLHP033PS). At this point a few 0.5-1 ml aliquots were put in cryovials and stored at -80°C. The remaining virus solution was transferred to Thermo Scientific Nalgene Oak Ridge High-Speed PPCO Centrifuge Tubes and the tubes were put into a pre-chilled rotor. The tubes had to be balanced and filled to more than 90% capacity, or they would collapse under high centrifugal forces, so Hank’s Balanced Salt Solution (HyClone #SH3026801) was used to balance the tubes. Centrifugation speed had to be adjusted for the rotor type and size used. The speed goal was for maximum centrifugal force to be between 90,000 and 100,000g. The rotor used in Hotta et al. 2009 was a Sorvall T-865 with a K-factor of 243 at 30,000 rpm, which took 2 hours to pellet the virus. The rotor available at the Virginia-Maryland College of Veterinary Medicine was a SA-600, which had a K-factor of 457 at 16,000 rpm. The following rotor conversion formula was used to calculate the time it would take to pellet the virus using the SA-600 rotor:

\[ \frac{T1}{K1} = \frac{T2}{K2} \]

where

- T1: time to pellet in the “new” rotor
- K1: K-factor of the “new” rotor
- T2: time to pellet in the “old” rotor
- K2: K-factor of the “old” rotor

Solving for T1, the SA-600 rotor took 3 hours and 46 minutes to pellet the virus. After centrifugation, the walls of the centrifuge and outside of the rotor were wiped down with 10% bleach followed by 70% ethanol in case there were any invisible spills. The rotor or just the tubes were carefully transferred to a biosafety cabinet. The viral pellet was visible as a tiny white spot, which was circled on the outside of the tube with a permanent marker. The supernatant was carefully aspirated off and discarded into labelled 10% bleach container immediately after centrifugation, because the viral pellet was fragile and easy to detach. Fifty µl of chilled HBSS was added to the pellet and tubes were incubated overnight at 4°C.

The next day the viral pellet was re-suspended in the HBSS by pipetting up and down at least 20 times. The solution was then transferred to a cryovial for long-term storage. Due to residual media from the wall of the tube, the volume per tube was typically 80-100 µl. The virus was aliquoted at 20 µl per cryovial and the cryovials were placed into polypropylene 15-ml conical tubes for double containment. Virus was stored at -80°C for up to six months. Freeze-thaw was avoided because of the temperature-sensitive nature of the virus.
3.2.2 Cell culture

See Chapter 2.

3.2.2.1 Embryonic stem cell or induced pluripotent stem cell culture

Twenty-four hours before subculture of ESCs or iPSCs, a feeder layer of irradiated mouse embryonic fibroblasts (IR-MEFs) was put in culture using “Starting cell culture” procedure above. 1x10^6 cells were put in a 10-cm dish or an equivalent value in a different sized dish. After 24 hours, the ESCs or iPSCs were put in culture on top of the IR-MEFs, using “Starting cell culture” procedure above, but using appropriate media. ESC media consisted of 90% DMEM/High glucose with L-glutamine (HyClone #SH30022.01) + 10% HyClone ES-qualified FBS (HyClone #SH30070.03) + 50 µg/ml Gentamycin (Lonza #17-5182) + 1X Gibco MEM Non-Essential Amino Acids (NEAA; ThermoFisher #11140050) + 10,000 U/ml Leukemia Inhibitory Factor (LIF; Stemgent #03-0011) + 0.1% β-Mercaptoethanol (Sigma #M7522). iPSC media consisted of 75% Knockout DMEM (ThermoFisher #10829-018) + 25% Knockout Serum Replacement (ThermoFisher #10828-101) + 50 µg/ml Gentamycin (Lonza #17-5182) + 1X MEM NEAA (ThermoFisher #11140050) + 10,000 U/ml LIF (Stemgent #03-0011).

To passage ESCs or iPSCs, IR-MEF feeder cells were put into culture 24 hours before subculturing ESCs or iPSCs. The next day, the ESC- or iPSC-containing culture dish was placed in the cell culture hood and the media was removed. The dish was washed with PBS then pre-warmed 0.25% trypsin was added, 1 ml per 25 cm^2 of surface area. The dish was placed in a 37°C incubator for no more than 5 minutes. An equivalent amount of serum-containing media (GM or ESC) was added to inactivate the trypsin. The dish was rotated to mix thoroughly and the media/cell mixture was transferred to a conical tube. The tube was left at room temperature for 10 minutes until a soft pellet formed at the bottom of the tube. This pellet contained mostly ESC or iPSC colonies. The supernatant was carefully discarded and the pellet was re-suspended in a small volume of ES or iPSC medium. The re-suspended cells were transferred to a culture dish with just enough media added to cover the surface of the dish. The dish was placed in the 37°C incubator for 15 minutes, during which time IR-MEFs attached to the dish but ESCs or iPSCs did not. The dish was transferred back into the hood. The supernatant was transferred to a clean tube and the tube was centrifuged at 120g for 5 minutes. The supernatant was discarded and twice the volume of the pellet of pre-warmed 0.25% trypsin was added to the tube. The tube was placed in the incubator for 5 minutes. An equivalent amount of serum-containing media was added to the tube and the tube was centrifuged at 120g for 5 minutes. Supernatant was discarded and cell pellet was re-suspended in culture media. Cells could be counted at this stage (see “Counting cells” section below). Cells were then added dropwise over pre-plated feeder cells.

3.2.3 Titration of virus

25,000 cells, of the cell type to be reprogrammed into iPSCs, were put in a single well of a 24-well plate in 500 µl of GM. The two cell types used were Oct4-GFP Mouse Embryonic
Fibroblasts (PrimCells #PCEMM03) and Porcine Embryonic Fibroblasts (made in our lab, see section 3.2.5). Figure 3.4 diagrams the plate design for titration. The plate was put in an incubator overnight. They next day the GM was removed and replaced with 1 ml of transfection media, which consisted of GM + 8 µg/ml Polybrene (Millipore TR-1003-G). The appropriate amount of virus was then added to each well. Only disposable plastic pipettes were used and were discarded into specially marked containers. The plate was put in the incubator for 8-16 hours. After 8-16 hours the virus-containing media was discarded into plastic bottle with 10% bleach and fresh, pre-warmed GM + 1 µg/ml doxycycline (Clontech #631311) was put on the cells. The cells were placed in incubator for 48 hours. Two days later, the media was aspirated off and was replaced with fresh GM + 1 µg/ml doxycycline. The next day immunofluorescence was performed (see “Immunofluorescence” procedure below). Since doxycycline has a half-life of 24 hours, it had to be added to new media right before addition to cells, and it had to be replaced every 48 hours.

![Control, Control + polybrene, 2nd Ab Control, 4 ul conc., 2 ul conc., 1 ul conc., 0.1 ul conc., 0.01 ul conc.]

**Figure 3.4: Plate design for virus titration.** The use of serial dilutions of virus allows determination of the potency of the virus. Addition of the virus followed by addition of doxycycline initiates the expression of the reprogramming factors from the 4F2A vector. Immunofluorescence for one of the four reprogramming factors identifies which cells contain the 4F2A factor and can be used as an approximation for the number of viral particles that are successfully transfected into that cell type when given that volume of concentrated virus.

After immunofluorescence, the Viral Titer was calculated using the following equation:

**Equation 3:**  
Viral titer (IU ml⁻¹) = [Infected cell # per well] × [GFP⁺ %/100] / [Amount of virus used (ml)]

A virus dilution that yielded infectivity between 5% and 25% was used for titer calculation. Cell cultures with an infectivity of greater than 50% probably contained multiple integrations and would have underestimated the viral titer. The viral titer was then used to calculate the Multiplicity of Infection (MOI) using the following equation:

**Equation 4:**  
MOI = [Viral titer (IU ml⁻¹)] × [Amount of virus used for infection (ml)] / [Target cell #]
3.2.4 iPSC production

In GM, 100,000 cells to be reprogrammed were put per well of a 6-well plate coated with matrigel (Corning #354230). The two cell types used were Oct4-GFP Mouse Embryonic Fibroblasts (PrimCells #PCEMM03) and Porcine Embryonic Fibroblasts (made in our lab, see section 3.2.5). The next day, transfection media was made by combining GM + 8 µg/ml Polybrene (Millipore #TR-1003-G) + virus. The MOI, viral titer, and target cell number for that particular cell type was used to solve Equation 4 (from “virus titration” section) to determine the amount of virus to use. For cellular reprogramming to iPSCs, a MOI of one was used. The plate was put in the CO₂ incubator at 5% CO₂ and 37°C (mouse) or 38.5 °C (pig) for 8-16 hours. The virus-containing media was removed and properly discarded and was replaced with iPS media with 1 µg/ml doxycycline (Clontech #631311). The media was changed every other day until colonies were large enough to pick.

Only disposable plastic pipettes were used when working with live virus and pipettes and discarded media were properly disposed of in “lentivirus”-labelled containers with 10% bleach. The hood and all instruments used to handle the virus in the hood were sprayed down with 10% bleach and let sit for 10 minutes before wiping everything down with 70% ethanol. This was only necessary through the first three media changes.

To pick colonies, a sterile glass picking tool was used to detach the colony under a dissecting microscope in a sterile hood. A 10 µl pipette set to 5 µl was used to pick up the colony and transfer it to a well of a 96-well plate containing 15 µl PBS. This was repeated for all colonies, transferring them into separate wells. In each well containing a colony, 20 µl of 0.25% trypsin was added and was incubated for 5 minutes at 37°C. The contents of each well were pipetted up and down twenty times to break up the colonies and were transferred to wells of a 24-well plate pre-plated with IR-MEFs. When colonies were getting close to touching each other, they were passaged at 1:4 and/or frozen at 5x10⁵ or 1x10⁶ cells per cryovial using the protocol from the “iPSC culture” section above.

3.2.5 Establishment of porcine fibroblast lines

The reproductive tract of a pig between thirty and sixty days pregnant was obtained from a slaughterhouse. The tract was rinsed with Nolvasan solution and was aseptically opened down the anti-mesometrial border. The feto-placental units were extracted and the placenta, including the amnion, was opened. The fetuses were extracted and placed in sterile 15 cm dishes. The sex and crown-rump length was recorded for each fetus and was used for estimation of gestation age using Table 3.2. The head, abdominal and thoracic cavity contents were removed. The remaining tissue was cut into 1cm squares and was transferred to 50 ml tubes with 30 ml Betadine. The tubes were wiped down with 70% ethanol and transferred into the cell culture hood. The Betadine was removed and the tissue was transferred into fresh 50 ml tubes with 30 ml of 70% ethanol for two minutes. The ethanol was removed and replaced with fresh 30 ml of
70% ethanol. After two minutes, the ethanol was removed and the tissue pieces were washed three times with 40 ml PBS. The tissue pieces were transferred to a 15 cm dish containing 10 ml of PBS. Using sterile razor blades, the tissue was minced into 1mm cubes. The tissue was transferred to a 50 ml tube with 50 ml PBS and the cubes were allowed to pellet by gravity. This was repeated five times. After the final wash, the cubes were re-suspended in enough GM, with twice the amount of gentamycin, to cover the bottom of a 15 cm culture plate and were transferred to the plate. The cell outgrowths were harvested when 70% confluent and split 1:5. The tissue pieces were put back in culture for extended outgrowth, and the outgrowths were split 1:5. Cells were frozen at 1x10⁶ cells per vial (see “freezing cells” protocol).

<table>
<thead>
<tr>
<th>DAY OF GESTATION</th>
<th>CROWN-TO-RUMP LENGTH (CM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2.5</td>
</tr>
<tr>
<td>40</td>
<td>4.8</td>
</tr>
<tr>
<td>50</td>
<td>8.2</td>
</tr>
<tr>
<td>60</td>
<td>11.9</td>
</tr>
<tr>
<td>70</td>
<td>15.8</td>
</tr>
<tr>
<td>80</td>
<td>17.6</td>
</tr>
</tbody>
</table>

Table 3.2: Guide for estimation of pig fetus gestational age by crown-to-rump length. For derivation of cell lines, day 30-60 fetuses are preferable.

### 3.2.6 Immunofluorescence

See Chapter 2 for protocol.

Primary antibodies used were Oct4 (SantaCruz #8629, 1:100 dilution), Sox2 (SantaCruz #17320; 1:100 dilution), Klf4 (SantaCruz #1905; 1:200 dilution), cMyc (SantaCruz #788; 1:100 dilution), Nanog (SantaCruz #33760, 1:200 dilution), Nanog (PeproTech #500-P236; 1:100 dilution), Tra-1-60 (Millipore #MAB4360; 1:100 dilution), Tra-1-81 (Millipore #MAB4381; 1:100 dilution), SSEA1 (SantaCruz #21702; 1:200 dilution), CD44 (Biolegend #103001; 1:500 dilution).

Secondary antibodies used were Donkey anti-Goat IgG (ThermoFisher #A11055; 1:400 dilution), Donkey anti-Rabbit IgG (ThermoFisher #A21206; 1:400 dilution), Goat anti-Mouse IgM-TR (SantaCruz #2983; 1:80 dilution), and Rabbit anti-Rat IgG (Jackson ImmunoResearch #312-545-003; 1:200 dilution).

### 3.2.7 Alkaline phosphatase test

Stemgent Alkaline Phosphatase Staining Kit II (Stemgent #00-0055) was used and protocol followed.
3.2.8 Polymerase chain reaction (PCR)

If necessary, RNA was reverse transcribed into DNA using a cDNA synthesis kit (BioRad #170-8890). RNA and DNA stored at -20°C. A Taq PCR Master Mix Kit (Qiagen #201443) was used to prepare the DNA for PCR amplification. For a negative control, an additional 1 µl of H2O was used in place of DNA. Samples were then put in the thermocycler for amplification. Gel electrophoresis using a Bio-Rad Power Pac 200 and a Bio-Rad Mini-Sub GT setup was necessary to visualize the PCR products. First, the percent agarose gel was chosen based on the size DNA to be visualized. Common percent agarose gels and their corresponding DNA appropriateness are listed in Table 1 in the appendix. The gel was prepared by combining PCR Agarose powder (Bio-Rad #161-3103) with 100 ml diluted 1x Tris/Borate/EDTA (TBE). For example, to get a 1% agarose gel, 1 gram of agarose powder was combined with 100 ml diluted 1x TBE. Diluted 1x TBE was made by combining 5x TBE with distilled H2O at a 1:4 ratio. 5x TBE was made by combining 27.5 g Boric Acid (Fisher Scientific #BP168.1), 3.72 g Ethylenediaminetetraacetic acid (EDTA) (Sigma #E-6511), and 53.0 g TrisBase (Fisher Scientific #BP152-1) in 1 liter of DI water. The agarose mixture was heated until the powder dissolved, but before it began boiling. A comb was placed in the gel container, and the agarose was poured in until approximately 1-2 cm thick. The gel was allowed 30 minutes at room temperature to set. Ladders were chosen based on the size of the DNA band expected. Six µl of 6x Blue Loading Dye (Sigma #G2526-5ML) was added to each 20 µl PCR sample. 6ul of each sample and the ladders were loaded into the gel. Voltage was calculated using the following equation:

Equation 5: \[(\text{length between electrodes}) \times (\text{voltage/cm}) = (15 \text{ cm}) \times (5 \text{ volts/cm}) = 75 \text{ volts}\]

Gel electrophoresis was ran at 75 volts until the dye was two-thirds of the way through the gel. Five to 10 µl of Ethidium Bromide (Bio-Rad #161-0433) was added to distilled H2O in a container. The gel was transferred to the container, the lid was closed, and the container was placed on a Barnstead/Thermolyne Roto Mix shaking plate for 15 minutes. Then the gel was visualized using a UVP Benchtop UV transilluminator.

3.2.9 Embryoid Body Formation

iPSCs were expanded on IR-MEFs then trypsinized and separated from IR MEFs (see “Cell culture” section above). iPSCs were re-plated at a density of 2x10^6 cells per 10-cm non-adherent petri dish in a medium composed of half growth media and half iPSC media without Lif. Porcine iPSCs were also given a one-time dose of 0.25 ug doxycycline / 1 ml media, allowing the cells to slowly deplete the small amount of doxycycline originally administered. Without weaning off the doxycycline, the piPSCs die. Over the next few days to a week the iPSCs aggregated together and differentiated to form embryoid bodies. Embryoid bodies could then be transferred to adherent culture plates, where they attach to the plate and differentiated cells grow out of them.
### 3.2.10 Secondary piPSC formation

Following 4 days in non-adherent petri dishes using the protocol in 3.2.9, the embryoid bodies were transferred to adherent culture plates in growth media. Once the plates were 70-80% confluent with the embryoid body outgrowths, the cells were trypsinized and re-plated. Embryoid body outgrowths adhere very tightly to the plate and require either prolonged exposure to trypsin, which adversely affects their ability to subsequently plate down, or the use of cell scrapers, which result in damage and loss of some of the cells but does not affect their ability to plate down. The following day the media was changed to iPS media with doxycycline and LIF. Media was refreshed every other day. Reprograming cells were trypsinized and re-plated onto IR-MEFs on day 5. This was necessary to provide a substrate for the colonies to attach to since they will not attach to the plates and will die.

### 3.3 Results

#### 3.3.1 Establishment of lentiviral reprogramming vector

##### 3.3.1.1 Approximate check for plasmids by PCR

Following initial expansion of the 4F2A and rtTA primers in Stab13 bacteria, three colonies were picked for each primer in addition to samples from the control and PCR was performed to confirm the presence of the plasmids in the bacteria (Figure 3.5). Visualization of the PCR results using gel electrophoresis showed that all three 4F2A-containing colonies produced a band at approximately 2200 base pairs, which corresponded to the fragment of the 4F2A vector amplified during PCR. All three rtTA-containing colonies also produced a band at approximately 1408 base pairs, which corresponded to the fragment of the rtTA vector amplified during PCR. Environmental bacteria used as the control was negative for both the 4F2A and rtTA plasmids. Since all three replicates of rtTA and 4F2A were PCR positive for the plasmid, rtTA#1 and 4F2A#1 were arbitrarily chosen for expansion and plasmid extraction.

![Figure 3.5: PCR confirmation of presence of reprogramming plasmids in bacteria prior to extraction. (a) Gel electrophoresis. (b) Primer sequences. Three replicates were performed, all of which were positive. The negative controls used appropriate primers without picked colonies.](image-url)
3.3.1.2 Precise check of plasmids by sequencing

To confirm that the plasmids had an intact genomic sequence, samples were sent to the Virginia Bioinformatics Institute (VBI) at Virginia Tech for Sanger sequencing. Primers MC10, KW19, and KW16 did not work, so new primers were designed (KW24, KW25, and KW26, respectively) and samples were resubmitted to VBI for sequencing. There were three base pair substitutions in 4F2A and two insertions in rtTA. These changes did not impair function.

Figure 3.6: Schematic of reprogramming plasmids with sequencing primers. (a) 4F2A plasmid. Doxycycline-inducible polycistronic lentiviral vector encoding mouse pluripotency transcription factors Oct4, Sox2, Klf4, and cMyc. (b) rtTA plasmid. Companion vector encoding a constitutively expressed reverse tetracycline transactivator. Images (a) and (b) made in SnapGene. (c) Primer sequences.
3.3.1.3 Virus titration

After sequencing, the reprogramming plasmids were combined with viral packaging plasmids Tat, Rev, PSPAX2, and PMD2 for virus production in 293T cells. The calculations for titration of the virus are in Table 3.3 below.

As a reminder,

\[
\text{Viral titer (IU ml}^{-1}\text{)} = \frac{\text{[Infected cell # per well] \times [GFP+ %/100]}}{\text{[Amount of virus used (ml)]}}
\]

* Only a virus dilution that yielded infectivity between 5% and 25% was used for titer calculation.

\[
\text{MOI} = \frac{\text{[Viral titer (IU ml}^{-1}\text{)]} \times \text{[Amount of virus used for infection (ml)]}}{\text{[Target cell #]}}
\]

With cellular reprogramming, a MOI of 1 is recommended. To achieve that in mouse embryonic fibroblasts, 0.00966 ml or 9.66 ul of concentrated virus was used for every 25,000 cells to be infected. Since there were viral concentrations (1 ul concentrated and 2 ul concentrated) that yielded an infectivity between 5 and 25 percent, the MOIs calculated from both were averaged to produce 0.00966. For an MOI of 1 in porcine embryonic fibroblasts, 0.05569 ml or 55.69 ul was used for every 25,000 cells to be infected.
3.3.2 iPSC production

3.3.2.1 Establishment of porcine fibroblast lines

Since the lentiviral vector being used for cellular reprogramming had previously only been used on mouse and human cells, we decided to reprogram mouse cells in addition to our species of interest, pigs. The mouse embryonic fibroblasts were ordered from PrimCells (#PCEMM03). They had a GFP reporter linked to the Oct4 gene, allowing visual identification of reprogramming cells by their green color change. The porcine embryonic fibroblasts were made in our lab from approximately day 45 fetuses (see Table 3.2 for conversion and section 3.2.5 for methodology).

<table>
<thead>
<tr>
<th>SEX</th>
<th>CROWN-TO-RUMP LENGTH (CM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIG #1 Male</td>
<td>6.8</td>
</tr>
<tr>
<td>PIG #2 Female</td>
<td>6.9</td>
</tr>
<tr>
<td>PIG #3 Female</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Table 3.4: Pig fetuses used to establish porcine fibroblast lines. Based on table 3.2, the crown-to-rump lengths indicate that these fetuses were day 40 and 50 of gestation, which is an ideal age at which to establish cell lines.

Figure 3.7: Generation of porcine fibroblast cells. Cellular outgrowths seen within three days of plating tissue pieces. Cells passaged one prior to freezing.

3.3.2.2 Reprogramming

The prepared lentivirus was added to embryonic fibroblasts to reprogram them into induced pluripotent stem cells. The mouse cells began to show signs of reprogramming at day 5 and had colonies large enough to pick by day 23. The porcine cells also began to show signs of reprogramming at day 5, but had colonies large enough to pick by day 12.
Figure 3.8: Reprogramming of MEFs to miPSCs. The cells were maintained in “iPS” media supplemented with doxycycline and LIF for the entirety of reprogramming. The colonies were large enough to pick and expand by day 23. The control cells were Oct4-GFP MEFs maintained in growth media and not exposed to the reprogramming virus.
Colonies were successfully picked, trypsinized, and re-seeded on irradiated mouse embryonic fibroblasts to form multiple new colonies. Both mouse and pig induced pluripotent stem cells could be passaged as single cells with minimal cell death between passaging. They could also be frozen in liquid nitrogen and subsequently put back in culture with minimal cell death. Colonies from both species had a rounded, naïve-like appearance and proliferated under the influence of LIF, without supplementation of FGF. The piPSCs, however, required continued supplementation of doxycycline in the media in order to maintain pluripotency. Without doxycycline the cells died or differentiated. Upon removal of doxycycline from the miPSCs, they continued to proliferate as before.

3.3.3 Characterization of mouse induced pluripotent stem cells

Visually, the miPSCs appeared and behaved like pluripotent cells. They were small, quickly dividing cells with a high nucleus:cytoplasm ratio that grew in rounded colonies. To confirm their pluripotency, the following assays were performed: immunofluorescence, alkaline phosphatase activity, PCR, and embryoid body formation.

Immunofluorescence was performed to look for cellular expression of pluripotent-specific factors: Oct4, Sox2, and Nanog. Oct4 and Sox2 were strongly positive in miPSCs and negative...
in the IR-MEF feeder cells. There was a lot of background staining associated with the Nanog antibody, but preferential expression in the miPSCs in comparison to the IR-MEFs is evident. Immunofluorescence was also done for factors associated with primed pluripotent cells: Tra-1-60 and Tra-1-81. The miPSCs were negative for both, supporting their naïve appearance.

**Figure 3.11: Immunofluorescence for characterization of pluripotency of miPSCs.**
(a) Oct4. (b) Sox2. (c) Nanog. (d) Tra-1-60. (e) Tra-1-81. Blue = dapi stain for nuclei.

Increased alkaline phosphatase activity indicates an increased telomere length and increased telomerase activity and is used as an indirect sign of pluripotency [71,161,162]. The miPSCs were strongly and distinctly positive for alkaline phosphatase. Alkaline phosphatase activity was also assessed in mouse ESCs as a control to assure the validity of the kit used.

**Figure 3.12: Alkaline phosphatase activity of mouse cells** (a) miPSCs. Blue = dapi stain for cell nuclei. (b) mESCs. Both cell types on feeder layer of IR-MEFs.

In addition to immunofluorescence, up-regulation of pluripotency markers Oct4, Sox2, Klf4, cMyc, and Nanog was demonstrated through PCR. Three controls were used. GAPDH was used as a positive control, since it is ubiquitously expressed in all cell types. The negative control also used the GAPDH primers, but the sample had no cDNA added. Finally, there was a control for the presence of the exogenous reprogramming vector in which primers amplified a stretch of the vector beginning at the end of the Oct4 sequence, going through the 2A sequence and ending in the following Sox2 sequence. The primers for Oct4, Sox2, Klf4, and cMyc were designed to
include part of the five prime or three prime untranslated region so that they would only amplify endogenously-produced products and not the sequences from the exogenous reprogramming vector. The PCR was repeated with cDNA from mESCs for comparison and to evaluate the efficacy of the primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
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<tbody>
<tr>
<td>mouse endogenous Oct4 forward</td>
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</tr>
<tr>
<td>mouse endogenous Oct4 reverse</td>
<td>ctccacctcacaacgggttctc</td>
</tr>
<tr>
<td>mouse endogenous Sox2 forward</td>
<td>accagcgcaggtsgacacgtac</td>
</tr>
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<td>mouse endogenous Sox2 reverse</td>
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<td>mouse endogenous Klf4 reverse</td>
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<tr>
<td>mouse endogenous cMyc forward</td>
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<td>mouse endogenous cMyc reverse</td>
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<td>mouse GAPDH reverse</td>
<td>agatgccgtcctcaccacccccctt</td>
</tr>
<tr>
<td>exogenous forward</td>
<td>gtagactgcacagtggccag</td>
</tr>
<tr>
<td>exogenous reverse</td>
<td>gggaccgccttgcctgtat</td>
</tr>
</tbody>
</table>

Figure 3.13: PCR for characterization of pluripotency in mouse cells. (a) miPSCs (b) mouse ESCs. GAPDH was positive control. No DNA was negative control. Used 100 bp ladder (New England Biolabs #N3231S). (c) Primer sequences.
Following transfer to a non-adherent petri dish, the miPSCs aggregated together and differentiated to form embryoid bodies. PCR was performed to show expression of at least one marker from each of the three germ layers: endoderm, ectoderm, and mesoderm. The embryoid bodies were positive for GATA4 (endoderm), GFAP (ectoderm), Pax6 (ectoderm), Col2A1 (mesoderm), and CD34 (mesoderm).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse Gata4</td>
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<tr>
<td>mouse Gata4</td>
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<tr>
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<td>mouse Pax6</td>
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</tr>
<tr>
<td>mouse CD34</td>
<td>cagcttttctcttgtagggc</td>
</tr>
</tbody>
</table>

Figure 3.14: miPSC embryoid bodies. (a) Formation of EBs in nonadherent petri dish with cellular outgrowths following transfer to adherent culture dish. (b) PCR for germ layer expression. 100 bp ladder (New England Biolabs #N3231S).
3.3.4 Characterization of porcine induced pluripotent stem cells

Similar to the miPSCs, the piPSCs appeared to be pluripotent in that they were also small, quickly dividing cells with a high nucleus:cytoplasm ratio that grew in rounded colonies. To further characterize their pluripotency the following assays were performed: immunofluorescence, alkaline phosphatase activity, PCR, and embryoid body formation.

Immunofluorescence was performed for Oct4, Sox2, Nanog, Tra-1-60, and Tra-1-81. Oct4, Sox2, and Nanog were strongly positive. However, since the cells required continual supplementation of doxycycline in the media, it is not possible to distinguish if the expression is from endogenous expression or expression from the exogenous reprogramming vector. Tra-1-60 and Tra-1-81 were both negative, which provides additional support for a naïve cell type. However, SSEA1 was also negative indicating that the cells may not be completely naïve.

Figure 3.15: Immunofluorescence for characterization of pluripotency for piPSCs. (a) Oct4. (b) Sox2. (c) Nanog. (d) Tra-1-60. (e) Tra-1-81. (f) SSEA1. Blue = dapi stain for cell nuclei.
The piPSCs were only weakly positive for alkaline phosphatase activity.

Following transfer to a non-adherent petri dish and weaning off doxycyline, the piPSCs aggregated together and differentiated to form embryoid bodies. PCR was performed to show expression of at least one marker from each of the three germ layers: endoderm, ectoderm, and mesoderm. GAPDH was included as a positive control. The embryoid bodies were positive for CRABP2 (endoderm), Nicastrin (ectoderm), Enolase (mesoderm), and Desmin (mesoderm). PCR for the same germ line layer markers was also performed for piPSCs and PEFs.

**Figure 3.16:** Alkaline phosphatase activity of piPSCs.

**Figure 3.17:** piPSC embryoid bodies. (a) Formation of free floating EBs in nonadherent petri dish. (b) Cellular outgrowths after transferring EBs to adherent culture dish.
Figure 3.18: PCRs for germ layer markers in pig cells. (a) pEBs. (b) piPSCs. (c) PEFs. GAPDH was positive control. No DNA was negative control. 100 base pair ladder (New England Biolabs #N3231S). (d) Primer sequences.
In addition to immunofluorescence, expression of pluripotency markers Oct4, Sox2, Klf4, cMyc, and Nanog was demonstrated through PCR. Two controls were used. GAPDH was used as a positive control, since it is ubiquitously expressed in all cell types. The negative control also used the GAPDH primers, but the sample had no cDNA added. PCRs were done to look at up-regulation of endogenous expression as well as exogenous expression from the reprogramming vector. The primers for endogenous Oct4, Sox2, Klf4, and cMyc were designed to include part of the five prime or three prime untranslated region so that they would only amplify endogenously-produced products and not the sequences from the exogenous reprogramming vector.

PCRs for endogenous expression were done using cDNA from the piPSCs, pig testes tissue, PEFs, and pEBs for comparison and to evaluate the efficacy of the primers. Since there are no available pig ESCs, pig testes tissue was used as a control to make sure that the primers worked. There was good expression from Oct4, Sox2, Klf4, cMyc, Nanog, and GAPDH from the pig testes tissue. The piPSCs showed expression of all of the endogenous genes as well, with expression of most genes not being quite as strong as in the pig testes. PEFs expressed GAPDH, cMyc, and a small amount of Klf4. The pEBs expressed GAPDH, cMyc, and a small amount of Klf4 and Sox2.
Table 3.19: PCRs for endogenous expression of pluripotency factors in pig cells. (a) Pig testes tissue. (b) piPSCs. (c) PEFs. (d) pEBS. GAPDH was positive control. No DNA was negative control that used the GAPDH primer, with no DNA added to the reaction mix. 100 base pair ladder (New England Biolabs #N3231S). (e) Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pig Oct4 forward</td>
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<td>123</td>
</tr>
<tr>
<td>pig Oct4 reverse</td>
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</tr>
<tr>
<td>pig Sox2 forward</td>
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</tr>
<tr>
<td>pig Sox2 reverse</td>
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<td></td>
</tr>
<tr>
<td>pig Klf4 forward</td>
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<td>pig Klf4 reverse</td>
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</tr>
<tr>
<td>pig cMyc forward</td>
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</tr>
<tr>
<td>pig Nanog forward</td>
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</tr>
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<td>pig GAPDH reverse</td>
<td>atgaggtccaccaccccttgtt</td>
<td></td>
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</table>
Expression of the mouse exogenous reprogramming factors Oct4, Sox2, Klf4, and cMyc was evaluated in piPSCs and pEBs. Since doxycycline had to be supplied to maintain pluripotency, it was expected that there would be exogenous expression in the piPSCs. All four exogenous factors were expressed in the piPSCS. PCR for exogenous expression was done in the pEBs as a control. Doxycycline was pulled during formation of the pEBs, meaning there shouldn’t be exogenous factor expression. The primers against the mouse factors were previously shown not to bind to the porcine gene sequences for Oct4, Sox2, Klf4, and cMyc on SnapGene. There was a small amount of expression of from the exogenous Oct4 primers in the pEBs.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
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<tr>
<td>exogenous Sox2 forward</td>
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<td>pig GAPDH reverse</td>
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</table>

Figure 3.20: PCRs for exogenous expression of pluripotency factors in pig cells. (a) piPSCs. (b) pEBS. GAPDH was positive control. No DNA was negative control. 100 base pair ladder (New England Biolabs #N3231S). (c) Primer sequences.
3.3.5 Production and characterization of secondary porcine induced pluripotent stem cells

Since a permanently integrating lentiviral vector was used for cellular reprogramming, we wanted to assess whether secondary piPSCs could be made. If they could, we also wanted to evaluate how closely they resemble the primary piPSCS. The timeline in figure 3.21 outlines the process by which this was done. The images in figure 3.22 illustrate the cellular changes. Cellular changes associated with reprogramming, such as decrease in cell size and swirling of cells, began as early as day 2 and colonies were first visible on day 5. This is significantly faster than the initial reprogramming, where cellular changes were seen starting around day 5 and colonies were apparent at day 10. This process was done three times to confirm repeatability of the secondary reprogramming system. All three replicates produced very similar rates of reprogramming.

**Figure 3.21: Timeline for production of secondary piPSCs.** Samples were as follows: (1) Alkaline phosphatase (2) Immunofluorescence
To test whether the secondary piPSCs are dependent upon dox like their primary piPSC progenitors, we took day 16 secondary piPSCs and split them into three groups on day 0. The first group did not receive any doxycycline. The second group received a half dose of doxycycline on day 0 and none in the media change on day 2. This was done to help wean the cells off of doxycycline in order to make the transition less traumatic. The third group received doxycycline all four days (day 0-3) and served as a control. Cells not given doxycycline never plated down on the IR-MEFs. Cells weaned off dox plated down and formed colonies during the first 2 days, but after the media change and dox removal they differentiated or died. Cells that continuously received doxycycline continued to grow and form colonies. This shows that the secondary piPSCs, like the primary piPSCs, are dependent on doxycycline to maintain their pluripotency.

Figure 3.22: Production of secondary piPSCs. Secondary piPSCs were passaged and re-plated on a feeder layers of IR-MEFs on days 5 and 10. Doxycycline was added to the culture media the entire time.
Alkaline phosphatase activity was assessed on days 1, 4, 7, 10, 13, and 16. Cells with a lot of alkaline phosphatase activity stain red. While a yellow color change was detected on days 10 and 13, presence of a red color change did not appear until day 16 and the color change there was mild and indistinct. This is similar to alkaline phosphatase activity seen with the primary piPSCs.

Figure 3.23: Doxycycline removal from secondary piPSCs. There were 3 treatments groups: (1) No doxycycline present (2) Doxycycline weaned off over 2 days (3) Doxycycline always present.

Alkaline phosphatase activity was assessed on days 1, 4, 7, 10, 13, and 16. Cells with a lot of alkaline phosphatase activity stain red. While a yellow color change was detected on days 10 and 13, presence of a red color change did not appear until day 16 and the color change there was mild and indistinct. This is similar to alkaline phosphatase activity seen with the primary piPSCs.

Figure 3.24: Alkaline phosphatase activity of secondary piPSCs. Secondary piPSCs were passaged and re-plated on a feeder layer of IR-MEFs on days 5 and 10.
Immunofluorescence was done on the secondary piPSCs on day 1, 4, 7, 10, 13, and 16 of reprogramming for Nanog and Cd44. Nanog is a marker of pluripotency that is not included in the reprogramming vector and thus expression is not complicated by the continued addition of doxycycline. Cd44 is a fibroblast marker. While the exact cell types that grew out of the plated embryoid bodies are unknown, preliminary testing with this antibody showed some expression in the embryoid body outgrowths. During the course of reprogramming, CD44 expression is hypothesized to decrease while Nanog expression should increase. Nanog was not expressed by the embryoid outgrowth cells on day 1, was weakly expressed by day 4, and was strongly expressed from day 7 onwards. Cd44 was expressed by the outgrowth cells on day 1, was expressed by some cells on day 4, and was only expressed in the IR-MEFs from day 7 onwards.

Figure 3.25: Immunofluorescence for Nanog and Cd44 for secondary piPSCs. Secondary piPSCs were passaged and re-plated on a feeder layer of IR-MEFs on days 5 and 10. Blue = dapi stain for cell nuclei.
3.4 Discussion

The porcine cells had different culture characteristics than the mouse cells. First, during production of primary induced pluripotent stem cells, porcine fibroblasts (PEFs) required more virus than mouse fibroblasts (MEFs): 9.66 µl of concentrated virus per 25,000 cells for MEFs vs. 55.69 µl per 25,000 cells for PEFs. Therefore, it seems that PEFs are more resistant to transfection with this viral reprogramming system. Second, the PEFs grew faster and reprogrammed faster than the MEFs. The MEFs took 23 days to reprogram, while the PEFs took 12 days. The more rapid reprogramming rate is likely due to the faster doubling time of the PEFs. The faster growth rate of the PEFs could indicate that the PEFs are less differentiated than the MEFs. Third, the porcine induced pluripotent stem cells (piPSCs) were more delicate to work with than the mouse induced pluripotent stem cells (miPSCs). The piPSCs did not form embryoid bodies very well and did not maintain their shape as well for immunofluorescence and alkaline phosphatase tests. Lastly, and most importantly, the piPSCs needed doxycycline to maintain pluripotency. When doxycycline was removed the cells either died or differentiated. In order to form embryoid bodies the piPSCs needed to be weaned off the doxycycline. This indicated that the piPSCs produced were not truly pluripotent. However, this reliance on exogenous reprogramming factors is consistent with piPSCs produced by other groups.

The pluripotency characterization assays revealed interesting characteristics of the piPSCs, as compared to the miPSC controls. First, immunofluorescence of the miPSCs and piPSCs revealed strong positives for pluripotency markers Oct4, Sox2, and Nanog and a lack of Tra-1-60 and Tra-1-81 expression. Tra-1-60 and Tra-1-81 are both markers of primed iPS cells. This finding, in conjunction with the rounded morphology of the miPSCs and piPSCs, the ability to passage them as single cells, and their dependence on LIF for maintenance in culture supports the conclusion that these are naïve iPSCs. However, the piPSCs did not show expression of SSEA1, a marker of naïve cells [73-77]. This could indicate that these piPSCs are not truly naïve or that they are naïve and do not express SSEA1. Due to a storage malfunction, the miPSCs were lost before SSEA1 immunofluorescence could be performed. Murine iPSCs are typically naïve and porcine iPSCs are typically primed type. However, both primed and naïve lines have been created for piPSCs [122-141]. It has been shown that species other than mice and humans do not consistently express SSEA1 [74], meaning the lack of SSEA1 expression does not preclude the piPSCs from being naïve. It is likely that they are somewhere in between a naïve and primed type. Second, alkaline phosphatase activity was very strong in the miPSCs and was comparable to the mESCs used as a positive control. Alkaline phosphatase activity was not as strong in the piPSCs as compared to the miPSCs. Elevated alkaline phosphatase activity indicates an increased telomere length and telomerase activity, which is a feature of pluripotent cells [71,161,162]. This decreased expression in the piPSCs could be due to them not being fully reprogrammed and being dependent on exogenous factor expression. Also, as with SSEA1 expression, pluripotent cells from species other than mice and human do not consistently have higher alkaline phosphatase expression [45,119,121]. Third, both the miPSCs and piPSCs
demonstrated endogenous expression of the pluripotency factors Oct4, Sox2, Klf4, cMyc, and Nanog. The primers were confirmed to work in positive controls (mESCs and porcine testes). Oct4 and Sox2 are the most supportive of pluripotency and the bands for those two factors were strong and distinct in both the miPSCs and piPSCs. Expression of porcine pluripotency factors were further evaluated in the PEFs and pEBs. The expression of cMyc and Klf4 in PEFs could mean that the PEFs have some degree of pluripotency or tumorigenicity, or that the primers are binding to an off-site area or Myc and Klf isoforms. The expression of cMyc and Klf4 in the pEBs to a similar degree as in the PEFs is an indication that when allowed to differentiate during embryoid body formation, the piPSCs preferentially differentiated into their cell type of origin. It has been demonstrated that, unless provided with certain culture conditions and/or exogenous factor suppression, induced pluripotent stem cells have a propensity to differentiate into their original cell type because they retain an epigenetic memory of that cell type [11-13]. Expression of exogenous Oct4, Sox2, Klf4, and cMyc was expected in the piPSCs since they were still dependent on doxycycline to maintain pluripotency. There should not have been any expression of these factors in the pEBs. However, there was a small amount of Oct4 expression, indicating that there was expression of the reprogramming vector, expression of endogenous Oct4 that is being picked up by the exogenous primers, or the primers were binding to another area of DNA resulting in transcription of a similarly sized product. The lack of expression of the other exogenous factors in figure 3.20b and the lack of endogenous Oct4 expression in figure 3.19d, indicate that the last theory is most probable. Finally, there was expression of at least one marker of each of the three germ layers in the embryoid bodies made from the miPSCs and the piPSCs. This capability to differentiate into cell types of all three embryonic germ layers, a criteria of pluripotency, provided further support that the generated iPSCs were pluripotent. Expression of these germ layer markers was explored further in the piPSCs. The lack of expression of the same germ layer markers in piPSCs showed both that piPSCs were pluripotent and that the designed PCR primers weren’t binding to off-target areas in the cells. There was almost identical PCR germ factor expression in the pEB outgrowths and PEFs. Additionally, both the murine and porcine embryoid body outgrowths were very uniform and morphologically resembled fibroblasts. This provides further support to the theory previously asserted in discussion of the PCR results that the EB cells have a propensity to differentiate into their original cell type.

The process of factor-based reprogramming is very inefficient, time consuming, and labor intensive. Creation of a secondary iPSC system, whereby the previously made iPSCs (primary iPSCs) could be differentiated into somatic cells then reprogrammed back into secondary iPSCs with exposure to doxycycline, creates a much simpler and more efficient model for the study of regenerative medicine. As expected, reprogramming to secondary piSPCs was significantly faster than the original reprogramming protocol. Secondary piPSC colonies were first seen on day 5, versus day 10 with primary piPSCs. The secondary piPSCs were also still dependent on doxycycline to maintain their pluripotency. This was not surprising since the culture conditions were not changed and no additional factors were given during reprogramming. The very weak alkaline phosphatase activity in the secondary piPSCs was very similar to the expression levels
in the primary piPSCs. Immunofluorescence was done for Nanog, a pluripotency marker, and Cd44, a fibroblast marker. As expected, Nanog was not expressed by the EB outgrowths, but expression increased during the reprogramming process. Once transferred to IR-MEFs, Nanog was only expressed by the secondary iPSC colonies that formed. The EB outgrowths uniformly expressed Cd44, but as the cells underwent reprogramming, the Cd44 expression became isolated to cells that were not undergoing morphological changes associated with reprogramming. Following transfer to IR-MEFs, only the IR-MEFs expressed Cd44 and the colonies of secondary piPSCs were negative. This supports a transition from differentiated fibroblast-like cells to pluripotent cells. Additional tests, such as PCR, could have been done to provide further support that the secondary piPSCs were comparable to the primary piPSCs, but the project was ended before these tests could be conducted.

3.5 Conclusions

Using a doxycycline inducible lentiviral reprogramming system we were able to successfully produce both murine and porcine iPS lines. With the porcine iPSCs we were also able to create secondary piPSCs that appear to be equivalent to the primary piPSCs, but are a much more efficient system to work with. The piPSCs are between naïve and primed type, displaying some but not all characteristics of naïve cells. Unfortunately, the piPSCs are also not fully pluripotent because they rely on doxycycline to maintain pluripotency.

The piPSCs established here have potential for a variety of uses for both in vitro and in vivo regenerative medicine studies in the future. For example, the secondary piPSCs system could be used to study the process of reprogramming. Efforts could be continued to produce truly pluripotent cells that aren’t dependent on exogenous reprogramming factors to maintain pluripotency. This could be done through supplementation of different additives to the media [148,164] or through the knockdown of proposed roadblocks to reprogramming, such as Mbd3 [149,175]. Another use for this system would be the development of directed differentiation protocols to guide the differentiation of piPSCs into desired cell types. The embryoid body protocol used here could be modified by adding growth factors to the media to direct differentiation towards germ layers or potentially specific cell types. There are many proposed protocols for this, but results vary greatly [129, 142-147]. The differentiated cells could then be used for disease modeling. For example, piPSCs directed towards becoming cardiomyocytes could then be injected into the hearts of pigs that have undergone myocardial infarctions.

Even though the piPSCs produced here are still dependent on the expression exogenous reprogramming factors in order to maintain pluripotency, there are many potential applications for them in the future. Pigs are the most similar animal model for human diseases because of their morphological, physiological, anatomical, and immunological similarities. However, pig models are not as developed as the more commonly used mouse models. Further research with the piPSCs created here could help better develop pigs as a disease model in addition to making advancements in the area of regenerative medicine.
References:


APPENDIX

Figure 1: PCR ladders. (a) Lambda DNA – Hind III digest ladder. New England Biolabs #N30125. (b) φX174 DNA – Hae III digest ladder. New England Biolabs #N30265. (c) 100 base pair ladder. New England Biolabs #N3231S.

<table>
<thead>
<tr>
<th>% AGAROSE</th>
<th>RANGE OF DNA (KB)</th>
</tr>
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<tbody>
<tr>
<td>0.3</td>
<td>5-60</td>
</tr>
<tr>
<td>0.6</td>
<td>1-20</td>
</tr>
<tr>
<td>0.7</td>
<td>0.8-10</td>
</tr>
<tr>
<td>0.9</td>
<td>0.5-7.0</td>
</tr>
<tr>
<td>1.2</td>
<td>0.4-6.0</td>
</tr>
<tr>
<td>1.5</td>
<td>0.2-3.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.1-2.0</td>
</tr>
</tbody>
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Table 2: Guide for selection of agarose gel for electrophoresis. Based on size of DNA to be separated.