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The Partial Reprogramming of Mammalian Somatic Cells by Pluripotent Cell and Oocyte Extracts

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### Publication Date

2013

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UNIVERSITY OF CALIFORNIA  
RIVERSIDE

The Partial Reprogramming of Mammalian Somatic Cells by Pluripotent Cell and Oocyte  
Extracts

A Dissertation submitted in partial satisfaction  
of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

David Daniel Parker

August 2013

Committee Members:

Dr. Frank Sauer, Chairperson

Dr. Prue Talbot

Dr. Russ Hille

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2013

The Dissertation of David Daniel Parker is approved:

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Committee Chairperson

University of California, Riverside

**My Committee:**

Frank Sauer - Bold ideas require risk and vision, thanks for encouraging me to try something big instead of safe, simple, or meaningless science. The elegance of approach, unique way of analyzing experiments and situations, I owe to your guidance. My time at UCR was made more fruitful by limited financial worries as compared to other students, and I thank you for your concern.

Prue Talbot – Thank you for investing your time and effort, I enjoyed several long conversations with you, and I wish I had spoken with you sooner and more often.

Jolinda Traugh – You provided a unique point of view for my project and I thank you for your helpful comments and encouragement.

Russell Hille – When I needed help, you came to my aid. Thanks for being a stand-up guy.

**The Sauer Lab:**

Silvia Sauer – Thank you for advice, human support, and hard work.

Stephane Bertani – Our debates have led me to a well-rounded view of scientific philosophy and humanity. Thanks for all your help; you are a lifelong friend to me.

Brian Gadd – You succeeded where others would have failed through intelligence, hard work, and dedication to science. Your hard work got me through some of my hardest

times, and when I was desperate you were always there for me. My appreciation cannot be measured.

Joshua Halford – My introduction to the lab was a result of your initial challenges to prove myself. I also owe you for some of the finest times in California, and I could always depend on your help when I needed it.

Alice Kan – Thanks for being a friend and helping me when I needed it. I always felt comfortable and welcomed by both you and Josh.

Tristan Copeland – Your work and interest motivated me more than you realize. Thanks for your help, dedication, and genuine interest. I consider you a true friend.

Alexandra Koetter- You helped me to deal with many challenges and people. Your imprint is left on me and on UCR forever.

Stephen Sakai – When times were tough I could depend on your help. Thanks my friend.

Ninuo Xia, Flora Psi, Dawai, thanks for the days that unite us in smiles and hard work.

**Family:**

Mary Parker (mom) – I owe my creative ability to you for spoiling me and nurturing me as a kid. Your support got me through some of the hardest times. Thanks for your help for all my years; I could not have a better mother.

David Parker (dad) – Your hard work enabled me to go to school and showed me good work ethic. I do not tell you often enough how much you mean to me. I could not have a better father.

Chad Parker (brother) – By marching your own path, you left me proud and free from worry. And I could always focus on my work and school knowing you were with mom and dad. I could not have a better brother.

Brittany Parker (sister) – You are a good person, and I never have to worry for your choices in life. I am anxious to see you grow up, and I am very proud of you. I could not have a better sister.

Judith Louviere (aunt) – Somehow, I feel like you were secretly pushing me when times were hard. I will never ever forget you.

Krista Sao – My love, my treasure. With you by my side, I can do anything. Your love and hard work kept me sane, as it will through all the coming years!

Bruno Rodriguez, Matthew Goitia

Grandma and Grandpa

**Others:**

Michael Doughty – You gave me my first stint in the lab, and I took that experience to heart.

Cheryl Offner – Your initial encouragement in science, gave me the spark I needed.

Dawn Loyola – Through all the pain and suffering, I knew one person at UCR genuinely cared about me. Thanks for minimizing my worries!

Kara Oswood, Kathy Redd, Melissa Gomez

**For Materials and Equipment:**

The California Institute of Regenerative Medicine (CIRM) funded UCR Stem Cell Core

The Ernest Martinez Laboratory: microscope use and materials

The Joao Pedra Laboratory: microscope use and materials

The Guillermo Aguilar Laboratory: FITC-dextrans

**References**

Extract reprogramming is challenging and there are few laboratories working on this technique. I would like to thank those, whom pioneered extract-based reprogramming, and I hope my references do you justice.



## **Dedication**

I dedicate this to my wonderful family; the hardest thing about my PhD was the time away from you. I hope you know how much you truly mean to me. I will always love and miss my mother, father, brother, sister, and Aunt Judy.

And to Krista, your love made this time amazing for me. Muah I win! :-P

## ABSTRACT OF THE DISSERTATION

The Partial Reprogramming of Mammalian Somatic Cells by Pluripotent Cell and Oocyte Extracts

by

David Daniel Parker

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology  
University of California, Riverside, August 2013  
Dr. Frank Sauer, Chairperson

During mammalian development pluripotent stem cells produce a myriad of structurally and functionally distinct somatic cells. The reprogramming of somatic into pluripotent cells is fundamental to the development of patient-specific cell sources applicable to biomedical science and regenerative medicine. Different experimental approaches can reprogram somatic into pluripotent cells: the induced pluripotent stem cell (iPSC) technique, somatic cell nuclear transfer (SCNT), and cell-cell fusion. However, the factors and mechanisms underlying somatic cell reprogramming remain elusive. Extract cell reprogramming is a suitable system to identify factors and mechanisms responsible for somatic cell reprogramming because it is amenable to biochemical assays and manipulations that can identify factors and therefore mechanisms involved in the reprogramming process. One prerequisite for the biochemical dissection of cell reprogramming is the development of a large scale reprogramming system, which uses potent but inexpensive extract sources to reprogram large numbers of somatic cells. Here, I describe the development of a cell extract-based reprogramming system, which

uses protein extract isolated from the oocytes of salmon (*Oncorhynchus tshawytscha*). The developed system supports the digitonin-mediated permeabilization and survival of somatic cells as well as the uptake and retention of large molecules such as proteins within somatic cells. Salmon oocyte extract can at least partially reprogram mouse somatic cells into pluripotent cells. The established extract-based reprogramming system provides evidence that salmon oocytes are a suitable cell source for the identification of factors and the dissection of mechanisms involved in somatic cell reprogramming.

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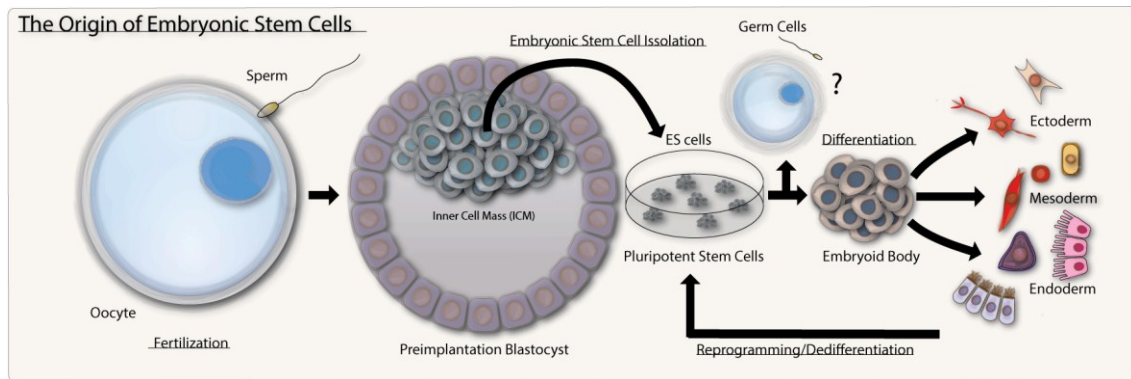
## Part I. History and Approaches of Somatic Cell Reprogramming

### Introduction

Embryonic stem cells (ESCs) are present in and extracted from the inner cell mass (ICM) of the preimplantation blastocyst (**Figure 0.1**).<sup>1,2,3</sup> They possess the qualities of self-renewal and pluripotency, as well as an indefinite proliferative capacity.<sup>1,2,3</sup> *In vitro* ESCs can be maintained in an undifferentiated state, yet grow rapidly as a source of uncommitted cells capable of differentiating into nearly any somatic cell lineage found in an adult organism (**Figure 0.1**). These attributes endow stem cells with enormous potential for developmental science,<sup>4</sup> toxicological research,<sup>5,6</sup> drug development,<sup>7</sup> and regenerative medicine, ideally interrupting the progression of human disease.<sup>6,8,9</sup>

Early embryos are totipotent, meaning they develop into an entire organism as well as extra embryonic tissue required for that organism's development.<sup>10</sup> The ICM is a large cache of ESCs found within the developing embryo prior to its implantation.<sup>1,11</sup> During mammalian development ESCs give rise to the three germ layers (i.e. ectoderm, endoderm, and mesoderm).<sup>12</sup> In mammalian development, these layers establish nearly every specialized cell in the adult organism (**Figure 0.1**).<sup>12</sup> This specialization can be recapitulated *in vitro* by subjecting ESCs to various culture conditions and/or treatment with various chemicals, creating highly specialized cells, which resemble those generated through the natural differentiation processes (**Figure 0.1**).<sup>4</sup> The artificially differentiated cells are currently being used for research and may one day serve as a resource for regenerative medicine, replacing damaged or diseased tissue.<sup>8</sup> Nevertheless, two key

problems remain: **1.)** the controversial use of embryos as a source for human pluripotent stem cells,<sup>13</sup> and **2.)** the potential immunorejection of differentiated cells and tissues, derived from embryos that are not of isogenic origin.<sup>14</sup> Therefore, a source for pluripotent stem cells other than human embryos is required, which generates cells that would not be subjected to immunorejection. One route toward this goal is known as somatic cell “reprogramming”, whereas a differentiated somatic cell “program” is reverted into an embryonic stem cell-like “program” (**Figure 0.1**).<sup>15</sup> Although it has been highlighted in recent years, somatic cell reprogramming was first achieved over sixty years ago using a technique called somatic cell nuclear transfer (SCNT).<sup>16,17</sup>



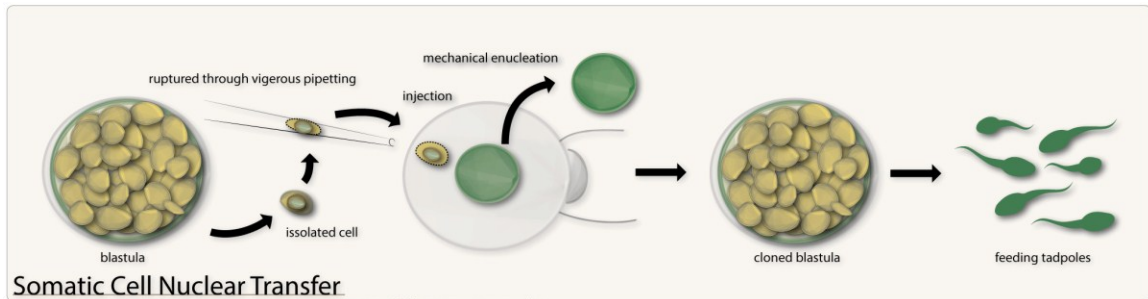
**Figure 0.1** Origins of pluripotent cells. Following fertilization, embryonic stem cells (ESCs) are isolated from the inner cell mass (ICM) of the preimplantation blastocyst.<sup>1,11</sup> ESC are characterized by self-renewal, rapid growth, and pluripotency, and can differentiate into specialized cells from the three germ layers<sup>4</sup>. Differentiated or somatic cells can be reprogrammed from a differentiated state toward one of pluripotency which resembles the state of ESCs.<sup>15</sup>

## The Birth of Reprogramming – Briggs and King

During the first half of the 20<sup>th</sup> century, the developed or differentiated nucleus of somatic cells was poorly understood<sup>18</sup> and the underlying genetic code responsible for development was still a mystery. Whether somatic cells contained the information required to generate a full organism or whether genetic information underwent permanent and irreversible changes throughout development remained unknown.<sup>18</sup> To better understand the undifferentiated state and development, Robert Briggs and Thomas J King developed the technique known today as SCNT (**Figure 0.2**).<sup>16</sup> During SCNT, a somatic cell nucleus is deposited into an enucleated oocyte, typically through microinjection.<sup>19</sup> In their pioneering efforts, Briggs and King mechanically enucleated an oocyte recipient from the Northern leopard frog *Rana pipiens* and injected a ruptured donor cell (intact nucleus) from the blastula-stage of the same species. This technique yielded developing blastulae and in some cases tadpoles (**Figure 0.2**).<sup>16</sup> Although Briggs and King generated a reproductive clone from blastulae, they were unsuccessful at obtaining clones using differentiated somatic cells leading them to believe that nuclei are irreversibly changed throughout development.<sup>16,20</sup> Even so, this experiment provided an initial insight into the reprogramming process and was acknowledged for its wider implications, as realized by John Gurdon.<sup>21</sup>

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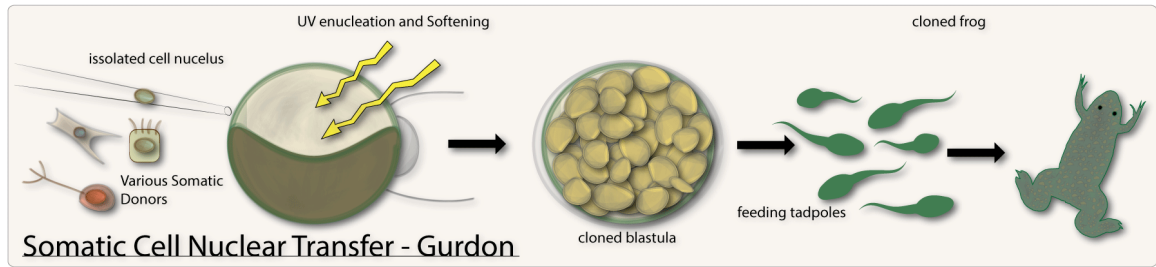
**Figure 0.2** SCNT as achieved by Briggs and King (1952). Cells were removed from various regions of the early blastulae *Rana pipiens*, ruptured by vigorous pipetting, then injected into a mechanically enucleated oocyte.<sup>16</sup> The oocyte then developed into irregular blastulae, and in rare cases normal blastulae and then feeding tadpoles.<sup>16</sup>

### Somatic Cell Nuclear Transfer - Gurdon's Frogs

In 1962, John Gurdon used oocytes from the African clawed frog *Xenopus laevis* as a cellular recipient for SCNT; coincidentally these oocytes were exposed to ultraviolet light resulting in the break-down of the jelly exterior and the nucleus of the oocytes (**Figure 0.3**).<sup>17,18</sup> As nuclear donors Gurdon chose intestinal epithelium cells from tadpoles of the same species, the tadpoles contained a genetic marker, absent from the oocytes, and allowed the identification of off-spring originating from the somatic cells (**Figure 0.3**).<sup>17,18</sup> The resultant embryos developed into blastocysts, tadpoles, and in some cases adult frogs (**Figure 0.3**).<sup>21</sup> These experiments were the first demonstration that SCNT can be used to generate a complete organism utilizing a differentiated somatic cell nuclear donor.<sup>22</sup> They also revealed somatic cell nuclear plasticity and indicated that differentiated somatic cells can be reprogrammed by an oocyte into a pluripotent cell that can generate the myriad of different cell types present in a multicellular organism.

Initially, these results were met with skepticism,<sup>18</sup> but in subsequent experiments using donor nuclei of various origins and genetic composition, Dr. Gurdon proved the validity of his previous results and began to identify a central dogma of reprogramming; highly differentiated, developed nuclei are more resistant to reprogramming than lesser differentiated cells.<sup>18</sup>

Observable changes in the nucleus upon injection eventually led to interest in the molecular events responsible for the reprogramming process.<sup>21,23,24</sup> When nuclei from various species were injected into *Xenopus laevis* eggs, they underwent vast structural changes.<sup>24</sup> For example, after being injected into *X. laevis* eggs, nuclei from human HeLa cells, a cultured cervical cancer line, undergo definable changes including nuclear enlargement and chromatin dispersal.<sup>24</sup> Although the injected HeLa nuclei do not form a true embryo, the observed changes in nuclear architecture supported the existence of specific mechanisms and activities involved in reprogramming. Upon injection, HeLa nuclei synthesize RNA and release proteins while enlarging and incorporating oocyte specific proteins (including histones) and RNA.<sup>24</sup> These changes coincided with a significant increase in transcription, the transcripts of which would take decades to identify (see **Extract-Based Reprogramming** below).<sup>22</sup> These experiments pointed toward the inevitable prospect of mammalian cloning, spawning a new field which seeks to convert a mammalian somatic nucleus toward a totipotent state and the creation of viable cloned organisms.



**Figure 0.3** SCNT as performed by John Gurdon (1962). Various differentiated nuclear donors from the species *Xenopus laevis* were injected into UV treated, enucleated eggs of the same species resulting in feeding tadpoles and full-grown frogs.<sup>17,18</sup>

### Somatic Cell Nuclear Transfer - Dolly the Sheep

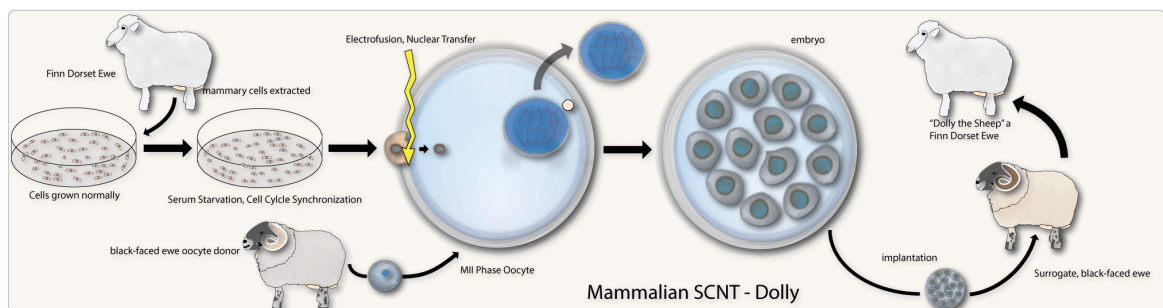
Following the success of SCNT in *X. laevis*, there were many attempts to perform similar experiments in mammals.<sup>18</sup> During the decades following Gurdon's work in frog these attempts were unsuccessful leading to the belief that mammalian somatic cell nuclei do not have the same plastic reprogrammable nature as of the nuclei of amphibians or are changed irreversibly throughout development.<sup>13,18</sup> Experimental and natural challenges distinguish *X. laevis* and mammalian systems. To overcome these challenges, many technical refinements were required in terms of microscaling and surgical implantation.<sup>18</sup> SCNT in *X. laevis* benefits from a relatively large oocyte recipient, approximately ten times that of mammals.<sup>18,25</sup> Moreover, amphibians, such as frogs, do not require a mother or surrogate to progress through development as with most mammals. Aside from technical challenges, natural challenges also govern the success of SCNT in mammals. In early mammalian SCNT experiments, enucleated early embryos or zygotes were used as

recipients, as zygotic material was considered more capable of progressing an organism through development and survival thereafter.<sup>18</sup> On its own, the use of oocytes as recipients improved the success of mammalian SCNT, however only when donor nuclei originated from early developmental donors such as blastocysts reminiscent of early work by Briggs and King.<sup>18,26</sup>

In 1996, Keith Campbell and Ian Wilmut made a critical breakthrough in SCNT using sheep as a model system by considering the cell cycle in both donor and recipient cells.<sup>27,28</sup> By synchronizing the cell cycle of both donor cells and recipient oocytes prior to SCNT, the cells of the resulting embryo became prone to proliferate and developed into a complete sheep. Oocytes at metaphase of the 2<sup>nd</sup> meiotic division (MII) are best able to reprogram somatic nuclei during mammalian SCNT, for reasons which are not entirely clear.<sup>29</sup> Some researchers suspect that the MII oocyte is simply easier to manipulate,<sup>29</sup> but MII oocytes also have the highest level of cyclin-dependent kinase 1 (CDK1), also known as Maturation Promoting Factor (MPF), which is responsible for chromosome condensation and breakdown of the somatic cell nucleus.<sup>30</sup> CDK1 is slowly reduced during cell division, in theory allowing somatic nuclei to progress and divide normally.<sup>27</sup> Donor nuclei are also synchronized to the diploid G<sub>0</sub> or G<sub>1</sub> cell cycle phase which seems compatible with the MII oocyte allowing proper cell cycle progression.<sup>30</sup> Campbell and Wilmut used an adult Finn Dorset ewe nuclear donor in the form of cultured mammary cells,<sup>28</sup> which had been serum starved toward G<sub>0</sub> phase (**Figure 0.4**),<sup>28</sup> and MII oocytes originating from an adult Scottish Blackface ewe (**Figure 0.4**).<sup>28</sup> The chromatin was removed from the oocytes and the enucleated oocytes were directly

fused to a mammary cell facilitating somatic nuclear delivery.<sup>28</sup> The resultant embryo was then placed into an adult Scottish Blackface ewe surrogate and a lamb, 6LL3, was born 148 days later.<sup>28</sup> This lamb would grow to become famous as “Dolly” the sheep (Figure 0.4).<sup>28</sup>

Dolly was succeeded by many clones thereafter, using similar methods, including: cow,<sup>31</sup> cat,<sup>32</sup> dog,<sup>33</sup> horse,<sup>34</sup> mouse,<sup>35</sup> rabbit,<sup>36</sup> monkey,<sup>37</sup> and many others.<sup>38</sup> Each revealed the conserved nature of oocyte-mediated reprogramming and reinforced the concept of nuclear plasticity in mammals. Considering the success of animal cloning, human cloning has been attempted with limited success.<sup>39,40</sup> The creation of human clones by using SCNT for either therapeutic or reproductive purposes would serve as a major breakthrough while at the same time leading to both ethical and moral dilemmas. One way to overcome these concerns would be in the generation of cellular material, as opposed to embryos, made possible by the discovery of embryonic stem cells.



**Figure 0.4** SCNT in mammals (1997). Somatic cells were isolated from the mammary tissue of a “Finn Dorset ewe”, after a brief time in culture these cells were arrested in the  $G_0$  cell cycle phase through serum starvation.<sup>28</sup> Then a  $G_0$  nucleus was delivered through cell electro-fusion to an MII phase oocyte originating in from a Scottish black-faced ewe donor. Subsequently, the developing embryo was implanted in a surrogate black-faced ewe, which eventually gave birth to a Finn Dorset Ewe – Dolly the sheep.<sup>28</sup>

## **A Change in Approach**

Malignant germ cell tumors known as teratocarcinomas contain undifferentiated cells among an array of differentiated cells culminating in the overall tumor mass.<sup>41,42</sup> Embryonic carcinoma cells (ECCs) derived from teratocarcinoma tumors are capable of rapid-growth, self-renewal, and differentiation into the three germ layers.<sup>43</sup> These functional features have made ECCs central to early developmental studies, especially in mouse.<sup>41,42</sup> In terms of differentiation, ECCs in general and humans ECCs in particular have limited, lineage preferred developmental potential.<sup>42</sup> However, because of germ line tumor origin and an irregular karyotype, ECCs represent an incomplete or even misleading model for mammalian embryonic development.<sup>42</sup> A model for recapitulating true embryonic development was thus needed confirming or refining studies founded in ECCs.<sup>1,42</sup>

Mouse ESCs (mESCs) were first isolated by Martin Evans, Matthew Kaufman, and Gail R. Martin from the ICM of 4-6 day old mouse blastocysts.<sup>1,2</sup> When plated onto feeder cells, they formed large colonies reminiscent of ECC morphology.<sup>21</sup> Upon subcutaneous injection into mice, they can form teratomas, which contain differentiated cells representing (or derived) from all three germ layers. Feeder cells were crucial to the cultivation of ESCs, as the feeders provide exogenous factors to maintain self-renewal [leukemia inhibitory factor (LIF)] and inhibit differentiation [bone morphogenic proteins (BMPs)].<sup>8</sup> Research using mESCs has advanced our understanding of developmental

biology and provided an indispensable tool for the assessment of gene function *in vivo*.<sup>44</sup> Through the use of an engineered DNA, modifications or disruptions of specific genes (i.e. “knockout”) can be introduced into cultured mESCs,<sup>45</sup> The mESCs, which incorporate modified, knockout genes, can be selected on the basis of drug resistance. Selected mESCs can be injected into mouse blastocysts for transplantation into surrogate mothers.<sup>45</sup> The resultant chimeric mice are then bred with wild-type mice until heterozygous mice are born which are then can be bred to produce homozygous mice.<sup>45</sup> Typically, the effects of knockouts are evident through phenotypical changes, thereby allowing the characterization of the genes function.<sup>45</sup>

It took much longer for human ESCs (hESCs) to impact science; unlike mouse embryonic stem cells, there is a limited availability of human embryos for research and culture conditions of hESCs are different from those of mESCs.<sup>42</sup> Ethical and moral questions as well as legal hurdles prevent the use of fertilized eggs specifically for research;<sup>39,40</sup> therefore, initial experiments relied on donated embryos produced through *in vitro* fertilization (IVF). Jamie Thompson manipulated these embryos through immunosurgery to isolate the ICM by first targeting the trophectoderm, an extra embryonic tissue, with nonspecific antibodies followed by incubation with sera complement to lyse these cells thereafter.<sup>3,46,47</sup> The surviving ICM cells were cultivated *in vitro* and identified as hESCs. Although initial differences in isolation and culture conditions limited derivation of hESCs,<sup>42</sup> they relied on culture conditions similar to mESCs and hESCs, require basic fibroblast growth factor bFGF instead of LIF,<sup>48</sup> and have traditionally been cultured on feeder cells or media derived from mouse embryonic

fibroblasts (MEFs), though much effort has been made to culture ESCs under “feeder-free” conditions.<sup>49</sup>

Both mESCs and hESCs share the characteristic features of ESCs: rapid growth, self-renewal, and differentiation and express pluripotent gene markers such as *Oct4*, *Sox2*, and *Nanog*.<sup>42</sup> In addition, ESCs express specific surface markers such as stage-specific embryonic antigen 1 (SSEA1) in mESCs; SSEA3 and SSEA4 in hESCs.<sup>42,50</sup> A more definitive approach, is the assessment of differentiation capacity *in vivo*.<sup>51</sup> To assess the pluripotency of ESCs, the teratoma assay has been developed. When subcutaneously injected into mice, mESCs and hESCs form tumors which contain cells derived from all three germ layers.<sup>8,42</sup> Limited developmental potential as evident by the inability of cells to form three germ layers has been termed multipotency.<sup>10,52</sup> The differentiation of ESCs into cell and tissue types from all three germ layers denotes their pluripotent potential as opposed to multipotency or other forms of limited developmental potential. ESCs also share an epigenetic signature.<sup>53</sup> The promoter of pluripotent genes is demethylated, and decorated with histone modifications associated with transcriptional activation.<sup>53</sup> At somatic genes just the opposite is observed with methylated promoter DNA and repressive histone modifications.<sup>53</sup> Intermediate, early developmental genes share both active and repressive epigenetic marks in a state termed bivalency.<sup>50,54</sup>

The isolation of hESCs, while considered controversial, changed developmental science in a fundamental way.<sup>8,13,52</sup> With the discovery of hESCs arose the possibility to reprogram human somatic cells *in vitro*, toward a state of pluripotency by using SCNT. If



cultured cells could be reprogrammed toward a pluripotent state, then the moral ambiguity of using human embryos or pluripotent human embryos generated by SCNT could be avoided and cells which have all the characteristic properties of embryonic stem cells (i.e. rapid growth, self-renewal, pluripotency) could be generated from somatic donors.<sup>8</sup> ESCs also set a benchmark by which the success of all reprogramming experiments can be judged by comparing the morphological and molecular properties of cells obtained through cell reprogramming with the properties of ESCs.<sup>55</sup> If successful, pluripotent cells generated by cell reprogramming could then be used for research purposes or, one day, for regenerative medicine.

### **Reprogramming through Cell Fusion**

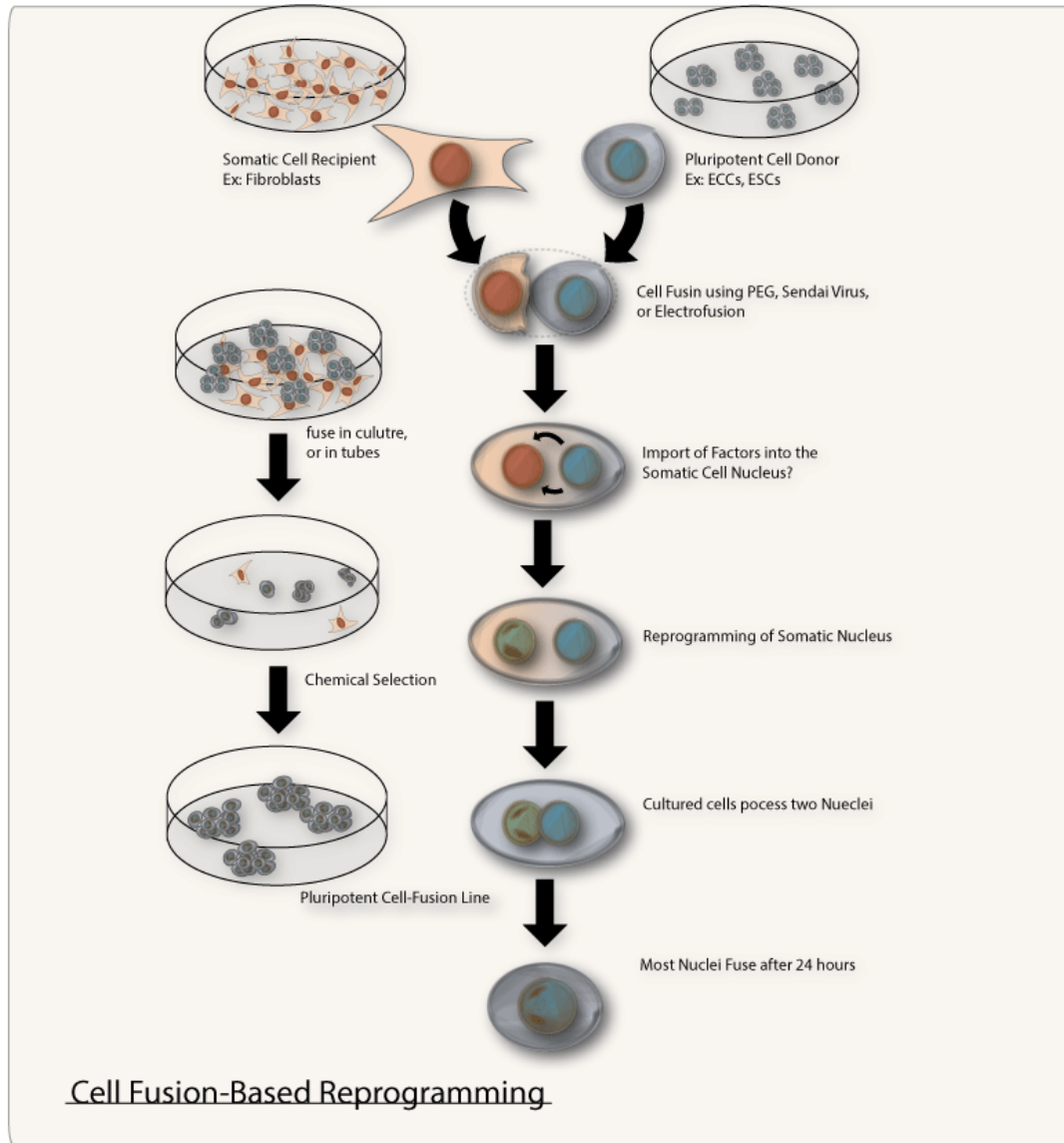
The first documented procedure for cell reprogramming was through artificial cell fusion. Cell fusion has been known to occur in specific cell types of the human body such as muscle progenitor cells.<sup>56</sup> Cell-cell fusion can be achieved artificially following brief exposure to poly-ethylene-glycol (PEG),<sup>57</sup> Sendai virus,<sup>58</sup> or through the use of an electrical current (**Figure 0.5**).<sup>59</sup> This technique has revolutionized human medicine through the creation of monoclonal antibodies.<sup>60</sup> By fusing Myeloma cells with antibody producing cells from the spleen, the resultant hybrid cells can produce antibody indefinitely.<sup>60</sup> This technique has been used for reprogramming since the 1970s, when ECCs were fused to thymocytes resulting in ECC-like hybrids which greatly resembled ECCs and were capable of tumor differentiation.<sup>58</sup>

However, the implications of this work as a model for cell reprogramming were neglected until a series of works from the team of Takashi Tada.<sup>59,61,62</sup> Tada's laboratory established systems by which mouse ESCs and embryonic germ cells (EGCs) could be fused to somatic cells,<sup>59,61,62</sup> generating hybrid cells with features reminiscent of the pluripotent state. Cells associated with an early developmental identity, such as EGCs, ECCs,<sup>63</sup> and ESCs can be fused to somatic cells, resulting in hybrid cells which exhibit properties representative of the corresponding earlier developmental type (**Figure 0.5**).<sup>59,61,62</sup> ESC-somatic cell hybrids express marker genes found in ESCs,<sup>61,62</sup> maintain differentiation capacity,<sup>64</sup> and form colonies.<sup>62,65</sup> Thus, these studies provide the first evidence that somatic cells can be reprogrammed by cultured pluripotent cells toward a pluripotent-like state *in vitro*.

Cells which contain two nuclei or more nuclei are called heterokaryons.<sup>66</sup> Hybrid cells begin as heterokaryons and after the nuclei fuse form an irregular karyotype with time in culture.<sup>64</sup> Although they possess an irregular karyotype, upon injection reprogrammed hybrid cells can contribute to tissue formation in mouse chimeras, supporting the pluripotency of the hybrid cells.<sup>67</sup> An important characteristic of fusion based cell reprogramming are changes in gene expression of somatic cell recipients, whereas markers of pluripotency are expressed even if the pluripotent fusion donor originates from a different species than the somatic recipient.<sup>63</sup> ESC marker genes, *Oct4* and *Nanog* are upregulated when somatic cells are fused to ESCs, while the somatic marker gene *Thy1* is downregulated.<sup>62</sup> *Oct4* and *Nanog* are thought to be central for self-renewal and pluripotency in omnipotent cells.<sup>64,68</sup> Concurrent with these expression

changes are specific changes to histone modification pattern of the hybrid cells.<sup>62</sup> The hybridization of ESCs with somatic cells leads to a global increase in the acetylation of histone H3 and H4.<sup>62</sup> Generally, histone acetylation is associated with transcriptional activation whereas lack of acetylation is associated with transcriptional repression.<sup>62</sup> In hybrid cells, the expression of *Oct4* has been linked to enrichment of H3K4 dimethylation (as detected by ChiP) at the *Oct4* gene promoter, an epigenetic mark associated with transcriptional activation,<sup>62</sup> Fusion based reprogramming experiments pointed towards the importance of epigenetics to the reprogramming process, thus explaining current efforts to dissect epigenetic mechanisms in cell and other types of reprogramming.<sup>57</sup>

Fusion-based reprogramming was used for the first successful reprogramming of human cells.<sup>57</sup> Human BJ neonatal fibroblasts carrying a chemical resistance under the control of the *OCT4* promoter were fused with human stem cells (hESCs) and the resultant hybrids were isolated in culture through chemical selection.<sup>57</sup> When injected into mice these reprogrammed hESC-BJ fibroblast hybrids differentiated into teratomas, containing cells derived from all three germ layers.<sup>57</sup> Moreover, the hybrid cells expressed pluripotency-associated marker genes found in hESCs.<sup>57</sup> Together, the results revealed that pluripotent human cells can be generated by cell fusion. Although beneficial to reprogramming studies, it is important to note that human pluripotent cells generated by cell fusion are limited in medical applications by concerns of tumorigenicity originating from the irregular karyotype of hybrid cells.<sup>69</sup>



**Figure 0.5** Cell reprogramming through cell fusion. Somatic cell and pluripotent cells are centrifuged together, then as a pellet, exposed to PEG,<sup>57</sup> Sendai Virus,<sup>70</sup> or electrical current.<sup>59</sup> Factors from the pluripotent nuclei are thought to enter the somatic cell nuclei, thereby reprogramming or remodeling it toward a state of pluripotency.<sup>64,71</sup> Nuclei are initially separate within fused cells, and then merge in successive cell divisions in culture leading to a reprogrammed cell type.<sup>64</sup> Somatic cells and stem cells can then be removed through isolation or chemical selection.<sup>57</sup>

## IPSC-based Reprogramming

In order to assess the importance of various genes in ESCs, genes can be exogenously added to somatic cells through electroporation as well as chemical or viral transfection.<sup>72</sup> This approach has been employed in attempts to identify factors important to the pluripotent state and somatic cell reprogramming. To date not one cell reprogramming factor has been identified using a single-gene, forced gene expression approach not even when crucial, known regulators such as *Oct4* and *Nanog* were tested.<sup>73</sup> Because the “single-gene” approach cannot recapitulate and identify gene networks can be missed,<sup>68</sup> the failure of the single gene approach suggested that multiple genes are required to induce reprogramming.

A major breakthrough came from experiments in the lab of Shinya Yamanaka.<sup>73,74</sup> Yamanaka and colleagues tried to identify the minimal set of factors required from somatic cell reprogramming. Yamanaka and colleagues used transgenic mouse embryonic feeder cells (MEFs) as factor recipients, which carried an *Fbx15* promoter (an *Oct4* target) coupled to neomycin resistance and beta galactosidase.<sup>73</sup> The reporter gene allowed for the selection and detection of reprogrammed cells. A set of 24 factors was chosen based on their involvement in the pluripotent state, and not a single factor alone activated *Fbx15* alone. However, upon transfection of all 24 factors, mouse embryonic fibroblasts (MEFs) underwent dramatic changes, forming colonies; these cells underwent reprogramming and were called induced pluripotent stem cells (iPSCs).<sup>73,74</sup> The 24 genes would be narrowed down to four: *Oct4*, *Sox2*, *c-Myc*, and *Klf4*.<sup>73,74</sup> **(Fig 5)**. The

production of iPSCs the first form of reprogramming achieved without using materials from oocytes, ESC, EGC, or ECC material. These results have since been duplicated in human cells,<sup>74</sup> and stimulated an entire field of iPSC-based reprogramming.<sup>75</sup>

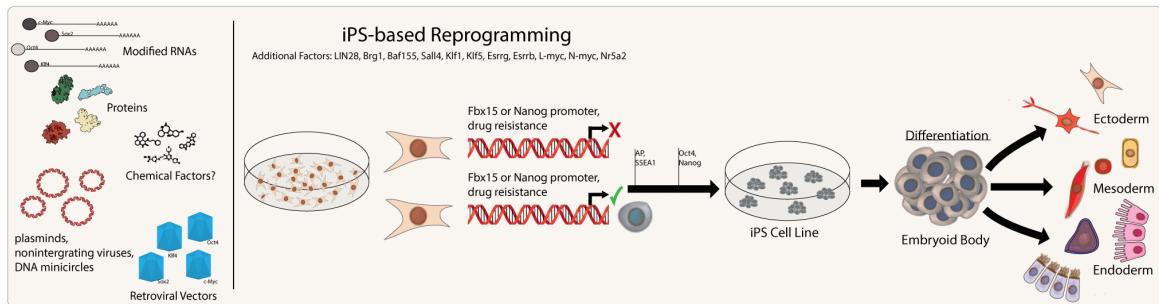
As with other reprogramming techniques, iPSCs have been subjected to various tests of pluripotency.<sup>73,74</sup> One test is morphology: similar to ESC-somatic cell hybrids, iPSCs morphology (3D colonies with defined edges) closely resembles that of ESCs.<sup>73,74</sup> Most iPSCs grow and proliferate at a similar rate to ESCs,<sup>73,74</sup> and can differentiate into three germ layers in culture and in teratomas.<sup>73,74</sup> The global gene expression patterns of iPSCs greatly resemble ESCs,<sup>73,74</sup> Some iPSCs are also able to give rise to mice using the tetraploid complementation method, a stringent method of confirming pluripotency.<sup>76</sup> Briefly, at the two cells stage, embryos are electrically fused forming a tetraploid blastocyst. A tetraploid blastocyst, while capable of contributing to extra embryonic tissue such as placenta, is incapable of forming viable offspring. This blastocyst will only continue through development with the contribution of exogenous, diploid tissue.<sup>76</sup> In such an experiment ESCs are able to contribute to the developing mouse, as they are pluripotent and differentiate into tissue from each of the three germ layers as well as germ cells.<sup>76</sup> Viral transduced iPSCs are considered near-equivalent to ESCs for their ability to form blastocysts and adult mice.<sup>76</sup>

Traditionally, iPSC reprogramming relies on the use of retrovirus to deliver the four factors.<sup>73,74</sup> Retroviruses randomly integrate into the genome, which may lead to ectopic, aberrant gene expression following reprogramming. Since *c-Myc* and *Klf4* are

proto-oncoproteins and the other reprogramming factors regulate cell proliferation, tumor formation is a major concern for using iPSCs in regenerative medicine.<sup>75,77</sup> Therefore efforts have been made to avoid the use of retroviral vectors and identification of reprogramming factors other than the four originally identified factors.<sup>78</sup> Alternate methods have been described which utilize non-integrating viruses,<sup>79,80</sup> plasmids,<sup>81</sup> DNA mini circles,<sup>82</sup> stabilized RNAs,<sup>83</sup> or delivered proteins,<sup>84</sup> and attempt chemical replacement.<sup>85</sup> The efficiency of each method varies greatly.<sup>86,87,88,89</sup> In addition, to addressing the efficiency of reprogramming it is vital to assay the extent, i.e. quality and safety of iPSC-based reprogramming.

While their resemblance to ESCs is remarkable, general differences have emerged even among quality iPSC lines. Closer examination revealed differences in gene expression, and iPSCs tend to “remember” or “recall” their somatic origin by expressing genes of the somatic cell donor following iPSC treatments.<sup>90</sup> This phenomenon has been linked to incomplete changes in DNA methylation patterns following reprogramming.<sup>89,90,91</sup> As compared to ESCs, somatic genes in iPSCs are not properly methylated.<sup>90</sup> When iPSCs and ESCs are derived from B6 mice, an inbred strain, then injected into adult B6 mice, only B6 iPSCs face immunorejection, while B6 ESCs form teratomas.<sup>14</sup> Therefore, an alternate source of cells or additional measures of quality may be required to verify iPSCs, prior to their implementation as donors in regenerative medicine. To achieve suitably reprogramming, an alternate approach or better factors are required; identification of the factors involved in other cell reprogramming methods such as SCNT may allow refinement of the iPSC method, thereby addressing issues of quality

and perhaps efficiency making iPSCs practical and safer for applications of regenerative medicine.



**Figure 0.6** iPSC based reprogramming. Various methods have been used to reprogram somatic cells using four transcription factors (as well as variants and other factors), these include the use of artificially stabilized RNAs, membrane crossing proteins, plasmids, non-integrating viruses, and the original retroviral vectors. Somatic cells are treated with these factors, and reprogrammed cells can be selected through drug resistance. Colonies reprogrammed by the iPSC-method first express alkaline phosphatase, then the surface marker SSEA1, followed by endogenous pluripotent genes. iPSCs have met various tests of pluripotency and are routinely differentiated into various tissues.

### Extract-Based Reprogramming

The foundation of extract-based reprogramming was established through the extract treatment of 293T cell recipients.<sup>92,93</sup> As a cell lineage, the origins of 293T are rather complex. HEK 293 cells were initially isolated from human embryonic kidney (HEK) and immortalized by use of adenovirus<sup>94</sup> Following viral transfection to express the Simian virus 40 large T antigen<sup>95</sup>, HEK 293 cells received the designation 293T.<sup>96</sup> While originally considered a model for kidney development, HEK 293 and 293T cells may actually represent premature neurons.<sup>97</sup> Two studies demonstrate the reprogramming power of extracts, one demonstrated that human Jurkat cell (immortalized T



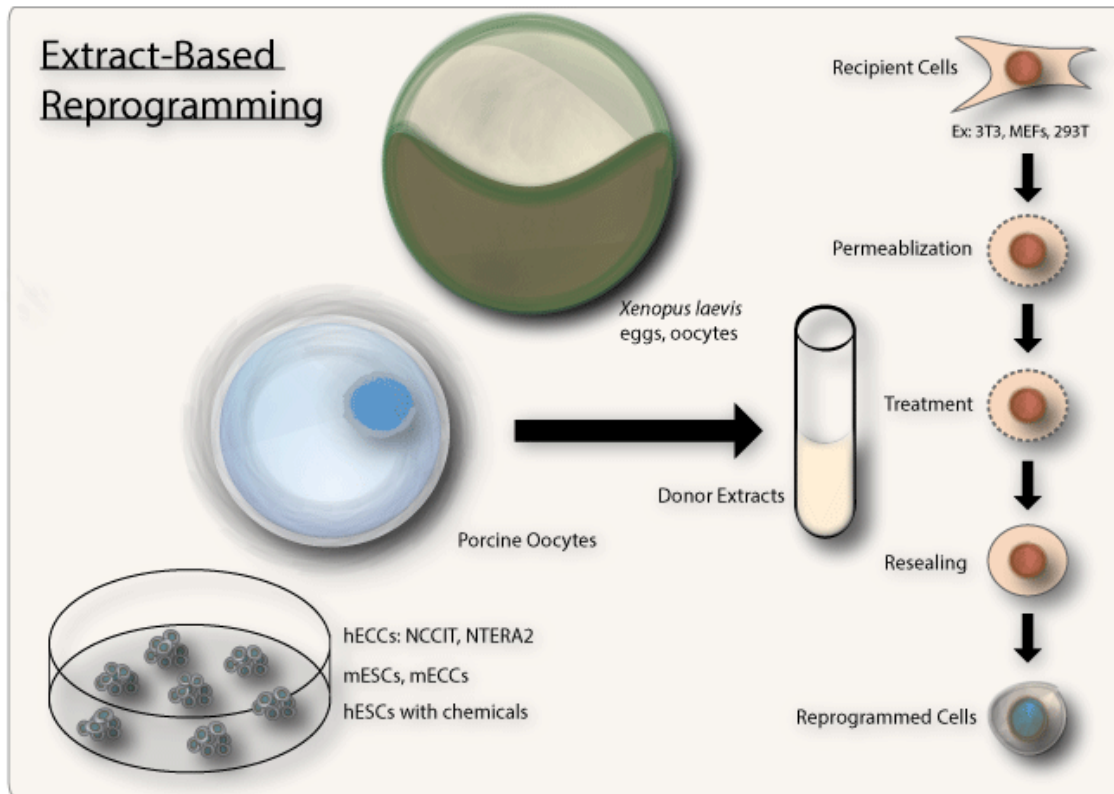
lymphocytes) extracts can transform permeabilized 293T cells toward a pattern of T-cell gene-expression,<sup>93</sup> and another demonstrated *X. laevis* oocyte/egg extracts can induce chromatin remodeling.<sup>98</sup>

In somatic cell nuclear transfer, proteins from the oocyte have direct access to the somatic cell nucleus.<sup>99</sup> In extract-based reprogramming, the plasma membrane represents a barrier to exogenous protein delivery.<sup>100</sup> Therefore, to facilitate access to the somatic cell nucleus, the plasma membrane must be sufficiently permeabilized without killing the recipient. Two permeabilization agents have been successfully used: streptolysin O (SLO) and digitonin, a pore forming toxin and a weak detergent respectively.<sup>101</sup> Both agents allow a wide range of proteins to enter the permeabilized cells: Permeabilized cells can then be treated with protein extracts of interest, but must be resealed for survival. Cells can be resealed with an inhibitor of SLO or digitonin such as CaCl<sub>2</sub>

*X. laevis* oocyte/egg extracts are often used for treatments because of their success in interspecies SCNT.<sup>19,102</sup> 293T cells treated with *X. laevis* egg extracts demonstrate significant changes in both morphology and gene expression such as activation of the pluripotency marker *OCT4* expression.<sup>92</sup> Oocyte, egg, and even blastula extracts from *X. laevis* can induce cell reprogramming, suggesting that the “machinery” required for reprogramming activity is maintained at least until the early stages of embryogenesis.<sup>92</sup> If evolutionarily conserved, similar or even identical machinery may play a role in mammalian reprogramming.

ESCs have been successfully applied as protein donors in extract-based reprogramming. Extracts from ESCs can reprogram permeabilized somatic cells toward an ESC-like state as indicated by changes in morphology, gene expression, epigenetic modifications, and differentiation capacity.<sup>97,103</sup> In addition extracts from ECCs are able to at least partially reprogram 293T cells as evident by the pluripotent marker gene expression, epigenetic modifications, and a limited differentiation capacity of reprogrammed cells.<sup>97</sup> However, while the pluripotent properties of carcinoma cells are retained in treated 293T cells, undesirable properties such as carcinogenic gene expression are essentially lost upon extract treatment.<sup>97</sup>

Extract-based reprogramming of somatic cells can be used to identify the factors and dissect the mechanisms of the reprogramming activity found in oocytes, eggs, ESCs, and ECCs (**Figure 0.7**). Using extracts from oocytes or eggs, the molecular process underlying SCNT can be studied in the form of extract-treated cells. Extracts from ESCs and ECCs can be used to dissect the factors and mechanism underlying cell-cell fusion. Factors involved in cell reprogramming can be identified by purifying the protein activity.<sup>92,104</sup> The identification factors and mechanism underlying responsible for extract based reprogramming, presents an important contribution to reprogramming studies, and may one day serve to refine the iPSC method or lead to alternate approaches to reprogramming.



**Figure 0.7** Cell reprogramming using extracts. Donor extracts can be prepared from oocyte sources, such as porcine MII oocytes or *X. laevis* oocytes and eggs. Cultured pluripotent cells such as mESCs, hESCs, hECs, and mECCs. Recipient cells can vary in origin but require permeabilization, treatment within extract (0.5-3hours), and resealing with  $\text{CaCl}_2$  supplemented DMEM prior to analysis.

## Conclusion

The conversion of somatic cells toward a pluripotent-state can be achieved through the various techniques outlined above. However each technique designed to achieve somatic cell reprogramming has advantages and limitations. While SCNT is the founding form of somatic cell reprogramming<sup>17</sup> and one of the most well studied,<sup>19</sup>

technical and material limitations render SCNT difficult in mammalian systems.<sup>19</sup> Although, the fusion of both pluripotent and somatic cells efficiently forms pluripotent hybrids,<sup>105</sup> hybrid cell lines are difficult to isolate, genetically unstable with genetic contributions from two cell lines, and can form tumors.<sup>65</sup> iPSC-based reprogramming can be used to generate cells, which are remarkably similar to ESCs,<sup>76,77</sup>; however problems have emerged such as immunorejection,<sup>14</sup> tumorigenicity,<sup>106</sup> irregular gene expression,<sup>91</sup> and aberrant epigenetic signatures.<sup>89</sup> Although technically challenging extract-based reprogramming of somatic cells has the potential to identify and dissect naturally occurring reprogramming factors and mechanisms from a wide range of sources including: oocytes, eggs, and cell lines originating from a variety of species.

## **Part II. Activities Associated with Extract-based Reprogramming**

### **Introduction**

A variety of biological activities and processes have been demonstrated in several extract-based reprogramming systems (**Table 0.1**). A principal difference among each system is the source of donor extract which is applied to an assortment of mammalian somatic cell recipients. Sources of extract include pluripotent cell lines such as ESCs and ECCs, as well as oocytes and eggs donors from several species, and each extract source affects cellular recipients differently (**Table 0.1**).

Activities and processes include: colony aggregation,<sup>92</sup> pluripotent gene expression,<sup>92,97</sup> somatic cell gene repression,<sup>25,97</sup> epigenetic modifications,<sup>97,107,108</sup> and surface marker changes.<sup>25</sup> Additionally, extract-based reprogramming has facilitated experimental works addressing the role of epigenetic and nuclear structural changes in cell reprogramming. However, individually observable activities and processes can vary based on the reprogramming system. For example, factors within amphibian oocyte extracts induce reprogramming activities in mammalian cells which are distinctly different from activates induced by whole-cell (ESC, ECC) extracts in the same cells (**Table 0.1**).

Efforts to identify factors involved in extract-based reprogramming have been met with limited success. Proteins have evolved as a main effector in in extract based reprogramming. In extracts treated with enzymes that specifically degrade nucleic acids,

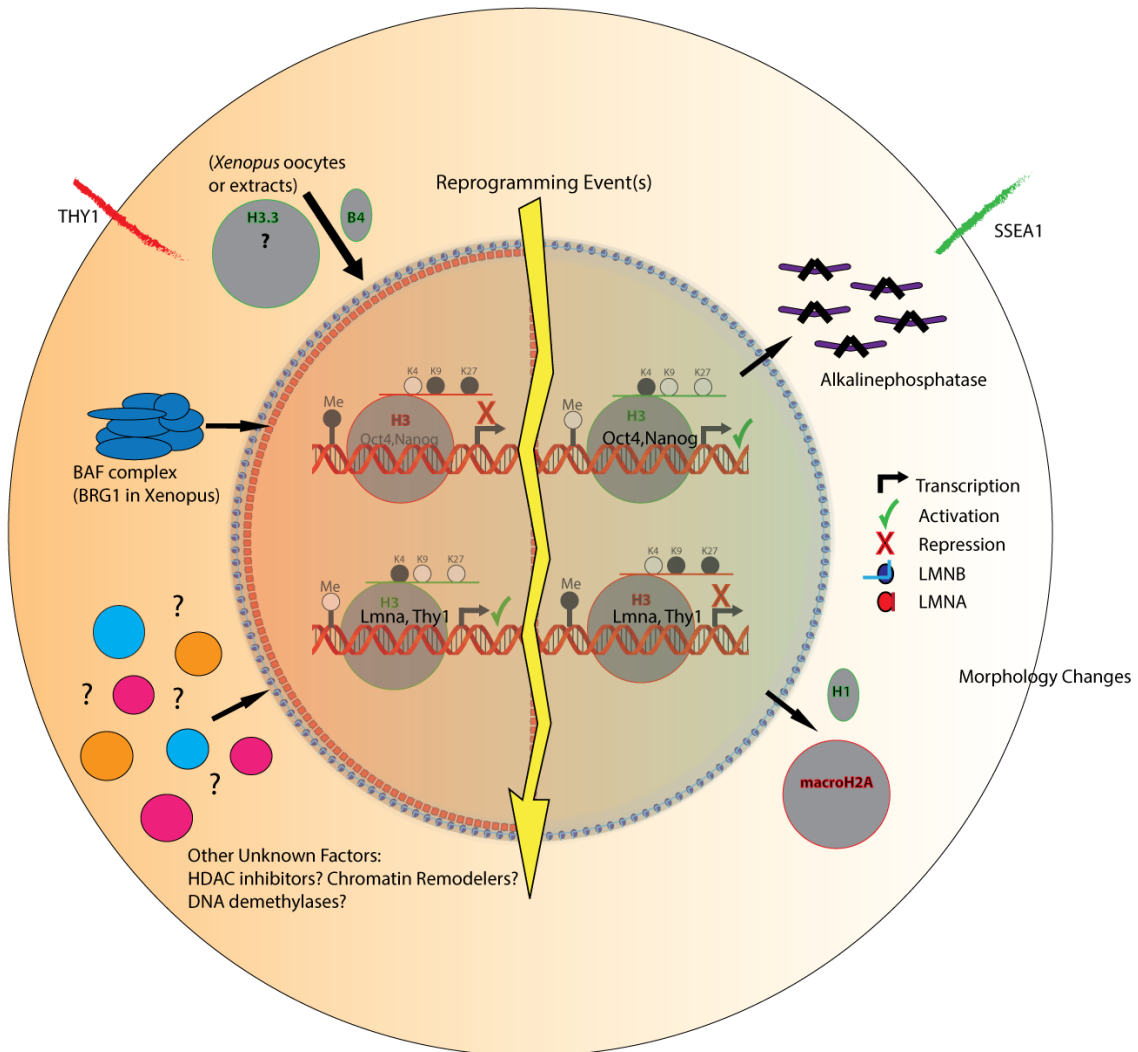
both colony formation and the expression of *Oct4* are maintained; while both activities are lost when cells were treated with the addition of proteases.

Extract Donor	Recipient Cell	morphology	Upregulated	Downregulated	AP activity	Nuclear Structure	Epigenetic (+)	Differentiation	Factor	Chemical	Referend
X. laevis eggs	293T	cell clusters, spheres	<i>OCT4, GCAP</i>	<i>OCT4, GCAP</i>	expressed ( <i>GCAP</i> )			neuronal, adipogenic, osteogenic, and endothelial differentiation	BRG1		(Hansis et al., 2004)
NCCIT (ECCs)	293T	NCCIT-type	genome-wide with <i>OCT4, NANOG, REK1</i>	genome-wide <i>LMNA</i>		loss of LMNA/C	pluripotent promoter demethylation. Histone modifications	protein implicated (DNase, RNAse, heating, trypsin)			(Taranger et al., 2005)
Mouse ESCs	3T3	distinct colonies	<i>Oct4</i>	<i>Lmna</i>	activity						(Taranger et al., 2005)
X. laevis eggs	porcine fibroblasts	small colonies	<i>Oct4</i> unspliced @ 23C spliced @ 37C			incorporation of Lamin LIII	B4 incorporation, global loss of Ace-H3K9	B4, Lamin LIII			(Miyamoto et al., 2007) (Bru et al., 2008)
X. laevis ovary/oocyte	293T					loss of LMNA/C					
46C, J1 (mESCs)	293T	colonies	<i>Nanog, Oct4, Sox2, Klf4</i>	<i>LMNA</i>		loss of Lamin A/C	loss of repressive marks on pluripotent promoters				(Bru et al., 2008)
X. laevis eggs	bovine cummulus		<i>OCT4, SOX2, NANOG</i>			incorporation of Lamin LIII	B4 incorporation	TBP loss			(Miyamoto et al., 2008)
porcine GV oocytes	porcine fibroblasts	colonies	<i>NANOG</i>	<i>COL5A2, FBN2, and TH1</i>			global loss of Ace-H3K9, upstream <i>NANOG</i> demethylation/upstream <i>NANOG</i> demethylation		TSA		(Miyamoto et al., 2009)
porcine MII oocytes	porcine fibroblasts		<i>POU5F1, SOX2</i>				upstream <i>NANOG</i> demethylation				(Miyamoto et al., 2009)
C57/BL6 (mESCs), E14 mESCs	mouse dermal skin fibroblasts	defined colonies	genome-wide with <i>Nanog, Oct4, Sox2</i>		activity		<i>Oct4, Nanog</i> promoter demethylation H3K4 enrichment @ <i>Oct4, Nanog</i> H3K27 loss @ <i>Oct4, Nanog</i>	chimeras and teratomas and multiple examples of ectodermal, endodermal, mesodermal			(Cho et al., 2010)
mESCs	HFFs	defined colonies	<i>OCT4, NANOG, SOX2, KLF4, TP53</i> and <i>CDKN1A</i>	<i>HDM2</i>		LAMIN A/C loss	loss of H3K9 acetyl, limited demethylation <i>Oct4, Nanog</i> promoters	neuronal differentiation			(Han et al., 2010)
axolotl oocyte	MCF7, HCC1954		<i>NANOG</i> (in cts also)								(Allegretti et al., 2011)
F9 (ECC)	MEFs	no visible change	<i>Oct4, Rex1</i>								(Singhal et al., 2010)
mESC C/J7	293T	colonies	<i>Oct4, Nanog</i>	<i>LMNA</i>							Ch. 1
NT2 (ECCS)	293T	bulges at high confluency	<i>Oct4, Nanog</i>								Ch. 1
salmon oocytes	3T3	colonies at 37°C colonies at 10°C	<i>Oct4</i>				activity in defined colonies, no activity in irregular colonies				Ch. 4

**Table 0.1** Extract based cell reprogramming in published works. Somatic recipients such as MEFs, 293T, and 3T3 cells have been treated with amphibian (green), mammalian (white), and fish (orange) extracts eliciting various activities including: morphological changes, gene activation, gene repression, nuclear structural changes, and implicated epigenetic modifications. While same species extract experiments have revealed multiple, extensive activities, interspecies experiments elicit limited forms of reprogramming.



## Extract Reprogramming Activities



**Figure 0.8** Activities associated with extract-based reprogramming. When permeabilized somatic cells are exposed to extracts, factors reprogram somatic nuclei toward a state of pluripotency. While many factors are unknown, components of the BAF complex play a role,<sup>104</sup> as well as the histone variant B4.<sup>109</sup> Genes associated with pluripotency are reprogrammed from an epigenetic state of inactivation toward an active state.<sup>103,108</sup> Genes associated with the differentiated state are driven toward an inactive epigenetic. This results in pluripotent gene expression,<sup>103</sup> and the expression of SSEA-1,<sup>103</sup> and alkaline phosphatase,<sup>92,103</sup> markers of pluripotency.

## Cell Morphology Changes

An observable change during cell reprogramming experiments is the formation of colonies by cells which normally exhibit a flat adherent morphology. Because pluripotent cells grow in colonies, colony formation is used as a benchmark for cell reprogramming experiments and often correlates with other reprogramming activities such as pluripotent marker expression.<sup>110</sup>

In mammalian cells treated with *Xenopus laevis* eggs, morphological changes often occur within 4-7 days following treatment.<sup>109,92</sup> Treated 293T cells first form colonies which eventually form large spheroid cell clusters that resemble embryoid bodies, spherical clusters of spontaneously differentiation stem cells.<sup>92</sup> Interestingly, when *X. laevis* egg extracts are applied to porcine cells, colony formation is rapid occurring after only four days.<sup>109</sup> In both cases, colony formation coincided with reprogramming activities such as the expression of pluripotent marker genes<sup>92,109</sup>

293T cells treated with human NCCIT (ECC) extracts form flat colonies, which resemble the NCCIT cell donor and this morphological change becomes more pronounced with time in culture.<sup>97</sup> This morphological change is concurrent with multiple reprogramming activities, such as global changes in gene expression and epigenetic signatures associated pluripotent cells (**Table 1**).<sup>97</sup> In some extract-based reprogramming experiments, morphological changes are not evident such as in some preparations of extracts.<sup>104</sup> In those cases, other reprogramming activities occur in extract treated cells, but they are limited when compared to cells which can form colonies.

## Changes in Gene Expression

Depending on the extract source, a variety of gene expression changes have been reported in extract treated cells (**Table 0.1**). Similar to colony formation, *Oct4* expression is used as an indicator of cell reprogramming.<sup>92,109,111,112</sup> In 293T cells treated with *X. laevis* egg extracts, *Oct4* expression occurs six days following treatment.<sup>92</sup> *Oct4* expression can also be induced by *X. laevis* blastula extract donors (stages 3-8) up until the midblastula transition,<sup>92</sup> supporting that reprogramming potential is retained during early embryogenesis. In porcine kidney cells treated with *X. laevis* oocyte extract, *Oct4* expression has also been observed however both spliced and unspliced forms are transcribed depending upon treatment conditions.<sup>109</sup> Similarly Byrne et al. 2003 noted the presence of both spliced and unspliced *Oct4* during interspecies SCNT experiments using human HeLa nuclei as donors.<sup>102</sup>

Other genes central to cell pluripotency have been studied in extract reprogramming experiments. Bovine cumulus cells treated with *X. laevis* extract express several pluripotency marker genes, notably the bovine orthologs for *Nanog*, *Sox2*, and *Klf4*.<sup>113</sup> while other pluripotent marker genes, such as *Rex1*, were notably absent.<sup>113</sup> Expression of pluripotent marker genes other than *Oct4* have not been reported in cells treated with *X. laevis* egg extract but have been reported in interspecies SCNT experiments and therefore are expected to occur in extract treated cells.<sup>114</sup> However, the differential activation of pluripotent genes in the two experimental systems could be a consequence of species specific differences or experimental limitations. The ability of

mammalian extracts to elicit global gene expressional changes has been better characterized.<sup>97,103</sup>

NCCIT ECC whole-cell extract can induce global gene expression changes in 293T cells. Upon treatment, 293T cells express *Oct4*, as well as many genes associated with the pluripotent state.<sup>97</sup> When compared to untreated cells and the extract donor, treated cells have a gene expression pattern closely resembling the NCCIT donor, with the notable exception of tumor suppressors and oncogenes.<sup>97</sup> Therefore, NCCIT extracts selectively induce pluripotent gene expression, with limited induction of the cancer genotype.<sup>97</sup>

Whole mESC extract can induce pluripotent gene expression in mouse 3T3 cells,<sup>97</sup> MEFs,<sup>107</sup> and cardiac fibroblasts.<sup>103</sup> In the case of mouse cardiac fibroblasts treated with mESC extracts, colony formation was induced 4-7 days following treatment, and colonies were maintained in culture. After “45-55 days” in culture,<sup>103</sup> cells expressed pluripotent genes, such as *Oct4* and *Nanog*, and exhibited an expression pattern nearly identical to their donor mESC line.<sup>103</sup> Such an expression pattern change is comparable to the one observed in iPSCs,<sup>73</sup> suggesting that extract-based reprogramming can generate pluripotent cells resembling ESCs and iPSCs.

Not only are mESC extracts capable of inducing transcription of pluripotent genes in differentiated mouse cells, they are also able to induce pluripotent gene expression in differentiated human 293T cells. Upon treatment with mESC extract, human 293T cells expressed pluripotent marker genes;<sup>107</sup> and this expression was associated with epigenetic

changes of the DNA methylation and histone modification patterns of the cells (see below).<sup>107</sup> A similar approach has been attempted using hESC extracts and human fetal fibroblast recipients; resulting cells were reprogrammed and expressed pluripotent genes, but at levels that were well below the levels observed in control hESCs.<sup>115</sup> The combination of histone deacetylase and DNA methyltransferase inhibitors was able to increase pluripotent gene expression in treated cells closer to the levels in hESCs, indicating that histone deacetylation and DNA methylation interfere with reprogramming.<sup>115</sup> Epigenetic factors present in the somatic cells or cell extracts may limit pluripotent gene expression and overcoming these barriers may depend on factors in or added to extracts.

While reprogramming of gene expression can be demonstrated following *X. laevis* egg, mESC, ECC, and hESC extract treatment, the factors responsible these gene expression changes remain elusive. Considering genome-wide expressional changes, epigenetic mechanisms, which have long been suspected to play important roles in reprogramming, may be responsible.<sup>116</sup> While pluripotent gene expression has been a focus of most extract-based reprogramming studies, repression of somatic gene expression plays an important role in the reprogramming process. Epigenetic mechanism may play important roles in repression of somatic gene repression during reprogramming.<sup>90</sup>

## DNA Demethylation

DNA methylation, specifically at cytosine-phosphate-guanine (CpG) dinucleotides in promoter regions, has been associated with transcriptional repression (citations). In ESCs, the promoters of genes such as *Oct4* and *Nanog* are demethylated, consistent with activation *Oct4* and *Nanog* expression in ESs and early embryos.<sup>117</sup> By contrast, promoters of genes associated with cell differentiation are methylated and inactive in early embryos and ESCs<sup>118,116</sup> Some gene promoters may remain methylated indefinitely, while others are demethylated during general or specific differentiation events.<sup>116,119</sup> During differentiation the promoter of genes required for pluripotency are methylated, seemingly ending their expression permanently.<sup>116</sup>

Reprogramming events can activate pluripotent genes and repress genes associated with differentiation.<sup>108</sup> To facilitate this activation, DNA is demethylated at pluripotent gene promoters<sup>116,120</sup> thereby leading to their activation,<sup>116,120</sup>. Upon injection of thymocyte nuclei into *X. laevis* oocytes, the *Oct4* promoter is demethylated in a manner not requiring cell division or DNA replication,<sup>120</sup> an activity which extends to naked, methylated plasmid DNA.<sup>120</sup> *Oct4* promoter demethylation activity has not been detected in cells treated with *X. laevis* egg extract,<sup>121</sup> although *Oct4* expression has been detected.<sup>92,109</sup> However, amphibian oocyte extracts have been shown to demethylate other gene promoters at tumor suppressor genes in extract-treated breast cancer cells.<sup>122</sup>

Upon treatment of somatic cells with ECC or mESC extract, *Oct4* and *Nanog* promoter regions undergo DNA demethylation concurrent with expression of both *Oct4*

and *Nanog* and subsequent protein expression.<sup>108</sup> Demethylation activity has also been observed during iPSC-based reprogramming.<sup>123</sup> While such methylation changes are expected to occur during treatment with *Xenopus* extract, it has only been reported to induce expression changes of specific marker genes, while the demethylation activity (at least of pluripotent genes) remains elusive.<sup>122</sup> The differential reprogramming of gene expression may be a result from ability of extract sources to demethylate pluripotent gene promoters, as this activity is often associated with the effectiveness of reprogramming extracts. In cells treated with hESC extracts supplemented with a DNA methyltransferase inhibitor in combination with an HDAC inhibitor, the expression of pluripotent genes was increased over cells treated with extract alone. These results support a role for histone acetylation and DNA demethylation for extract based cell reprogramming. In SCNT, global DNA demethylation has been observed in injected nuclei.<sup>124</sup> While this activity may include the demethylation of promoters at pluripotent genes, global demethylation remains poorly understood. Global changes in DNA methylation patterns are concurrent with the incorporation of histone variants or large-scale changes of histone modification patterns (see **Histone Modifications** and **Histone Variants** below),<sup>104, 108</sup> In iPSCs, loss of repressive histone modifications precedes DNA demethylation on pluripotent genes.<sup>125</sup> However, the coordination between histone modifications (or the incorporation of variants) and DNA demethylation during extract-based reprogramming remains unclear.

## Histone Modifications

Various histone modifications have been linked to changes in transcription.<sup>126,127</sup> Histone acetylation and L-specific methylation occurs at lysine residues within the histone tails and specific marks have been associated with transcriptional activation, such as methylation at lysine 4 of histone H3 (meH3K4), and acetylation at lysine 9 of histone H3 (acH3K9),<sup>128</sup> while other marks are associated with repression, such as trimethylation at lysine 9 of histone H3 (3meH3K9) and trimethylation at lysine 27 of histone H3 (3meH3K27).<sup>128</sup> Cell fusion-based reprogramming has linked multiple histone modifications to the cell reprogramming process,<sup>129</sup> however one limitation to this approach is the presence of the pluripotent nucleus and its histones. The extensive epigenetic changes, which occur during reprogramming, can be studied through extract-based cell reprogramming. Interestingly, histone modifications can depend on extract origin and species. When exposed to *X. laevis* extracts, porcine adult fibroblasts can be reprogrammed to express the porcine orthologs of *Oct4*,<sup>109</sup> in a temperature-dependent manner. Concurrent with this change, cells exhibit a global reduction in acH3K9.<sup>109</sup> While associated with repression and formation of heterochromatin, this modification may repress somatic gene expression.<sup>109</sup> These results however do not address specific promoters and the multiple histone modifications, which are occurring differentially in the genome following extract treatment.<sup>107</sup>

Following exposure to extracts from ESCs or ECCs, permeabilized mammalian somatic cells undergo changes in gene expression, which have been linked to specific



histone modifications at various gene promoters.<sup>97,107</sup> In 293T cells treated with NCCIT extract, an increase in acH3K9 and 3meH3K4 relative to control cells was observed at both the *Oct4* and *Nanog* gene promoters,<sup>108</sup> whereas markers associated with gene repression such as meH3K27 and meH3K9 were reduced.<sup>108</sup> However, it should be noted that these markers were observed four weeks following treatment. In 293T cells treated with mESC extracts, changes in some histone modifications were observed within 6 hours of treatment.<sup>107</sup> The reduction of repressive marks at the *Nanog* and *Cripto* promoters in 293T cells treated with mESC extract occurs only a few hours after treatment.<sup>107</sup> Taken together, reversal of histone modifications may be a prerequisite for the changes in gene expression associated with cell reprogramming.

### **Histone Variants**

During nuclear transfer experiments with *X. laevis* egg recipients and permeabilized endoderm donors from *X. laevis* embryos the expression of somatic cell marker genes (*MyoD*, *edd*) persists even after multiple nuclear transfers (reprogramming events) and with no linkage to known epigenetic phenomenon.<sup>124</sup> This persistent gene expression has been linked to the histone variant H3.3, H3.3 can remain associated with somatic promoters maintaining an active expression state<sup>124</sup>. H3.3 can also associate with pluripotent genes, when originating from recipient oocytes or eggs.<sup>130</sup> Various somatic nuclear donors are also resistant to cell reprogramming, rarely yielding viable clones through SCNT.<sup>18</sup> While it has been suggested that such resistance correlates with later

stages of differentiation,<sup>18</sup> the presence of a histone variant macroH2A correlates with resistance to SCNT-based reprogramming.<sup>131</sup>

While H3.3 and macroH2A have not been studied in extract-based reprogramming experiments, the *X. laevis* maternal, linker histone H1 variant B4 (H1oo in humans) has been studied in both SCNT and extract-based reprogramming experiments.<sup>98,114,132</sup> Recently, it has been demonstrated that eviction of linker histone H1 and the subsequent incorporation of histone variant B4 is linked to pluripotent gene expression in nuclei (originating from retinoic acid-differentiated ES cells) injected into *X. laevis* oocytes/eggs.<sup>114</sup> Similarly, during extract-based reprogramming histone B4 incorporates in the nuclei of mammalian somatic cell recipients in a temperature and energy independent fashion.<sup>109</sup>

Histone variants have received the most attention in SCNT-based systems,<sup>130</sup> playing distinct, but subtle roles in gene expression changes.<sup>114,131,124</sup> Thus, it appears likely that both modified histones and their variants may play similar roles in extract-based reprogramming experiments. Therefore extract-based reprogramming may reveal distinct roles for both native (recipient) and donor histones and variants, shedding light onto complex aspects of cellular reprogramming.

### **Nuclear Structural Changes**

The extensive changes which occur during the reprogramming of somatic cells require extensive changes to both somatic cellular function and structure. During differentiation stem cells begin to express Lamin A/C, a protein that is incorporated into

the nuclear scaffold throughout differentiation.<sup>133</sup> Extract-based reprogramming studies have demonstrated a loss of Lamin A/C from the nuclear structure,<sup>97,107</sup> and repression of Lamin A/C expression during the reprogramming process. Eviction of Lamin A/C occurs in 293T cells treated with either *Xenopus* egg or NCCIT whole-cell extracts.<sup>97,107</sup>, suggesting that the eviction of Lamin A/C during cell reprogramming is evolutionary conserved. While the precise function of Lamin A/C remains unknown, it is believed that Lamin A/C helps to facilitate chromatin conformation and gene expression in the pluripotent state.<sup>134</sup>

Another lamin, the *X. laevis* specific Lamin LIII, is also incorporated into *X. laevis* extract-treated porcine cells in an ATP-dependent manner.<sup>109</sup> Lamin LIII is expressed in cells during early development and is no longer expressed after differentiation.<sup>109</sup> Ultimately factors, which affect nuclear structural changes and the precise effects of such changes during reprogramming, remain elusive. Dramatic changes during reprogramming may require an entirely remodeled nucleus; Lamin LIII incorporation and loss of Lamin A/C may help to facilitate changes required for reprogramming of somatic into pluripotent cells.

### **Differentiation Capacity**

Colony formation, epigenetic changes, and marker gene expression all point toward the property that makes stem cells interesting and useful: pluripotency.<sup>12</sup> An important goal of cell reprogramming is not just the conversion of somatic cells toward a pluripotent state, but also their subsequent differentiation toward various somatic cell

lineages.<sup>15</sup> However, cell reprogramming is difficult *per se*, and the differentiation of pluripotent cells obtained by cell reprogramming appears to be even more challenging. In 293T cells treated with *Xenopus* egg extract, cells not only form colonies, but embryoid body-like cell clusters.<sup>92</sup> This result indicates reprogramming followed by a progression toward an early differentiated state. However, lineage-specific differentiation has not been described in *X. laevis* extract treated mammalian cells.<sup>92,113</sup>

In the case of NCCIT extract treated cells specific differentiation programs can be initiated, notably neuronal differentiation exemplified by the expression of *Nestin*, and NeuN and NF-200 surface markers.<sup>97</sup> Neurite protrusions, culminating in a neuron-like morphology were also observed.<sup>97</sup> Other forms of differentiation are also possible as made evident following the treatment of reprogrammed cells with growth factors and/or chemicals, leading to adipogenic cells (cells staining for Oil-Red-O), osteoblast cells (cells staining positive for alizarin red), and endothelial cells (cells expressing surface markers CD31 and CD144).<sup>97</sup> Therefore extract treated cell reprogramming can generate pluripotent cells, which can be differentiated along specific lineages.

One group has described multiple forms of differentiation of extract-treated cells.<sup>103</sup> Following treatment with mESC extracts, primary mouse cardiac fibroblasts form colonies capable of endoderm, mesoderm, and ectoderm differentiation.<sup>103</sup> Similarly, upon subcutaneous injection into mice, these cells differentiate into teratomas featuring cells derived from all three germ layers.<sup>103</sup> Furthermore, these cells contribute to the developing blastocysts and adult differentiated tissue as evident in the production

of chimeric mice.<sup>103</sup> Thus, extract-based reprogrammed cells can rival iPSC-based reprogramming.<sup>103</sup>

### **Additional Markers**

Aside from pluripotency marker gene expression, the presence of specific enzymes and surface antigens can be used as indicators of reprogramming and pluripotency.<sup>1,2,3,135</sup> One common marker is the enzyme alkaline phosphatase (AP).<sup>136,137</sup> AP has been identified as an early and essential marker of pluripotency, and its expression may precede other marker expression during the reprogramming process.<sup>138,139</sup> Both mESC and ECC extracts can induce AP activity in treated somatic cells.<sup>103</sup> While AP induction has not been reported in *X. laevis* oocyte/egg extract treated cells, the expression of another alkaline phosphatase *GCAP* – germ cell alkaline phosphatase – has been reported and shares close sequence identity to human placental alkaline phosphatase.<sup>92</sup>

ESCs and somatic cells also display specific surface antigens of their respective cell-type. In mESCs SSEA1 has been attributed to the pluripotent state and similar to AP, can be detected during early stages of cell reprogramming.<sup>137,140</sup> While the expression of cell surface markers has been neglected in extract-based reprogramming studies, mouse cardiac fibroblasts treated with mESC extracts form colonies which display SSEA1.<sup>103</sup> Therefore AP and SSEA1 can be used as pluripotency markers in extract-based reprogramming experiments.

## Identified Regulators

In fusion reprogramming experiments, proteins involved in reprogramming were identified by an RNA interference (RNAi) based “loss of function” approach.<sup>141</sup> Genes are inactivated by using short interfering RNA.<sup>141</sup> In extract-based reprogramming, factors involved in reprogramming have been identified by purifying or immunodepletion of proteins from extracts.<sup>92,104</sup> In *X. laevis* egg extracts, epigenetic regulators were immunodepleted prior to somatic cell treatment. The loss of one regulator, BRG1, repressed *OCT4* gene expression following extract treatment, indicating that BRG1 is involved in activating BRG1 expression. BRG1 is involved in ATP-dependent chromatin remodeling, and resembles a component of the SWI/SNF complex in yeast.<sup>92</sup>

The importance of BRG1 has been recently confirmed through the use of purified extracts from F9 mouse embryonic carcinoma cells.<sup>92,104</sup> When separated into nuclear and cytoplasmic fractions, nuclear protein retained reprogramming functionality, as accessed by pluripotent gene expression.<sup>104</sup> When this extract was subjected to size-exclusion chromatography, only the initial fraction (likely to contain large proteins and protein complexes) retained reprogramming activity. Within this fraction were various known regulators, such as *Oct4*, *Nanog*, *Klf4*, and components of the BAF complex, one of which was BRG1.<sup>104</sup> When added to the four genes known to induce iPSCs from somatic cells, components of the BAF complex were able to increase reprogramming efficiency, as measured through colony formation.<sup>104</sup> This increase coincided with an increase in various reprogramming activities such as promoter demethylation and histone

modifications associated with the pluripotent state such as 3meH3K4.<sup>104</sup> Therefore results of extract-reprogramming studies can be applied to improve iPSCs.

Proteomic analysis has been used to confirm translation of genes upregulated in 293T cells treated with NCCIT extract and identified factors which may be involved in reprogramming:<sup>142</sup> GRP78 a protein found in tumors and related to heat shock protein 70, TPM3 a protein involved in tumor cell motility and suppression of differentiation, and SAKS which is involved in proteolysis.<sup>142</sup> Their enrichment in extract treated cells may be indicative of various reprogramming activities; however their function in reprogramming still remains elusive.

Alteration of chromatin structure through histone modifications,<sup>115</sup> incorporation of histone variants,<sup>114,131,124</sup> and the action of chromatin remodeling complexes<sup>92,104</sup> have been shown to influence cellular reprogramming. However, because large-scale transcriptional changes occur during SCNT, the general RNA polymerase II transcription machinery (GTM) may also play a role in the reprogramming process. Notably, TATA binding protein (TBP) and the variant TBP-2 have been implicated in reprogramming.<sup>143</sup> TBP is exported from the somatic cell nucleus during extract reprogramming, and it is now believed that the *X. laevis* oocyte/egg specific variant, TBP-2, replaces mammalian TBP in the nucleus during interspecies SCNT.<sup>113,143</sup> Therefore, oocyte and egg specific GTM may influence the transcription of genes associated with the early stages of development.

During iPSC reprogramming, four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) are responsible for converting somatic cells toward a pluripotent-state.<sup>73,74</sup> It remains unclear if and what role these factors play in extract reprogramming. In oocyte and embryos from mouse, iPSC factors are not detectable in sufficient quantities to induce iPSC-based reprogramming, although alternate transcription factors or variants could substitute for the four iPSC factors.<sup>144</sup> In interspecies extract-based reprogramming experiments, the influence of iPSC factors is debatable. Both genetic and protein sequence analysis indicate some level of evolutionary conservation across species.<sup>145,146</sup> However, sequence-specific DNA binding of *Oct4* has changed throughout evolution.<sup>147,148</sup> Taken together, while mammalian *Oct4* is structurally similar to its evolutionary predecessors,<sup>145,146</sup> its target gene specificity changes significantly throughout evolution.<sup>147,148</sup> Because extensive transcriptional, epigenetic, and structural changes occur during reprogramming transcription factors, such as (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) should be considered for their role in the reprogramming process.



## Conclusion

Various techniques can be used for reprogramming somatic cells toward an ESC-like state.<sup>68</sup> While each technique has advantages and limitations, two approaches utilize naturally occurring material for reprogramming potential, SCNT and fusion-based reprogramming. Because extracts from oocytes, eggs, and cultured pluripotent cells can reprogram permeabilized somatic cells, extract-based reprogramming represents the best approach to identify and characterize the factors and mechanisms mediating cell reprogramming. Extract reprogramming overcomes limitations of SCNT by 1) reducing technical and material requirements 2) cells can be analyzed in a cultured, steady-state as opposed to developing embryos<sup>25</sup> and 3) multiple extract sources can be analyzed for reprogramming potential such as oocytes, eggs or embryonic sources represented by pluripotent, cultured cells. Extract-based reprogramming also addresses problems associated with fusion-based reprogramming by eliminating pluripotent donor cells and nuclei which could potential interfere with analysis of somatic cell recipients. Previous experiments have revealed expectations and challenges for the basis of studying extracts from a variety of sources. Through the extract-based reprogramming approach, many activities and processes have been identified, including colony formation, expression changes, epigenetic modifications, nuclear structural changes, and marker expression. Additionally, activities and processes which occur during extract-based can be corroborated with other forms of reprogramming and extract-reprogrammed cells can be compared to cultured pluripotent cell lines. While various regulators have been

implicated, a cost-effective experimental system is needed which can lead to the large-scale identification of reprogramming factors.

Despite the availability of published works (**Table 0.1**), the establishment of an extract-based reprogramming system remains technically challenging. This work seeks to refine and standardize digitonin-based extract reprogramming toward the identification of novel extracts with reprogramming potential. In **Chapter 1**, I describe my pilot experiments which demonstrate the reprogramming potential of both mouse CJ7 and human NTERA-2 cl.D1 cells, while also revealing limitations of extracts based cell reprogramming assays using ESCs and ECCs as extract donor. In **Chapter 2**, I describe the digitonin-mediated permeabilization of somatic cells, their subsequent survival, and effective working concentrations for the treatment of mouse 3T3 cells. Because treatments require the penetration and retention of large molecules, in **Chapter 3**, I describe large molecule assessment through the use of fluorescent-conjugates as molecular probes. Finally in **Chapter 4**, I describe the novel reprogramming capacity of extracts prepared from salmon oocytes. Salmon roe extract can induce the expression of the pluripotency markers induction of SSEA1, AP, and *Oct4* in mouse cells, thereby presenting a potential source of cell reprogramming protein.

## References

1. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells *Developmental Biology : Proceedings of the National Academy of Sciences* **78**, 7634–7638 (1981).
2. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
3. Thomson, J. a. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science* **282**, 1145–1147 (1998).
4. Bhattacharyya, S., Kumar, A. & Khanduja, K. L. The voyage of stem cell toward terminal differentiation : a brief overview *Biology of Stem Cells. Acta Biochimica et Biophysica Sinica* **44**, 463–475 (2012).
5. Wobus, A. M. & Löser, P. Present state and future perspectives of using pluripotent stem cells in toxicology research. *Archives of Toxicology* **85**, 79–117 (2011).
6. Drews, K., Jozefczuk, J., Prigione, A. & Adjaye, J. Human induced pluripotent stem cells--from mechanisms to clinical applications. *Journal of molecular medicine (Berlin, Germany)* **90**, 735–45 (2012).
7. Pouton, C. W. & Haynes, J. M. Embryonic stem cells as a source of models for drug discovery. *Nature Reviews Drug Discovery* **6**, 605–616 (2007).
8. Regenerative Medicine 2006. *Department of Health and Human Services*. (2006).
9. Volarevic, V. *et al.* Human stem cell research and regenerative medicine--present and future. *British medical bulletin* **99**, 155–68 (2011).
10. Mitalipov, S. & Wolf, D. Totipotency, pluripotency and nuclear reprogramming. *Advances in biochemical engineering/biotechnology* **114**, 185–99 (2009).
11. Thomson, J. a. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science* **282**, 1145–1147 (1998).
12. Gilbert, S. F. *Developmental Biology. 6th edition*. (Sunderland (MA): Sinauer Associates; 6th edition, 2000).

13. Power, C. & Rasko, J. E. J. Will Cell Reprogramming Resolve the Embryonic Stem Cell Controversy? A Narrative Review. *Annals of Internal Medicine* **155**, 114–121 (2011).
14. Zhao, T., Zhang, Z.-N., Rong, Z. & Xu, Y. Immunogenicity of induced pluripotent stem cells. *Nature* **474**, 212–5 (2011).
15. Lanzuolo, C. & Orlando, V. The function of the epigenome in cell reprogramming. *Cellular and molecular life sciences : CMLS* **64**, 1043–62 (2007).
16. Briggs, B. R. & King, T. J. Transplantation of Living Nuclei from Blastula Cells into Enucleated Frogs' Eggs. *Proceedings of the National Academy of Sciences* **38**, 455–463 (1952).
17. Gurdon, J. B. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *Journal of embryology and experimental morphology* **10**, 622–40 (1962).
18. Gurdon, J. B. & Byrne, J. a. The first half-century of nuclear transplantation. *Bioscience reports* **24**, 545–57 (2003).
19. Gurdon, J. B. & Wilmut, I. Nuclear transfer to eggs and oocytes. *Cold Spring Harbor perspectives in biology* **3**, (2011).
20. Armstrong, L., Lako, M., Dean, W. & Stojkovic, M. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem cells (Dayton, Ohio)* **24**, 805–14 (2006).
21. Gurdon, J. B. Nuclear reprogramming in eggs. *Nature medicine* **15**, 1141–4 (2009).
22. Koziol, M. J., Garrett, N. & Gurdon, J. B. Tpt1 activates transcription of oct4 and nanog in transplanted somatic nuclei. *Current biology : CB* **17**, 801–7 (2007).
23. Gurdon, J. B., Partington, G. a & De Robertis, E. M. Injected nuclei in frog oocytes: RNA synthesis and protein exchange. *Journal of embryology and experimental morphology* **36**, 541–53 (1976).
24. Gurdon, J. B. Injected nuclei in frog oocytes: fate, enlargement, and chromatin dispersal. *Journal of embryology and experimental morphology* **36**, 523–40 (1976).

25. Miyamoto, K. *et al.* Cell-free extracts from mammalian oocytes partially induce nuclear reprogramming in somatic cells. *Biology of reproduction* **80**, 935–43 (2009).
26. Meissner, A. & Jaenisch, R. Epigenetic reprogramming From frog cloning to customized human embryonic stem cells. *Science* **21**, 5–12 (2006).
27. Campbell, K. H., McWhir, J., Ritchie, W. a & Wilmut, I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**, 64–6 (1996).
28. Wilmut I, Schnieke AE, McWhir J, Kind AJ, C. K. Viable offspring derived from fetal and adult mammalian cells. *Nature* **386**, 810–813 (1997).
29. Yang, H. *et al.* High-efficiency somatic reprogramming induced by intact MII oocytes. *Cell research* **20**, 1034–42 (2010).
30. Campbell, K. H. S. *et al.* Somatic cell nuclear transfer: Past, present and future perspectives. *Theriogenology* **68S**, S214–31 (2007).
31. Forsberg, E. J. *et al.* Production of cloned cattle from in vitro systems. *Biology of reproduction* **67**, 327–33 (2002).
32. Pryor, J. *et al.* A cat cloned by nuclear transplantation. *Nature* **415**, 2002 (2002).
33. A dog's life. *Nature* **436**, 2005 (2005).
34. Woods, G. L. *et al.* A mule cloned from fetal cells by nuclear transfer. *Science* **301**, 1063 (2003).
35. Wakayama, T., Perry, a C., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–74 (1998).
36. Chesné, P. *et al.* Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nature biotechnology* **20**, 366–9 (2002).
37. Byrne, J. a *et al.* Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature* **450**, 497–502 (2007).
38. Rodriguez-Osorio, N., Urrego, R., Cibelli, J. B., Eilertsen, K. & Memili, E. Reprogramming mammalian somatic cells. *Theriogenology* **78**, 1869–86 (2012).
39. Gruen, L. & Grabel, L. Concise review: scientific and ethical roadblocks to human embryonic stem cell therapy. *Stem cells (Dayton, Ohio)* **24**, 2162–9 (2006).

40. Hall, V. & Stojkovic, M. The status of human nuclear transfer. *Stem cell reviews* **00**, 301–308 (2006).
41. Chambers, I. & Smith, A. Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene* **23**, 7150–60 (2004).
42. Yu, J. & Thomson, J. a. Pluripotent stem cell lines. *Genes & development* **22**, 1987–97 (2008).
43. Müller, R. & Lengerke, C. Patient-specific pluripotent stem cells: promises and challenges. *Nature reviews. Endocrinology* **5**, 195–203 (2009).
44. Willadsen, S. M. Nuclear transplantation in sheep embryos. *Nature* **320**, 63–65 (1986).
45. Hall, B., Limaye, A. & Kulkarni, A. B. Overview: generation of gene knockout mice. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.] Chapter 19*, Unit 19.12 19.12.1–17 (2009).
46. Solter, D. & Knowles, B. B. Immunosurgery of mouse blastocyst. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 5099–102 (1975).
47. Chen, A. E. & Melton, D. a. Derivation of human embryonic stem cells by immunosurgery. *Journal of visualized experiments : JoVE* 574 (2007). doi:10.3791/574
48. Efe, J. a & Ding, S. The evolving biology of small molecules: controlling cell fate and identity. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **366**, 2208–21 (2011).
49. Braam, S. R. *et al.* Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification. *Nature protocols* **3**, 1435–43 (2008).
50. Jaenisch, R. & Young, R. Review Stem Cells , the Molecular Circuitry of Pluripotency and Nuclear Reprogramming. *Cell* **132**, 567–582 (2008).
51. Wesselschmidt, R. L. The teratoma assay: an in vivo assessment of pluripotency. *Methods in molecular biology (Clifton, N.J.)* **767**, 231–41 (2011).
52. National Institutes of Health. Stem Cell Basics. *Introduction. In Stem Cell Information* 1–26

53. Yang, W., Zhou, Q. & Wang, X.-J. Regulation beyond genome sequences: DNA and histone methylation in embryonic stem cells. *Frontiers in Biology* **5**, 41–47 (2010).
54. Donovan, P. J. & Gearhart, J. The end of the beginning for pluripotent stem cells. *Nature* **414**, 92–7 (2001).
55. Chin, M. H. *et al.* Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell stem cell* **5**, 111–23 (2009).
56. Sanges, D., Lluís, F. & Cosma, M. P. Cell-Fusion-Mediated Reprogramming: Pluripotency or Transdifferentiation? Implications for Regenerative Medicine. *Advances in Experimental Medicine and Biology* **713**, 137–159 (2011).
57. Cowan, C. a, Atienza, J., Melton, D. a & Eggan, K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science (New York, N.Y.)* **309**, 1369–73 (2005).
58. Miller, R. A. & Ruddle, F. H. Pluripotent teratocarcinoma-thymus somatic cell hybrids. *Cell* **9**, 45–55 (1976).
59. Tada, M., Tada, T., Lefebvre, L., Barton, S. C. & Surani, M. a. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *The EMBO journal* **16**, 6510–20 (1997).
60. Alkan, S. Monoclonal antibodies: the story of a discovery that revolutionized science and medicine. *Nature Reviews Immunology* **4**, 3–6 (2004).
61. Tada, M., Takahama, Y., Abe, K., Nakatsuji, N. & Tada, T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Current biology : CB* **11**, 1553–8 (2001).
62. Kimura, H., Tada, M., Nakatsuji, N. & Tada, T. Histone Code Modifications on Pluripotential Nuclei of Reprogrammed Somatic Cells. *Molecular and Cellular Biology* **24**, 5710–5720 (2004).
63. Flaszka, M. *et al.* Reprogramming in inter-species embryonal carcinoma-somatic cell hybrids induces expression of pluripotency and differentiation markers. *Cloning and stem cells* **5**, 339–54 (2003).
64. Pereira, C. F. *et al.* Heterokaryon-based reprogramming of human B lymphocytes for pluripotency requires Oct4 but not Sox2. *PLoS genetics* **4**, e1000170 (2008).

65. Wong, C. C., Gaspar-Maia, A., Ramalho-Santos, M. & Reijo Pera, R. a. High-efficiency stem cell fusion-mediated assay reveals Sall4 as an enhancer of reprogramming. *PloS one* **3**, e1955 (2008).
66. Palermo, A. *et al.* Nuclear reprogramming in heterokaryons is rapid, extensive, and bidirectional. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 1431–40 (2009).
67. Do, J. T., Choi, H. W., Choi, Y. & Schöler, H. R. Pluripotent hybrid cells contribute to extraembryonic as well as embryonic tissues. *Stem cells and development* **20**, 1063–9 (2011).
68. Yamanaka, S. Pluripotency and nuclear reprogramming. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **363**, 2079–87 (2008).
69. Islam, M. Q., Islam, K. & Sharp, C. a. Epigenetic reprogramming of nonreplicating somatic cells for long-term proliferation by temporary cell-cell contact. *Stem cells and development* **16**, 253–68 (2007).
70. Miller, R. a & Ruddle, F. H. Properties of teratocarcinoma-thymus somatic cell hybrids. *Somatic cell genetics* **3**, 247–61 (1977).
71. Piccolo, F. M. *et al.* Using heterokaryons to understand pluripotency and reprogramming. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **366**, 2260–5 (2011).
72. Kim, T. K. & Eberwine, J. H. Mammalian cell transfection: the present and the future. *Analytical and bioanalytical chemistry* **397**, 3173–8 (2010).
73. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–76 (2006).
74. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–72 (2007).
75. Han, J. W. & Yoon, Y.-S. Induced pluripotent stem cells: emerging techniques for nuclear reprogramming. *Antioxidants & redox signaling* **15**, 1799–820 (2011).
76. Zhao, X. *et al.* iPS cells produce viable mice through tetraploid complementation. *Nature* **461**, 86–90 (2009).



77. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313–7 (2007).
78. Yamanaka, S. A fresh look at iPS cells. *Cell* **137**, 13–7 (2009).
79. Zhou, W. & Freed, C. R. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. *Stem cells (Dayton, Ohio)* **27**, 2667–74 (2009).
80. Woltjen, K. *et al.* piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* **458**, 766–70 (2009).
81. Yu, J. *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science (New York, N.Y.)* **324**, 797–801 (2009).
82. Jia, F. *et al.* A nonviral minicircle vector for deriving human iPS cells. *Nature methods* **7**, 197–9 (2010).
83. Warren, L. *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell stem cell* **7**, 618–30 (2010).
84. Kim, D. *et al.* Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell stem cell* **4**, 472–6 (2009).
85. Lin, T. *et al.* A chemical platform for improved induction of human iPSCs. *Nature methods* **6**, 805–8 (2009).
86. Zhang, B. *et al.* Estimating the quality of reprogrammed cells using ES cell differentiation expression patterns. *PloS one* **6**, e15336 (2011).
87. Wang, B. *et al.* Reprogramming efficiency and quality of induced Pluripotent Stem Cells (iPSCs) generated from muscle-derived fibroblasts of mdx mice at different ages. *PLoS currents* **3**, RRN1274 (2011).
88. Bock, C. *et al.* Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell* **144**, 439–52 (2011).
89. Lister, R. *et al.* Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* **471**, 68–73 (2011).
90. Ohi, Y. *et al.* Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. *Nature cell biology* **13**, 541–9 (2011).

91. Kim, K. *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285–90 (2010).
92. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Current biology : CB* **14**, 1475–80 (2004).
93. Håkelién, A.-M., Landsverk, H. B., Robl, J. M., Skålhegg, B. S. & Collas, P. Reprogramming fibroblasts to express T-cell functions using cell extracts. *Nature biotechnology* **20**, 460–6 (2002).
94. Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *The Journal of general virology* **36**, 59–74 (1977).
95. Dubridge, R. B. *et al.* Analysis of Mutation in Human Cells by Using an Epstein-Barr Virus Shuttle System. **7**, (1987).
96. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. Production of high-titer helper-free retroviruses by transient transfection. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 8392–6 (1993).
97. Taranger, C. K. *et al.* Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells. **16**, 5719–5735 (2005).
98. Dimitrov, S., Dasso, M. C. & Wolffe, a P. Remodeling sperm chromatin in *Xenopus laevis* egg extracts: the role of core histone phosphorylation and linker histone B4 in chromatin assembly. *The Journal of cell biology* **126**, 591–601 (1994).
99. Gurdon, J. B. & Melton, D. a. Nuclear reprogramming in cells. *Science (New York, N.Y.)* **322**, 1811–5 (2008).
100. Stephens, D. J. & Pepperkok, R. The many ways to cross the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4295–8 (2001).
101. Schulz, I. Permeabilizing cells: some methods and applications for the study of intracellular processes. *Methods in enzymology* **192**, 280–300 (1990).
102. Byrne, J. A., Simonsson, S., Western, P. S. & Gurdon, J. B. Nuclei of Adult Mammalian Somatic Cells Are Directly Reprogrammed to oct-4 Stem Cell Gene Expression by Amphibian Oocytes. *Current Biology* **13**, 1206–1213 (2003).

103. Cho, H.-J. *et al.* Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* **116**, 386–95 (2010).
104. Singhal, N. *et al.* Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. *Cell* **141**, 943–55 (2010).
105. Tada, T. Toti-/pluripotential stem cells and epigenetic modifications. *Neurodegenerative diseases* **3**, 32–7 (2006).
106. Ohm, J. E. *et al.* Cancer-related epigenome changes associated with reprogramming to induced pluripotent stem cells. *Cancer research* **70**, 7662–73 (2010).
107. Bru, T. *et al.* Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. *Experimental cell research* **314**, 2634–42 (2008).
108. Freberg, C. T., Dahl, J. A., Timoskainen, S. & Collas, P. Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract. *Molecular biology of the cell* **18**, 1543–53 (2007).
109. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by *Xenopus laevis* egg extracts. *Molecular reproduction and development* **74**, 1268–77 (2007).
110. Chan, E. M. *et al.* Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. *Nature biotechnology* **27**, 1033–7 (2009).
111. Häkkelien, A.-M. *et al.* Long-term in vitro, cell-type-specific genome-wide reprogramming of gene expression. *Experimental cell research* **309**, 32–47 (2005).
112. Xu, Y.-N. *et al.* ES cell extract-induced expression of pluripotent factors in somatic cells. *Anatomical record (Hoboken, N.J. : 2007)* **292**, 1229–34 (2009).
113. Miyamoto, K. *et al.* Reversible membrane permeabilization of mammalian cells treated with digitonin and its use for inducing nuclear reprogramming by *Xenopus* egg extracts. *Cloning and stem cells* **10**, 535–42 (2008).
114. Jullien, J., Astrand, C., Halley-Stott, R. P., Garrett, N. & Gurdon, J. B. Characterization of somatic cell nuclear reprogramming by oocytes in which a linker histone is required for pluripotency gene reactivation. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5483–8 (2010).

115. Han, J., Sachdev, P. S. & Sidhu, K. S. A combined epigenetic and non-genetic approach for reprogramming human somatic cells. *PloS one* **5**, e12297 (2010).
116. Han, J. W. & Yoon, Y. Epigenetic landscape of pluripotent stem cells. *Antioxidants & redox signaling* **17**, 205–23 (2012).
117. Yeo, S. *et al.* Characterization of DNA methylation change in stem cell marker genes during differentiation of human embryonic stem cells. *Biochemical and biophysical research communications* **359**, 536–42 (2007).
118. Reik, W., Dean, W. & Walter, J. Epigenetic reprogramming in mammalian development. *Science (New York, N.Y.)* **293**, 1089–93 (2001).
119. Bird, A. DNA methylation patterns and epigenetic memory. *Genes & development* **16**, 6–21 (2002).
120. Simonsson, S. & Gurdon, J. B. DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. *Nature cell biology* **6**, 984–90 (2004).
121. Ganier, O. *et al.* Synergic reprogramming of mammalian cells by combined exposure to mitotic *Xenopus* egg extracts and transcription factors. *Proceedings of the National Academy of Sciences* (2011). doi:10.1073/pnas.1100733108
122. Allegrucci, C. *et al.* Epigenetic reprogramming of breast cancer cells with oocyte extracts. *Molecular cancer* **10**, 7 (2011).
123. Cox, J. L. & Rizzino, A. Induced pluripotent stem cells: what lies beyond the paradigm shift. *Experimental biology and medicine (Maywood, N.J.)* **235**, 148–58 (2010).
124. Ng, R. K. & Gurdon, J. B. Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. *Nature cell biology* **10**, 102–9 (2008).
125. Cedar, H. & Bergman, Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nature reviews. Genetics* **10**, 295–304 (2009).
126. Lachner, M., O’Sullivan, R. J. & Jenuwein, T. An epigenetic road map for histone lysine methylation. *Journal of cell science* **116**, 2117–24 (2003).
127. Barski, A. *et al.* High-resolution profiling of histone methylations in the human genome. *Cell* **129**, 823–37 (2007).

128. Pasque, V., Jullien, J., Miyamoto, K., Halley-Stott, R. P. & Gurdon, J. B. Epigenetic factors influencing resistance to nuclear reprogramming. *Trends in genetics : TIG* **27**, 516–25 (2011).
129. Kimura, H., Tada, M., Nakatsuji, N. & Tada, T. Histone Code Modifications on Pluripotential Nuclei of Reprogrammed Somatic Cells Histone Code Modifications on Pluripotential Nuclei of Reprogrammed Somatic Cells. (2004). doi:10.1128/MCB.24.13.5710
130. Jullien, J. *et al.* HIRA dependent H3.3 deposition is required for transcriptional reprogramming following nuclear transfer to *Xenopus* oocytes. *Epigenetics & chromatin* **5**, 17 (2012).
131. Pasque, V., Gillich, A., Garrett, N. & Gurdon, J. B. Histone variant macroH2A confers resistance to nuclear reprogramming. *The EMBO journal* **30**, 2373–87 (2011).
132. Teranishi, T. *et al.* Rapid replacement of somatic linker histones with the oocyte-specific linker histone H1foo in nuclear transfer. *Developmental Biology* **266**, 76–86 (2004).
133. Constantinescu, D., Gray, H. L., Sammak, P. J., Schatten, G. P. & Csoka, A. B. Lamin A/C expression is a marker of mouse and human embryonic stem cell differentiation. *Stem cells* **24**, 177–85 (2006).
134. Li, M., Liu, G.-H. & Izpisua Belmonte, J. C. Navigating the epigenetic landscape of pluripotent stem cells. *Nature reviews. Molecular cell biology* **13**, 524–35 (2012).
135. Wobus, a M., Holzhausen, H., Jäkel, P. & Schöneich, J. Characterization of a pluripotent stem cell line derived from a mouse embryo. *Experimental cell research* **152**, 212–9 (1984).
136. O'Connor, M. D. *et al.* Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells. *Stem cells (Dayton, Ohio)* **26**, 1109–16 (2008).
137. Brambrink, T. *et al.* Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell stem cell* **2**, 151–9 (2008).
138. Brambrink, T., Foreman, R. & Welstead, G. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell stem cell* **2**, 151–159 (2008).

139. Silva, J. *et al.* Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS biology* **6**, e253 (2008).
140. Saric, T. & Hescheler, J. Stem cells and nuclear reprogramming. *Minimally invasive therapy & allied technologies : MITAT : official journal of the Society for Minimally Invasive Therapy* **17**, 64–78 (2008).
141. Bhutani, N. *et al.* Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature* **463**, 1042–7 (2010).
142. Pewsey, E. *et al.* Proteomics analysis of epithelial cells reprogrammed in cell-free extract. *Molecular & cellular proteomics : MCP* **8**, 1401–12 (2009).
143. Jullien, J., Pasque, V., Halley-Stott, R. P., Miyamoto, K. & Gurdon, J. B. Mechanisms of nuclear reprogramming by eggs and oocytes: a deterministic process? *Nature reviews. Molecular cell biology* **12**, 453–9 (2011).
144. Pfeiffer, M. J. *et al.* Proteomic analysis of mouse oocytes reveals 28 candidate factors of the “reprogrammome”. *Journal of proteome research* **10**, 2140–53 (2011).
145. Zhang, X., Ma, Y., Liu, X., Zhou, Q. & Wang, X.-J. Evolutionary and Functional Analysis of the Key Pluripotency Factor Oct4 and Its Family Proteins. *Journal of Genetics and Genomics* (2013). doi:10.1016/j.jgg.2013.04.011
146. Fuellen, G. & Struckmann, S. Evolution of gene regulation of pluripotency--the case for wiki tracks at genome browsers. *Biology direct* **5**, 67 (2010).
147. Kunarso, G. *et al.* Transposable elements have rewired the core regulatory network of human embryonic stem cells. *Nature genetics* **42**, 631–4 (2010).
148. Fernandez-Tresguerres, B. *et al.* Evolution of the mammalian embryonic pluripotency gene regulatory network. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 19955–60 (2010).

## **Chapter 1: Pilot Experiments**

### **Abstract:**

The selection of both extract donor and the somatic cell recipient is a fundamental challenge for devising an extract-based reprogramming system. Extracts prepared from human NTERA-2 cl.D1 and mouse CJ7 cells can elicit both morphological and gene expression changes in permeabilized human 293T cells. The pilot experiments described here illustrate the reprogramming potential of protein extracts obtained from cultured pluripotent cells, revealed experimental challenges, as well as cost and material limitations for protein extracts prepared from pluripotent cells. The experiments described here also emphasize the necessity for developing control assays for cell permeabilization as well as the uptake and retention of large molecules (**Chapter 2,3**), and resulted in the identification of a novel, pluripotent extract donor, which can provide large quantities of protein extract in a cost-efficient fashion (**Chapter 4**) for the extract-based reprogramming.

### **Introduction**

Extract-based reprogramming techniques present a unique opportunity to study the reprogramming potential of protein from various sources such as ESCs, ECCs, and oocyte/egg extracts.<sup>1,2,3</sup> Not only can this approach reveal previously unknown extract sources with reprogramming potential; these sources represent one of few routes by which factors responsible for reprogramming activities can be identified and the mechanisms underlying reprogramming can be elucidated.<sup>2,4</sup> Paramount for this

approach is the establishment of assay systems, which support the delivery of exogenous proteins into recipient cells without affecting cell viability.<sup>1,4</sup>

Extract-based reprogramming system consists of three major components (**Figure 1.1**): recipient cells (**Figure 1.2A**), a cell permeabilization/protein delivery assay(s), and a protein extract donor (**Figure 1.2B**). Each component is vital for the success of extract based cell reprogramming, and together the three components have to form a cohesive experimental unit. Thus, each component has to be chosen based on specific characteristics and its ability to work in combination with the other two components. (**Figure 1.1A**). One starting point is the selection of a somatic cell recipient. A variety of somatic cell recipients have been used in previous experiments including: human 293T cells,<sup>1,2,5</sup> mouse embryonic fibroblasts (MEFs),<sup>3</sup> and a number of primary cell lines from murine,<sup>6</sup> bovine,<sup>7</sup> porcine,<sup>7,8</sup> and even human sources.<sup>9</sup> Attempts to use primary lines reflect a major objective of reprogramming experiments: derivation of patient-specific stem cells from their own fully differentiated, adult somatic cells.<sup>10</sup> Consideration of recipient cell sources is also important, because depending upon origin, and differentiation state, cells respond differently to somatic cell reprogramming experiments.<sup>11</sup> However for extract-based reprogramming assays, an essential parameter is the selection of a recipient line, which can survive the harsh conditions of cell permeabilization and protein extract treatment.

Extract-based reprogramming was initially established in 293T cells, using *X. laevis* egg and early embryo extract donors.<sup>2</sup> 293T cells also respond to extracts



originating from both mESCs and ECCs.<sup>1,5</sup> Therefore, as a starting point, 293T cells represent a suitable extract recipient (**see Introduction**). In addition to 293T, I also tested two additional lines as extract recipients: human BJ neonatal fibroblasts and mouse 3T3 cells. BJ neonatal fibroblasts is a primary cell line isolated from human foreskin and have been used for iPSC-based reprogramming.<sup>12</sup> Mouse 3T3 cells originate from fibroblasts, which were extracted from early mouse embryos, and through the loss of cell cycle regulators such as p53 or p19ARF, became randomly immortalized after many generations in culture.<sup>13,14</sup> The 3T3 cell line is representative of the fibroblast, somatic cell lineage, and is both well characterized and homogenous.<sup>13,14</sup> Although 3T3 cells are not karyotypical, they have not been genetically modified to the extent of 293T cells.<sup>13,14</sup>

Another consideration for establishing an extract-based reprogramming system is methods for somatic cell permeabilization. The mammalian cytoplasmic cell membrane is impervious to protein and small molecules (except in specific cases such as facilitated diffusion).<sup>15,16</sup> Therefore cell reprogramming using protein extracts requires assays which facilitate both cell permeability (**Figure 1.3, Chapter 2**) and the delivery of large, exogenous proteins within the cell (**Chapter 3**). Three approaches have been described to permeabilize the cytoplasmic membrane: the use of electrical current (electroporation),<sup>17</sup> bacterial toxin pore formation,<sup>18</sup> and membrane solubilization using nonionic detergent.<sup>19</sup> While commonly employed for transfection, electrical current does not generate pores of sufficient size to permit entry of proteins larger than 1KDa.<sup>19</sup> Several bacterial toxins have been used to penetrate the cytoplasmic membrane: (I) alpha toxin, which forms small pores,<sup>15,19,18</sup>; (II) streptolysin O (SLO), which forms large pores through an

association with cholesterol in the plasma membrane.<sup>15,19,18</sup> SLO mediates the uptake of molecules larger than 200 kDa into cells, and thus allows the entry of a wide range of proteins within cell extracts into cells.<sup>15,19</sup>

Similar to SLO, the nonionic detergents, digitonin and saponin (though structurally different) can permeabilize cells in a cholesterol dependent manner. Although the precise mechanism remains unclear, digitonin is thought to act through association,<sup>20</sup> replacement, solubilization, and/or the sequestration<sup>21</sup> of cholesterol permitting the entry of large protein molecules into cells.<sup>8,22,23</sup> In the first extract based reprogramming experiments, SLO was used to facilitate reprogramming of human 293T cells using Jurkat whole-cell extracts.<sup>24</sup> The same approach was adopted for cell treatment using both NTERA2 cl. D1 (NT2) and NCCIT extracts.<sup>1,24,25</sup> Both SLO and digitonin (at low concentrations), are thought to permeabilize the cell membrane exclusively.<sup>26,27,28</sup> Reprogramming factors enter the cell by diffusion and exert their activity in the cytoplasm or after nuclear import in the somatic cell nucleus.<sup>29,30,31,32</sup> However, it should be noted that SLO activity is difficult to control and can vary in a batch dependent manner.<sup>8,33</sup> Later studies have employed digitonin as a permeabilization agent, although experimentally its activity also requires careful manipulation, despite its greater consistency over bacterial toxins.<sup>8,34</sup> In either case cells can be resealed through the addition of CaCl<sub>2</sub> which inhibits digitonin or SLO activity.<sup>4</sup> For my experiments, I used digitonin for cell permeabilization.

A variety of treatment times (1-10minutes) and concentrations (0-200ug/mL) have been used for digitonin-mediated permeabilization.<sup>2,8,34,35,36</sup> Permeabilization using digitonin, can be affected by cell cholesterol content, consequently it may vary by cell type.<sup>8</sup> For my initial treatments, I used a moderate treatment time (5 minutes) mandating a low concentration of digitonin as determined by previous work.<sup>34</sup> Multiple approaches have been used to assess digitonin-mediated cell permeabilization, and for my initial assessment I elected to use trypan blue (discussed further in **Chapter 2**). While ideal, the permeabilization of the entire sample population (100%) often compromises cell viability. Therefore, to maximize cell permeabilization without compromising cell viability, I elected to use digitonin concentrations which could permeablize 80% of treated cells, an approximation based on assessments in previously published studies<sup>1,8,34</sup>.

Pluripotent cells contain all factors involved in initiating and maintaining the pluripotent state. One approach to extract-based reprogramming is the replacement of somatic proteins with proteins originating from pluripotent cells. Recent studies have demonstrated that protein extracts isolated from ESCs (46C, J1)<sup>5</sup> and ECCs (NCCIT, F9)<sup>1,37</sup> can induce activities and process associated with at least partially somatic cell reprogramming. In this study, I tested two available pluripotent cell lines: a mESC line, CJ7 and a human ECC line, NT2. CJ7 is a germline competent mESC line.<sup>38,39</sup> The NT2 cell line is a subclone of a cultured testicular germ cell tumor, originally isolated from the lung metastasis of a human patient.<sup>40</sup> NT2 cells can be maintained in a pluripotent state without chemical stimulation, and have the capacity to differentiate toward multiple

lineages, in particular the neuronal lineage.<sup>40</sup> CJ7 and NT2 express factors required for establishing and maintaining the pluripotent cell state such as *Oct4* and *Nanog*.<sup>38,39</sup>

Various indicators have been used to assess the reprogramming potential of extracts. Therefore I decided to assess cells for two primary changes observed during extract-based reprogramming: colony formation and pluripotent gene expression. In pluripotent cells, *OCT4* is considered central to embryonic stem cell potency and prevents differentiation.<sup>41,42,43</sup> Therefore, reconstitution of *OCT4* expression is considered a significant marker for the success of reprogramming assays<sup>1,44,45,46</sup> *OCT4* expression has been primarily examined in ESCs and pluripotent cell lines; however, *OCT4* is also expressed in a tissue-specific fashion<sup>47</sup> in either non-pluripotent cell lines or embryonic carcinomas.<sup>47,48</sup> Pluripotent cells such as ESCs or ECCs express two *OCT4* variants: *OCT4A* (*POU5F1*) and *OCT4B* (*POU5F1B*).<sup>47,48</sup> While some non-pluripotent cells express *OCT4B* exclusively.<sup>48</sup> Although, *OCT4* splice variants have not been addressed in extract-based reprogramming experiments, nascent transcripts have been observed in cells treated with *Xenopus laevis* egg extracts and during the injection of somatic nuclei into *Xenopus laevis* egg.<sup>7,49</sup> Therefore, in this Chapter, I examined the differential expression of both *OCT4A* and *OCT4B* in extract treated cells, to determine if cells are reprogrammed to express *OCT4* in a fashion similar to pluripotent or non-pluripotent cells.

## **Materials and Methods:**

### **Somatic Cell Culture**

Human 293T were provided by Geoffrey Shouse of Xuan Liu's laboratory, human BJ neonatal fibroblasts were provided by the UCR stem cell core facility, and mouse 3T3 cells were graciously provided by the laboratory of Susan Kane. Each line was cultured under similar conditions. Cells were seeded in 100mm cell culture dishes and adherent cells were cultured in “**complete DMEM**”: high-glucose Dulbecco's Modified Eagle Medium (Mediatech) supplemented with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum (FBS), 4 mM L-glutamate (Mediatech), 1mM sodium pyruvate (Sigma), 0.1 U/ml penicillin, and 0.1 µg/ml streptomycin. Cells were cultured at 37°C with 5% CO<sub>2</sub> and subcultured every 3-5 days by trypsinization.

### **Pluripotent Cell Extract Donors**

Mouse CJ7 cells were cultured in six well culture plates coated with 0.1% gelatin porcine skin, type A (Sigma) as previously described.<sup>39</sup> Cells were maintained in “**complete KO DMEM**”: Knockout™ DMEM supplemented with 15% Knockout™ Serum Replacement, 2 mM L-Glutamine, 0.1 mM MEM nonessential amino acids, 0.1 U/ml penicillin, 0.1 µg/ml streptomycin, 0.55 mM β-mercaptoethanol (Sigma), and 1,000 U/ml ESGRO® [Leukemia Inhibitory Factor (LIF)]. Cells were cultured at 37°C with 5% CO<sub>2</sub>. Cells were subcultured every 3-4 days by trypsinization.

Human NT2 were obtained from American Type Culture Collection (ATCC). NT2 cells were cultivated in 150 mm culture dishes, in complete DMEM and cultured at 37°C with 5% CO<sub>2</sub>. NT2 cells were subcultured by gentle scraping.

### **Preparation of Human 293T Somatic Cells**

Only 293T cells at approximately 50-80% confluency were used for cell treatments. Cells were collected by trypsinization. Following collection, cells were maintained at 4°C between steps wherever possible. First, cells were washed twice in Phosphate Buffered Saline (PBS): for each wash step, pelleted cells were first resuspended in PBS, and then centrifuged at 300 x g for 5 minutes at 4°C in a swing-bucket rotor. Pelleted cells were then resuspended in freshly prepared and sterile-filtered Hank's Balanced Salt Solution (HBSS) containing 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, and 4.2 mM NaHCO<sub>3</sub>. Resuspended cells were quantified on a hemocytometer to achieve precise cell counts in aliquots. Resuspended cells were then centrifuged at 300 x g at 4°C for five minutes. Pelleted cells were then resuspended in HBSS and distributed into 1.5mL reaction tubes as individual aliquots – typically 1mL of HBSS containing 1-2x10<sup>5</sup> cells. Subsequently, cells were centrifuged in a barrel rotor at 300 x g for 10 minutes. Pelleted cells, termed “**prepared 293T cells**” were immediately used for digitonin treatments followed by permeability assessment (see **Trypan Blue Staining** below) or treatment with pluripotent cell extracts (see **Permeabilization and Extract Treatment**).

### **Trypan Blue Staining**

Trypan blue staining was used to assess cell permeabilization. Prepared 293T cells (see **above**) were treated in aliquots. Each aliquot of  $1 \times 10^5$  cells in 1.5mL reaction tubes was gently suspended in 100  $\mu$ L of either 0, 0.5, 1.5, 2.5, 3.5, 4.5, or 5.5  $\mu$ g/mL of digitonin in HBSS for 5 minutes. 900  $\mu$ L of HBSS was then added to reaction tubes to inactivate digitonin (through dilution). Aliquots were then centrifuged at 300 x g for 10 minutes. Following centrifugation, liquid was removed from treated cells. Cells were then incubated in 100  $\mu$ L of dilute trypan blue (0.4% trypan blue diluted 1:2 in HBSS) for 3 minutes. Cells were then counted on a hemocytometer; percentages were determined by comparing stained (blue) cells with the total cell number (blue plus unstained cells) for each treatment concentration.

### **Pluripotent Cell Extract Preparation**

Both CJ7 and NT2 cells were grown to 80% confluence, to maximize material for extract preparation. Each line was processed differently depending on source: CJ7 cells were briefly trypsinized inside six well cell culture plates, and then were resuspended in 10 mLs of complete KO-DMEM. NT2 cells were removed from cell culture dishes by gentle scraping. Each was deposited in 15mL conical tubes for centrifugation, cells were then pelleted at 300 x g, for 10 minutes 4°C. For washing steps, each line was resuspended in

10mL of PBS and centrifuged at 300 x g for 10 minutes at 4°C. After the final wash with PBS, cell were washed by suspension in detergent-free lysis buffer [20 mM HEPES pH 8.2, 50 mM NaCl, 5mM MgCl<sub>2</sub>, 1mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% protease inhibitor cocktail (Sigma)] and centrifuged for 10 minutes at 300 x g at 4°C. After centrifugation, all remaining liquid was removed from cell pellets, and pellet volume was estimated (~250 uL). Each pellet was matched with an equal volume of lysis buffer. Resuspended cells were allowed to swell on ice for 40 minutes then lysed by three rounds of sonication at 20% power. Lysis was verified by light microscopy, by which no intact cells were visible in extracts. Extracts were then centrifuged at 13,000 RPM in a table top centrifuge at 4C. Clarified portions of extract supernatant were retained and supplemented with an ATP regenerating system containing 1mM ATP (Sigma), 10mM creatine phosphate (Sigma), 25 µg/ml creatine kinase (Sigma) and 1mM of each ribonucleotide triphosphate (Roche). Finally, extracts were filtered through 0.2µm filter to eliminate any remaining intact cells.

### **Permeabilization and Extract Treatment of 293T cells**

293T cells were prepared as described (see **cell preparation** above). Cell pellets were gently resuspended in either 100µL of HBSS (for treatment control), or 100 µL of 2 µg/mL of digitonin for cell permeabilization. Cells were then incubated for 5 minutes, after which 900 µL was added to inactivate digitonin activity (by dilution). Cells were then centrifuged at 300 x g for 10 minutes. After centrifugation, all liquid was removed from cell pellets. Cells were then suspended in either 100 µL of detergent-free lysis (see **Pluripotent Cell Extract Preparation**) buffer, CJ7 (ESC) extract, or NT2 (ECC)



extract. Cells were then incubated for 1hr in a water bath incubator, with gentle tapping to keep cells in suspension. Following treatment, aliquots of cells including protein extract were resuspended in complete-KO DMEM supplemented with  $\text{CaCl}_2$  (which inhibits digitonin activity). Individual cell aliquots were plated into three wells of 24-well cell culture plates for the first week of culture, and then transferred to 6 well plates during expansion and subculture. All cells, including controls were cultivated in complete KO-DMEM.

### **Cell Photographs**

Reference pictures were taken on different instruments: human 293T, mouse 3T3, CJ7, and NT2 were photographed using a NikonD100, connected to a Meiji Techno 5400 Inverted Phase Contrast Microscope. Human BJ neonatal fibroblasts cells were photographed using the Eclipse Ti (Nikon, UCR stem cell core). Treated cells were photographed (in 24 well cell culture dishes) 1 week for (293T-mESCs) and 2 weeks (293T-lysis buffer, 293T-NT2) following extract treatment through the ocular of a Nikon Eclipse T5100 microscope using a Canon PowerShot SD780 IS.

### **RT-PCR**

#### **Isolation of RNA and reverse transcription**

Cells were either isolated by trypsinization (attached cells) or direct centrifugation (floating cells). Isolated cells were suspended in PBS and centrifuged at 300 x g to remove traces of DMEM. Then cells were resuspended in 1mL of TRIZOL reagent

(Invitrogen) for 5 minutes after which 200  $\mu$ L of chloroform was added to each reaction tube. Mixtures were then centrifuged at 13,000 x g for 10 minutes. The aqueous phase was isolated from each sample then resuspended in 500  $\mu$ L of isopropanol. RNA was then pelleted by centrifugation at 13,000 RPMs for 10 minutes. Pellets were then washed with 1mL of 70% EtOH and collected by centrifugation at 12,000 RPMs for 10 minutes. Pellets were allowed to dry for 10 minutes under a chemical hood. RNA was resuspended in 25  $\mu$ L aliquots. Samples were each treated using TURBO-DNASE-free kit (Ambion now Invitrogen) according to manufacturer's instructions. cDNA was prepared using SuperScript II (Invitrogen), according to manufacturer's instructions.

## **PCR**

PCR reactions were performed using 2X GoTaq Green Master Mix (Promega) with a total reaction volume of 25  $\mu$ L including: 0.5  $\mu$ L each of forward and reverse primers, 0.5  $\mu$ L of cDNA or gDNA (for primer validation). Products were amplified using the following conditions: 95 °C for 5 min followed by 40 cycles of 95°C for 15s, 60°C for 10s and 72°C for 20s.<sup>5</sup> GAPDH transcript was detected for standardization of cDNA pools.

## **PCR Primers**

All samples were free of gDNA, and primers used were exon spanning where possible as verified using Genbank (NCBI) and In-Silico PCR (UCSC genome browser). Some primers were available from previously published works.<sup>1,50</sup>

>[NM\\_000478.4](#) Homo sapiens alkaline phosphatase, liver/bone/kidney (ALPL)

transcript variant 1, mRNA

product length = 114

Forward primer 1 CCTACCAGCTCATGCATAACATC 23

Template 871 ..... 893

Reverse primer 1 TGGCTTTCTCGTCACTCTCATAAC 23

Template 984 ..... 962

>[NM\\_024865.2](#) Homo sapiens Nanog homeobox (NANOG), mRNA

product length = 151

Forward primer 1 TTTAATAACCTTGGCTGCCG 20

Template 1673 ..... 1692

Reverse primer 1 CCTCCCAATCCCAAACAATA 20

Template 1823 ..... 1804

>[NM\\_001257374.1](#) Homo sapiens lamin A/C (LMNA), transcript variant 4, mRNA

product length = 240

Forward primer 1 CTGTGGTTGAGGACGACGAG 20

Template 1351 ..... 1370

Reverse primer 1 TGCGGTAGCTGCGAGTGA 18

Template 1590 ..... 1573

>[NM\\_001198557.1](#) Homo sapiens lamin B1 (LMNB1), transcript variant 2, mRNA

product length = 163

Forward primer 1 AAGGCGAAGAAGAGAGGTTGAAG 23

Template 878 ..... 900

Reverse primer 1 GCGGAATGAGAGATGCTAACACT 23

Template 1040 ..... 1018

>[NM\\_002701.4](#) Homo sapiens POU class 5 homeobox 1 (POU5F1), transcript variant 1, mRNA

product length = 195

Forward primer 1 GATGGCGTACTGTGGGCCC 19

Template 270 ..... 288

Reverse primer 1 TGGGACTCCTCCGGGTTTTG 20

Template 464 ..... 445

>[NM\\_001159542.1](#) Homo sapiens POU class 5 homeobox 1B (POU5F1B), mRNA

product length = 652

Forward primer 1 CAGGCACTGTGTTTCATTGCT 20

Template 131 ..... 150

Reverse primer 1 CCAAATAGAACCCCCAGGAT 20

Template 782 ..... 763

>[NM\\_002046.4](#) Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1, mRNA

product length = 238

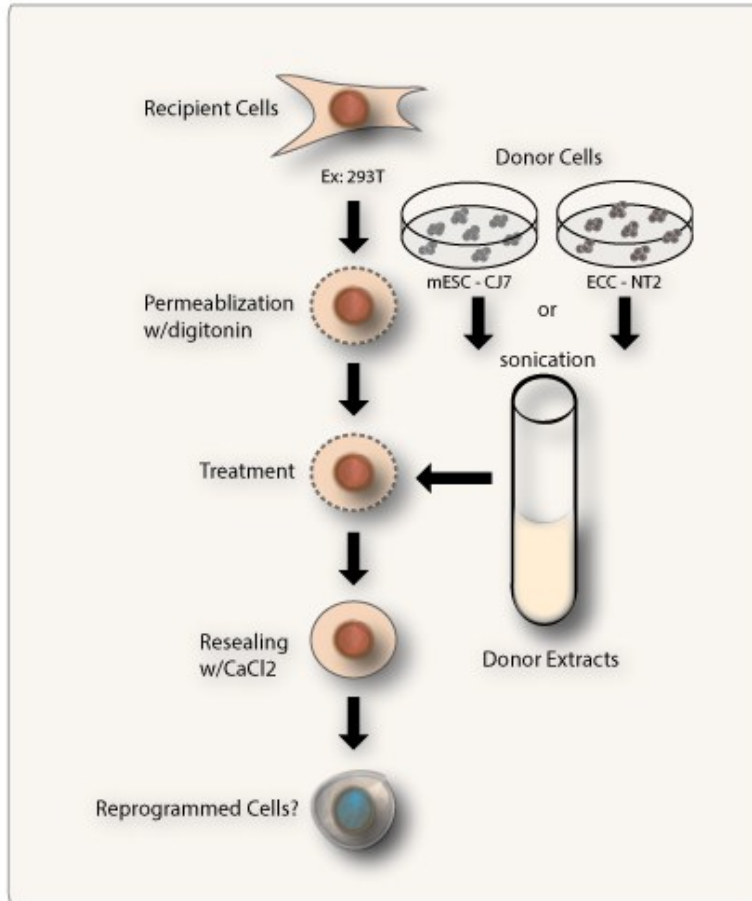
Forward primer 1 GAGTCAACGGATTTGGTCGT 20

Template 194 ..... 213

Reverse primer 1 TTGATTTTGGAGGGATCTCG 20

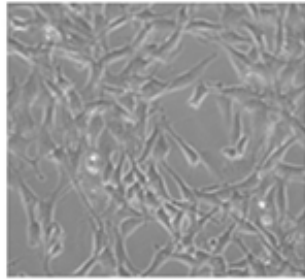
Template 431 ..... 412

## Results and Figures:



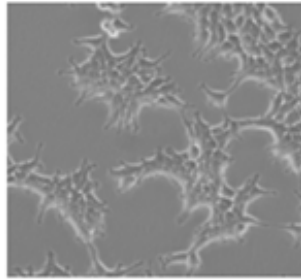
**Figure 1.1** Cell extract-based reprogramming experiments. Healthy somatic cell recipients are washed to remove calcium, and then exposed to bacterial toxins or nonionic detergents for permeabilization. Permeabilized cells are then treated with whole-cell (ESC, oocyte, egg, ECC) extracts for extensive time outside the incubator. Exogenous protein is then removed and cells are placed in resealing medium with CaCl<sub>2</sub> to inactivate permeabilizing agents. Cells can then be subjected to further analysis.

## A. Somatic Cell Recipients



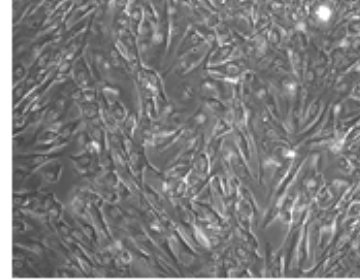
3T3

immortal mouse fibroblasts



293T

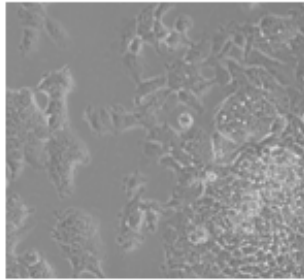
human embryonic kidney



BJ

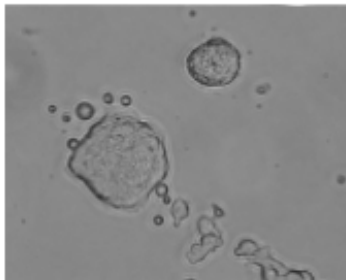
human neonatal fibroblasts

## B. Pluripotent Cell Donors



NTERA-2 cl.D1

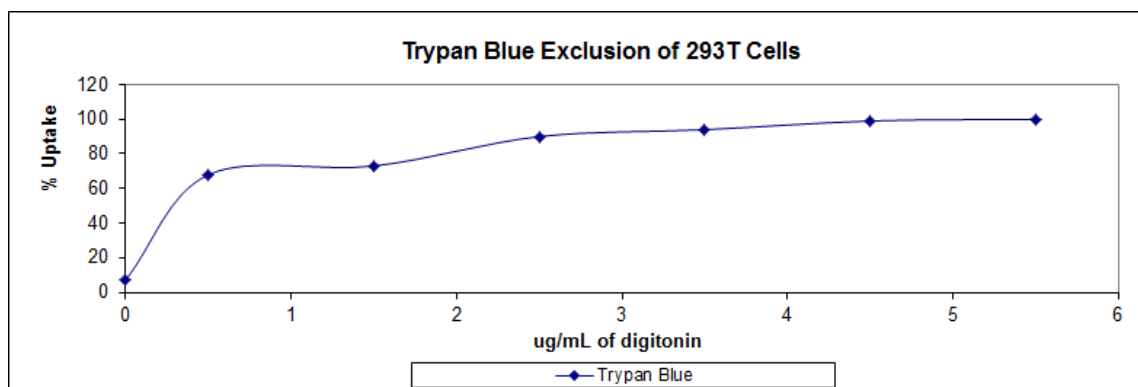
embryonic carcinoma (hECC)



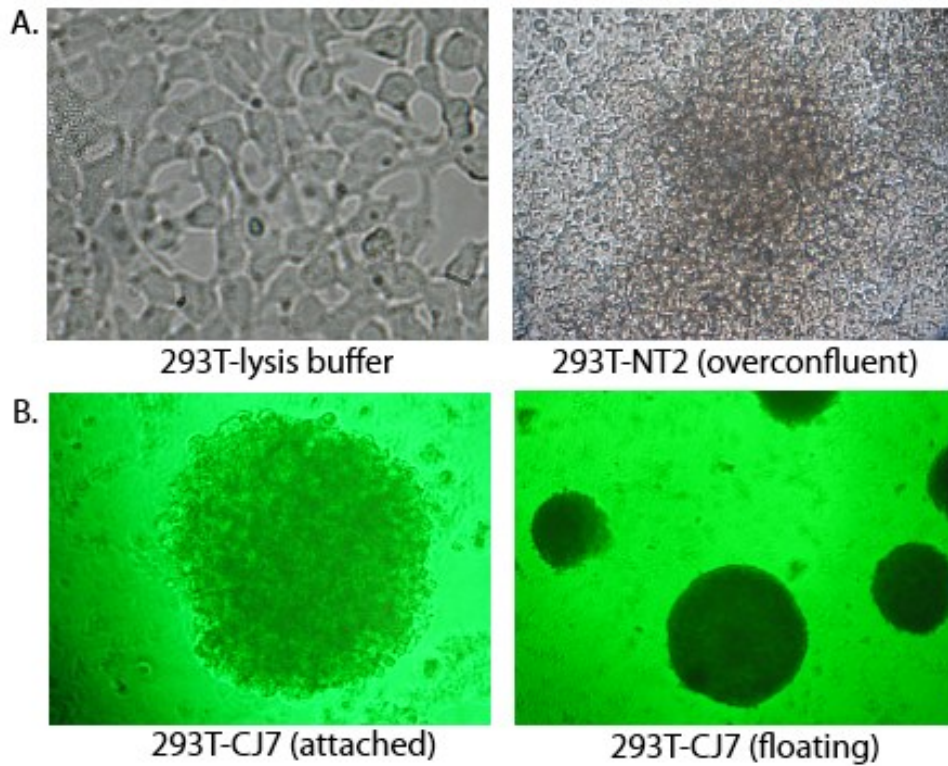
CJ7

mouse stem cell (mESC)

**Figure 1.2** Somatic cell recipients and pluripotent cell extract donors. Three somatic cell donors were used in this study: **A.** mouse 3T3 cells (**Chapters 2-4**), human 293T cells (**Chapter 1**) and human BJ neonatal fibroblasts (**Chapter 3**). Two pluripotent cell extract donors were used for pilot experiments: human NTERA-2 cl.D1 embryonic carcinoma, and mouse CJ7 colonies.

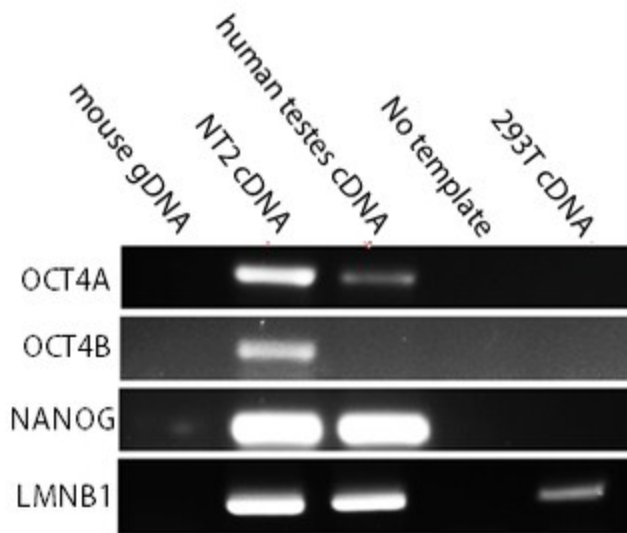


**Figure 1.3** Optimization of digitonin concentration for the permeabilization of 293T cells. Human 293T cells were treated with low concentrations of digitonin to determine its effect on small molecule uptake (trypan blue). Even at low concentrations, digitonin effectively permeabilized 293T cells.

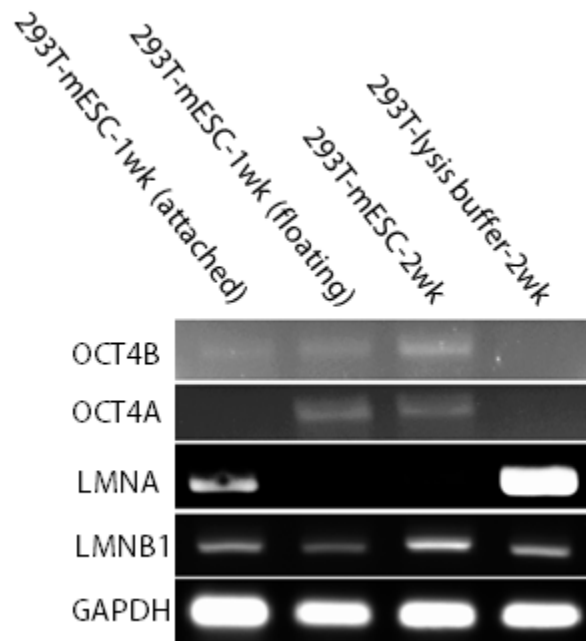


**Figure 1.4** Morphological changes following extract treatments. Recipient 293T cells were treated with lysis buffer and NTERA-2 cl.D1 or CJ7 whole cell extracts as indicated. **A.** Cells treated in lysis buffer only retained normal 3T3 cell morphology. Following treatment with NTERA-2 cl.D1 embryonic carcinoma extracts. Morphological changes were not evident, except in cases of higher confluence, where cells compacted into “bulges” or clusters, reminiscent of NTERA-2 cl.D1 cell morphology. **B.** In the case of treatment with mESC extracts, large proliferative colonies formed resembling mESCs or hESCs. Many cells floated as large, round clusters reminiscent of embryoid bodies.

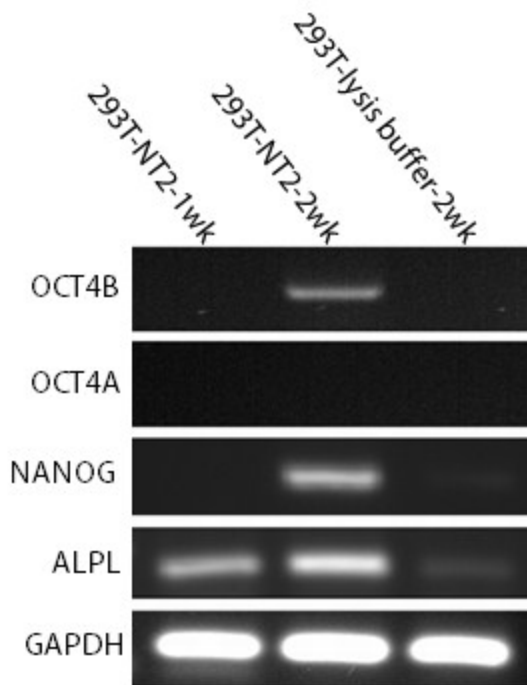




**Figure 1.5** Transcription of *OCT4* splice variants, *NANOG*, and *LMNB1* in various templates. Primers specific for *OCT4A*, *OCT4B*, and *NANOG* were successful in amplifying pluripotent genes exclusively in cDNA templates indicating selected primers were specific for cDNA (positive control). No products were amplified using mouse genomic DNA as a template (negative control) and additionally, only *LMNB1* was amplified in 293T cell cDNA.



**Figure 1.6** Expression of pluripotent markers in CJ7 extract treated 293T Cells. Cells treated with mouse CJ7 extract expressed the pluripotent gene *OCT4*. Initially cells were tested on the basis of attachment. After 1 week, *OCT4B* was detected in both floating and attached 293T cells treated with CJ7 extract. Both splice variants *OCT4A* and *OCT4B* were detected in initially floating cells and after two weeks when cells were attached exclusively. Additionally, the marker *LMNA* was detected in control cells, but reduced in both floating and attached cells (2wk). As expected both *GAPDH* and *LMNB1* were detected in all cells treated or untreated.



**Figure 1.7** Expression of pluripotent marker genes in NT2 extract treated 293T cells. NTERA-2 cl.D1 extract treated cells were analyzed for pluripotent marker expression at indicated time points. After 1 week of treatment, neither *OCT4A* nor *OCT4B* could be detected. However, after two weeks in culture, both *OCT4B* and *NANOG* were expressed in treated cells. Notably absent was *OCT4B*, the splice variant associated with pluripotent cells. Under each condition *GAPDH* was detectable.

### Morphological Features of Cultured Cells

Cell lines used as somatic recipients and pluripotent extract donors each had well-defined morphological features. Individually, each cell line [mouse 3T3 (**Chapter 2-4**), human 293T (**Chapter 1**) and human BJ neonatal fibroblasts (**Chapter 3**)] possessed fibroblast morphology: elongated, with multiple attachment points (**Figure 1.2A**). 3T3 and BJ neonatal fibroblasts cells formed flat monolayers while 293T cells which form flat clusters (**Figure 1.2A**). The morphology of the donor cells differed drastically from

recipient cells: both CJ7 and NT2 formed colonies; CJ7 colonies were clearly 3-dimensional, NT2 colonies were flat (**Figure 1.2B**).

### **Initial Trypan Blue Exclusion Assay**

Digitonin-mediated cell permeabilization of human 293T cells was optimized using trypan blue. Human 293T cell aliquots were treated with increasing amounts of digitonin (0.5-5.5  $\mu\text{g}/\text{mL}$ ) for 5 minutes, and then monitored for uptake of trypan blue. Even at low digitonin concentrations, the majority of treated cells were permeable to trypan blue (**Figure 3**). The digitonin concentration, which permeabilized 80% of cells (2  $\mu\text{g}/\text{mL}$ ), was used for cell permeabilization and extract treatment (**Figure 1.3**).

### **Cell Morphological Changes**

Somatic cell lines (human 293T, mouse 3T3, and human BJ neonatal fibroblasts) and donor pluripotent cell lines (human NT2 and mouse CJ7) exhibited morphology consistent with previously published works.<sup>39,40</sup> Following treatment the morphology of 293T cells resembled the one of untreated, cultured 293T cells, as exemplified in a monolayer of cells each with defined attachment points (**Figure 1.4A left**). When cells were treated with 2  $\mu\text{g}/\text{mL}$  digitonin, and incubated with pluripotent cell extracts, morphological changes associated with pluripotency were apparent in some treated samples (**Figure 1.4**). Although most NT2 extract treated 293T cells retained normal 293T morphology, 3-dimensional cell bugles were observed similar to the ones in cultures of NT2 cells at high confluency (**Figure 1.4A right**). However, it should be noted that such changes may occur as a result of clumping during treatment. 293T cells

treated with CJ7 extracts underwent a much more dramatic morphological change. After 1 week, large colonies were observed attached to the plate surrounded by floating spheres (**Figure 1.4B**). These colonies are reminiscent of ESC colonies, but are distinctly different from CJ7 cells, indicating the conversion of 293T cells toward 3-dimensional morphology associated with the pluripotent state.

### **Gene Expression Changes**

In 293T cells treated with CJ7 extracts, expression of the pluripotent marker gene *OCT4* was detected after one week of treatment. Attached cells, expressed the pluripotent cell specific variant *OCT4B* exclusively while floating cells expressed both *OCT4A* and the non-specific variant *OCT4B* (**Figure 1.6**). After 2 weeks of treatment, nearly all cells were attached and both *OCT4A* and *OCT4B* transcripts were detected (**Figure 1.6**).

Interestingly, cell populations, which expressed both transcriptional variants, also lost the somatic/early differentiation marker *LMNA* expression (see **Introduction**). In cells treated with NT2 extracts, only *ALPL*, a tissue-specific form of alkaline phosphatase, was detectable following one week of treatment (**Figure 1.7**). After two weeks of treatment, cells transcribed *ALPL*, *OCT4B*, and the pluripotent marker gene *NANOG*.

### **Discussion**

In my initial experiments, I sought to study the reprogramming potential of pluripotent cells. I used two lines: CJ7 and NT2 (**Figure 1.1**). Both pluripotent lines should provide extracts suitable for extract based reprogramming as extracts prepared from similar cell lines were successfully used in prior reprogramming experiments.<sup>1,6,37</sup>

Although a simple concept, the delivery of protein through the somatic cell membrane is difficult without compromising cell survival thereafter. For my initial approach, I determined a concentration of digitonin which permeabilized 80% of somatic cells (**Figure 1.3**). Here, I report the results of successful reprogramming experiments. In some treatments of 293T cells were adversely affected either through digitonin or extract toxicity as previously reported.<sup>1,25</sup> Therefore, for my pilot experiments I determined effective concentrations should not only emphasize cell permeabilization, they should include cell survival (**Chapter 2**). Without accounting for survival following digitonin treatment, lack of cell survival cannot be attributed to digitonin treatment or extract toxicity alone.

293T cells, which survived both permeabilization and extract treatment, showed morphological and molecular changes, which serve as indicators of reprogramming. Morphological change, were apparent only in extract in treated cells. Cells treated with CJ7 extract are termed 293T-mESC. Morphologically, 293T-mESC cells did not resemble cultured 293T cells (**Figure 1.2**) or buffer-treated controls (**Figure 1.3A**). 293T-mESCs formed large-round colonies one-week following treatment (**Figure 1.3B**). Among colonies, floating spheres were observed unattached to cell culture plates (**Figure 1.3B**). The loss of features associated with somatic cell origins (flat, adherent cells, with defined attachment points), and the formation of colonies, a notable feature of pluripotent cells, suggest 293T-mESCs are reprogrammed toward a pluripotent state. Although morphological changes indicate cell reprogramming, additional markers for pluripotency

such as gene expressional changes have to be analyzed to support somatic to pluripotent cell reprogramming.

To investigate molecular changes associated with reprogramming, I determined *OCT4* expression in extract-treated cells. Interestingly, dependent upon morphology *OCT4* marker gene expression differed between both attached and unattached cells (**Figure 1.6**). Attached colonies did not differ significantly from control, lysis buffer treated cells. Attached cells weakly transcribed *OCT4B* and showed solid transcription of the somatic marker gene *LMNA* (**Figure 1.6**). In floating cells however, both the *OCT4A* and *OCT4B* variants were transcribed indicating that floating cells were reprogrammed toward pluripotent gene expression. This expression coincided with loss of the somatic marker *LMNA* (**Figure 1.6**). After two weeks, cells treated with CJ7 extracts were found attached exclusively and expressed both *OCT4* transcriptional variants *OCT4A* and *OCT4B* (**Figure 1.6**). The morphological changes in treated cells as well as activation of *OCT4* expression and reduction of *LMNA* expression indicates that 293T somatic cells are converted toward a pluripotent state.<sup>1,48</sup> Expression based on attachment may reflect the effectiveness cell of permeabilization, with some cells retaining different amounts of pluripotent cell protein. Reprogrammed cells may also respond differently to treatment progressing at different rates following treatment, a phenomenon reported in iPS-based reprogramming methods.

In cells treated with NT2 extract, effects on cell morphology were not clearly defined as with CJ7 cell treatments (**Figure 1.3**). While both 293T and NT2 cell lines are

morphologically similar (**Figure 1.2**), at high confluence NT2 cells exhibit large 3-dimensional “bulges” whereas 293T cells form confluent monolayers. In 293T treated with NT2 extracts, confluent regions of cells NT2 at high confluence (**Figure 1.4A right**), with bulges similar to those observed in NT2 cell culture.

After 1 week expression of *ALPL* was the only transcriptional change detected. Following two weeks of treatment, the splice variant *OCT4B* was detectable along with the pluripotent cell gene marker *NANOG*. Interestingly, although both isoforms (*OCT4A* and *OCT4B*) are expressed in NT2 cells, only the splice variant *OCT4B* was observed in treated cells. Therefore *OCT4B* is expressed in cells treated with either mESC, or ECC, extracts, while the pluripotent variant *OCT4A* was transcribed solely in mESC, extract treated cells. These results indicate that while NT2 extracts are capable of eliciting *OCT4* expression, the splice variant activated is nonspecific for the pluripotent state, as it can also be found in somatic cells or carcinomas incapable of differentiation.<sup>48</sup>

From the onset, preliminary experiments revealed experimental concerns for both pluripotent donors: CJ7 and NT2. To perform reprogramming experiments, large quantities of donor and recipient cells are required. Even so, extracts can adversely affect cell survival depending on individual preparations. Additionally, media for culturing and maintaining ESCs, such as CJ7, is cost intensive limiting experimental potential. While NT2 extracts are cheaper to generate, they are limited in reprogramming potential as evident by limited morphological changes and pluripotent marker gene expression. Previous studies also revealed that NT2 extracts can induce neuronal gene expression.



Taken together NT2 extracts may induce reprogramming, toward a mixed-lineage/partially pluripotent state.

Because extract-based reprogramming is best established in 293T cells, it was the focus of my pilot experiments<sup>1,37,5,24</sup> However, because 293T cells may not be representative of a defined lineage the selection of marker genes could be problematic. Although not evident in my experiments (lysis buffer controls **Figure 1.6, 1.7**), 293T cells can also express *Oct4* based on culture conditions.<sup>51</sup> The testing of treatment conditions would complicate the manipulation of 293T cells. Therefore, I focused the development of my extract-treatment system on two well-defined fibroblast cell line mouse 3T3 cells (**Chapter 2-4**) and human BJ neonatal fibroblasts (**Chapter 3**).

Initial experiments revealed necessary controls required for extract-based reprogramming. Accounting for cell permeability and survival (**Chapter 2**) as well as large molecule penetration and retention (**Chapter 3**) is crucial toward establishing a stable and consistent extract-based reprogramming system. While CJ7 and NT2 extracts each demonstrated reprogramming potential, CJ7 cells may be cost-prohibitive and NT2-cells may not induce proper reprogramming. Therefore, in **Chapter 4**, I used an oocyte donor addressing both quantities for future experiments while highlighting activities associated with SCNT.

## References:

1. Taranger, C. K. *et al.* Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells. **16**, 5719–5735 (2005).
2. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Current biology : CB* **14**, 1475–80 (2004).
3. Singhal, N. *et al.* Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. *Cell* **141**, 943–55 (2010).
4. Agarwal, S. Cellular reprogramming. *Methods in enzymology* **420**, 265–83 (2006).
5. Bru, T. *et al.* Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. *Experimental cell research* **314**, 2634–42 (2008).
6. Cho, H.-J. *et al.* Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* **116**, 386–95 (2010).
7. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by *Xenopus laevis* egg extracts. *Molecular reproduction and development* **74**, 1268–77 (2007).
8. Miyamoto, K. *et al.* Reversible membrane permeabilization of mammalian cells treated with digitonin and its use for inducing nuclear reprogramming by *Xenopus* egg extracts. *Cloning and stem cells* **10**, 535–42 (2008).
9. Han, J., Sachdev, P. S. & Sidhu, K. S. A combined epigenetic and non-genetic approach for reprogramming human somatic cells. *PloS one* **5**, e12297 (2010).
10. Zhu, S. *et al.* Brief Report Reprogramming of Human Primary Somatic Cells by OCT4 and Chemical Compounds. *Stem Cell* **7**, 651–655 (2010).
11. Gurdon, J. B. & Byrne, J. a. The first half-century of nuclear transplantation. *Bioscience reports* **24**, 545–57 (2003).
12. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–72 (2007).

13. Sharpless, N. E. Preparation and immortalization of primary murine cells. *Cell and Tissue Culture: Assorted Techniques* 223–228 (2006).
14. Todaro, G. J. & Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *The Journal of cell biology* **17**, 299–313 (1963).
15. Giacomini, K. M. *et al.* Membrane transporters in drug development. *Nature reviews. Drug discovery* **9**, 215–36 (2010).
16. GM, C. *The Cell: A Molecular Approach. 2nd edition.* (Sinauer Associates, 2000).
17. Zaharoff, D. a, Henshaw, J. W., Mossop, B. & Yuan, F. Mechanistic analysis of electroporation-induced cellular uptake of macromolecules. *Experimental biology and medicine (Maywood, N.J.)* **233**, 94–105 (2008).
18. Bhakdi, S. *et al.* Staphylococcal alpha-toxin, streptolysin-O, and Escherichia coli hemolysin: prototypes of pore-forming bacterial cytolysins. *Archives of microbiology* **165**, 73–9 (1996).
19. Schulz, I. Permeabilizing cells: some methods and applications for the study of intracellular processes. *Methods in enzymology* **192**, 280–300 (1990).
20. Nishikawa, M., Nojima, S., Akiyama, T., Sankawa, U. & Inoue, K. Interaction of digitonin and its analogs with membrane cholesterol. *Journal of biochemistry* **96**, 1231–9 (1984).
21. Eilenberg, H., Klinger, E., Przeddecki, F. & Shechter, I. Inactivation and activation of various membranal enzymes of the cholesterol biosynthetic pathway by digitonin. *Journal of lipid research* **30**, 1127–35 (1989).
22. Adam, S. a, Marr, R. S. & Gerace, L. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *The Journal of cell biology* **111**, 807–16 (1990).
23. Schulz, I. Permeabilizing Cells: Some Methods and Applications for the Study of Intracellular Processes. **192**, 280–300 (1990).
24. Håkelién, A.-M., Landsverk, H. B., Robl, J. M., Skålhegg, B. S. & Collas, P. Reprogramming fibroblasts to express T-cell functions using cell extracts. *Nature biotechnology* **20**, 460–6 (2002).
25. Collas, P. Reprogramming Somatic Cells in Extract of Undifferentiated Carcinoma Cells. [www.collaslab.com](http://www.collaslab.com) 1–11

26. Kehlenbach, R. H. & Paschal, B. M. Analysis of Nuclear Protein Import and Export in Digitonin-Permeabilized Cells. (2006).
27. Stephens, D. J. & Pepperkok, R. The many ways to cross the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4295–8 (2001).
28. Wesche, J. & Olsnes, S. Selective Permeabilization of the Cell-Surface Membrane by Streptolysin O. 19–22 (2006).
29. Marfori, M. *et al.* Molecular basis for specificity of nuclear import and prediction of nuclear localization. *Biochimica et biophysica acta* **1813**, 1562–77 (2011).
30. Yasuhara, N. *et al.* Triggering neural differentiation of ES cells by subtype switching of importin-alpha. *Nature cell biology* **9**, 72–9 (2007).
31. D'Angelo, M., Gomez-Cavazos, J. & Mei, A. A change in nuclear pore complex composition regulates cell differentiation. *Developmental cell* **22**, 446–458 (2012).
32. Young, J. C., Major, A. T., Miyamoto, Y., Loveland, K. L. & Jans, D. a. Distinct effects of importin  $\alpha 2$  and  $\alpha 4$  on Oct3/4 localization and expression in mouse embryonic stem cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **25**, 3958–65 (2011).
33. Walev, I. *et al.* Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3185–90 (2001).
34. Karl, J. *et al.* GFAP phosphorylation studied in digitonin-permeabilized astrocytes: standardization of conditions. *Brain research* **853**, 32–40 (2000).
35. Tramontina, F. *et al.* Digitonin-permeabilization of astrocytes in culture monitored by trypan blue exclusion and loss of S100B by ELISA. *Brain research. Brain research protocols* **6**, 86–90 (2000).
36. Holden, P. & Horton, W. a. Crude subcellular fractionation of cultured mammalian cell lines. *BMC research notes* **2**, 243 (2009).
37. Freberg, C. T., Dahl, J. A., Timoskainen, S. & Collas, P. Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract. *Molecular biology of the cell* **18**, 1543–53 (2007).
38. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. & Brivanlou, A. H. Maintenance of pluripotency in human and mouse embryonic stem cells through

- activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature medicine* **10**, 55–63 (2004).
39. Bertani, S., Sauer, S., Bolotin, E. & Sauer, F. The noncoding RNA Mistral activates Hoxa6 and Hoxa7 expression and stem cell differentiation by recruiting MLL1 to chromatin. *Molecular cell* **43**, 1040–6 (2011).
  40. Andrews, P. W. TERA2 and Its NTERA2 Subline: Pluripotent Human Embryonal Carcinoma Cells. (2006).
  41. Hammachi, F. *et al.* Transcriptional activation by Oct4 is sufficient for the maintenance and induction of pluripotency. *Cell reports* **1**, 99–109 (2012).
  42. Pan, G. J., Chang, Z. Y., Schöler, H. R. & Pei, D. Stem cell pluripotency and transcription factor Oct4. *Cell research* **12**, 321–9 (2002).
  43. Yamanaka, S. Pluripotency and nuclear reprogramming. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **363**, 2079–87 (2008).
  44. Cowan, C. a, Atienza, J., Melton, D. a & Eggan, K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science (New York, N.Y.)* **309**, 1369–73 (2005).
  45. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–76 (2006).
  46. Boiani, M. *et al.* Variable Reprogramming of the Pluripotent Stem Cell Marker Oct4 in Mouse Clones: Distinct Developmental Potentials in Different Culture Environments. *Stem cells (Dayton, Ohio)* **23**, 1089–104 (2005).
  47. Liedtke, S., Stephan, M. & Kögler, G. Oct4 expression revisited: potential pitfalls for data misinterpretation in stem cell research. *Biological chemistry* **389**, 845–50 (2008).
  48. Atlasi, Y., Mowla, S. J., Ziaee, S. a M., Gokhale, P. J. & Andrews, P. W. OCT4 spliced variants are differentially expressed in human pluripotent and nonpluripotent cells. *Stem cells (Dayton, Ohio)* **26**, 3068–74 (2008).
  49. Simonsson, S. & Gurdon, J. B. DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. *Nature cell biology* **6**, 984–90 (2004).

50. Wang, X. & Dai, J. Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology. *Stem cells (Dayton, Ohio)* **28**, 885–93 (2010).
51. Debeb, B. G. *et al.* Characterizing cancer cells with cancer stem cell-like features in 293T human embryonic kidney cells. *Molecular cancer* **9**, 180 (2010).

## **Chapter 2: Mouse 3T3 Cell Permeabilization**

### **Abstract:**

Cell permeabilization is required to facilitate extract-based reprogramming. To establish effective treatment concentrations using digitonin, accurate assessment of cell permeability and cell survival are necessary. By using trypan blue as marker for permeabilization, I identified digitonin treatment concentrations, which efficiently permeabilized mouse 3T3 cells, without adversely affecting their survival in culture. I also tested an alternative, “in plate” approach for permeabilization and tested the inclusion of propidium iodide as marker for membrane permeability; both approaches while indicative proved ineffective for consistent cell permeabilization and assessments, respectively. The identification of optimal digitonin treatment concentrations and conditions ultimately were paramount for determining large-molecule penetration and retention (**Chapter 3**) and establishing a protein-extract based cell reprogramming (**Chapter 4**).

### **Introduction:**

Efficient cell permeabilization is a key step in extract-based reprogramming.<sup>1,2</sup> Permeabilization requires adaptation of cells from an optimal setting suitable for survival toward an adverse environment required for extract treatments.<sup>1,2</sup> These conditions mandate that cells are treated vigorously but remain viable. Therefore as a technique, cell

permeabilization is very challenging, requiring careful refinements.<sup>1,2</sup> Cell health, handling, and preparation are among a multitude of factors, which can greatly affect efficiency of cell permeabilization and cell survival.

The uptake of large molecules requires cell treatments with harsh agents such as streptolysin-O (SLO), a bacterial toxin, or digitonin, the nonionic detergent.<sup>3,4,5</sup> In our hands, SLO had inconsistent activity as previously reported.<sup>3,6</sup> Digitonin was effective for treatment of 293T cells, however precise control of digitonin treatment is required to maintain treatment consistency and cell survival thereafter.<sup>6</sup> Therefore I included control assays monitoring cell survival during the overall treatment process.

In fixed cells, detergents are often used for penetration of large molecules such as antibodies. Digitonin has been traditionally used as a “gentle” permeabilizing agent, in applications where maintenance of internal structures was required for analysis.<sup>7,8</sup> Indeed, digitonin treatment can be used to facilitate the uptake of large molecules such as antibodies into living cells without compromising large cellular structures such as the endoplasmic reticulum and the nucleus.<sup>4,9,10</sup> Because of mild activity at low concentrations, digitonin is preferred over detergents such as Triton X-100 which can cause extensive cellular damage and cell death.<sup>11</sup>

Nevertheless, even low concentrations of digitonin are not without consequence to cell integrity. Aside from opening large pores in the cell membrane, digitonin affects internal cell components such as the cytoskeleton as evident through the release of proteins such as actin and tubulin.<sup>12,13</sup> In addition, digitonin-treatment causes contents of



the cytosol leak out of the cell including enzymes such as lactate dehydrogenase (LDH) and glutathione S-transferase (GST).<sup>12</sup> Release of enzymes such as LDH, can limit the use of enzymatic assays that can determine cell viability following digitonin treatments.<sup>14,15,16</sup> The structural changes and depletion of intracellular components can detrimentally affect cell survival.<sup>17</sup> At high concentrations, digitonin can strip mitochondrial membranes,<sup>18,19</sup> a process which releases caspases, enzymes responsible for apoptotic signaling through proteolytic cleavage.<sup>20</sup> Because digitonin concentration can trigger the release of proteases, I decided to add protease inhibitors to mediate potential problems created by apoptotic mechanisms in digitonin permeabilized 3T3 cells.<sup>1</sup>

The cellular environment during permeabilization and subsequent treatments requires different considerations from that of normal, cultured cells.<sup>9</sup> Because digitonin-mediated pores permit access to cells, treatment buffers can enter and affect cell survival during treatment. Both acetate buffer and balanced salt solutions have been used in experiments using digitonin and SLO respectively.<sup>21,22</sup> Ionic balance may aid cell survival following permeabilization, sufficiently replicating or restoring the internal environment lost during cytosolic leakage.<sup>9</sup> Because HBSS (Hanks Balanced Salt Solution) was used to mediate digitonin treatment in some reprogramming experiments, I utilized the buffer for my approach in pilot experiments.<sup>1,23</sup> When reevaluating treatment conditions I elected to use “acetate transport buffer”, which is prevalent for long-term digitonin treatments.<sup>6,24,25</sup>

Multiple methods are required to assess the effect of digitonin concentration on cell permeability and survival. While digitonin treatment can compromise several enzymatic assays that monitor cell viability, alternate approaches can be used to assess both cell health and membrane integrity following treatment.<sup>14,15,16</sup> To identify the immediate impact of digitonin, morphological effects can be used to assess cell viability.<sup>17</sup> The most obvious phenotype after digitonin treatment (at high concentrations) is cell disruption as evident through shrinking and loss of integrity.<sup>26</sup> While indicative, such an approach has to be used in conjunction with other assays capable of detecting cell survival, because at toxic concentrations cell death without structural change may occur and treated cells no longer survive in culture.<sup>6</sup> Trypan blue, which is a determinant of cell health and viability, is well suited for assessing membrane integrity following digitonin treatment. Cells treated with high concentrations of SLO are able to survive in culture, despite a loss of membrane integrity as indicated by trypan blue inclusion.<sup>23</sup> Together trypan blue exclusion and survival are useful forms of assessing permeabilization and cell survival after digitonin treatment.

Methods for assessing cell membrane integrity have often been debated, specifically for their relevance in determining cell viability.<sup>27,28</sup> Therefore, I sought to assess membrane integrity by alternative means alongside the trypan blue exclusion assay. The uptake of various colorimetric dyes into permeabilized cells has been used to assess membrane integrity. Like trypan blue, colorimetric fluorescent dyes are membrane impermeant, only entering the cell upon loss of membrane integrity.<sup>29</sup> Unlike trypan blue

however, their color and appearance requires excitations from specific wavelengths of light for visualization. I accessed the incorporation of Propidium Iodide (PI) into permeabilized cells. PI binds DNA, and emits red light, at 617 nm when excited at 535 nm with a green laser. Typically a DNA counter stain such as Hoechst 33342 is used in combination with PI to locate cells which are not penetrated by PI.<sup>30</sup>

In **Chapter 1**, I detailed an initial approach relying on ~80% permeability, without accounting for cell survival following treatment. For the experiments described in this chapter, I adjusted treatment conditions (timing, protease inhibition, buffer composition) in an effort to maximize digitonin-mediated permeabilization of mouse 3T3 cells, while maintaining cell survival. Instead of relying on a fixed level of cell permeabilization, as in **Chapter 1**, I characterized cell permeabilization through trypan blue exclusion with additional consideration of cell survival. Additionally, I tested alternative assays for both treatment and permeability respectively.

## **Materials and Methods:**

### **Preparation of Digitonin**

Large stocks of digitonin (Sigma) were prepared to ensure standardization and consistency for all cell treatments. Stock solutions were prepared by dissolving digitonin in water at a concentration of 2mg/mL, and then freezing aliquots before use. Precipitate in stock solutions was dissolved by heating the digitonin stock at 95°C (as opposed to filtration).

### **Cell Preparation (Suspension Treatments)**

3T3 cells were cultured under standard conditions (see **Chapter 1**) in 100mm cell culture dishes. Cells were grown to 50% confluency, and then trypsinized until detachment was observed (after about 3 minutes). To inactivate trypsin, complete DMEM was gently added, and cells (plus media and trypsin) were transferred to 15mL tubes. Cells were pelleted at 300 x g for 5 minutes, and then remaining media was discarded. Cells were then gently suspended in 10mL of PBS and centrifuged at 300 x g for 5 minutes. This step was repeated, then cell pellets were resuspended in freshly prepared and filtered acetate transport buffer 20 mM HEPES at pH 7.4, 110 mM potassium acetate, 5mM sodium acetate, 2mM magnesium acetate, 1mM EGTA, 0.1mM DTT, 0.1mM PMSF, and protease inhibitor tablets (Roche)] and counted on a hemocytometer (for aliquots).<sup>6</sup> Cells were then centrifuged again at 300 x g, in a swing bucket rotor. Resuspended cells were then dispersed among 1.5mL reaction tubes, in  $1 \times 10^5$  aliquots. Aliquoted cells were then centrifuged for 10 minutes at 300 x g, and remaining acetate buffer was removed ensuring while ensuring cell pellets were left undisturbed. Aliquots of digitonin were thawed by boiling at 95°C for 5 minutes. After that, working solutions were prepared by diluting digitonin with acetate transport buffer. To facilitate permeabilization, 100uL working solutions (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50  $\mu\text{g}/\text{mL}$ ) were added to individual cell samples by gentle pipetting, using 200 $\mu\text{L}$  pipette tips which had been cut

(to minimize cell shearing). Cells were then incubated on ice for 1-2 minutes. To reduce digitonin activity, 900 $\mu$ L of acetate buffer was added to each individual aliquot. Then, cells were centrifuged at 300 x g, in a barrel rotor 10 minutes at 4°C. After centrifugation, acetate transport buffer solutions containing digitonin were gently removed from pellets ensuring no cell loss and pellets remained intact. Cells were then processed for **trypan blue assessment, cell survival, or propidium iodide treatment.**

### **Trypan Blue Assessment**

Each aliquot of cells was suspended in diluted trypan blue (0.4% trypan diluted 1:2 in acetate buffer) and incubated at room temperature for 3 minutes. 10 $\mu$ L of cell mixtures were then counted under a hemocytometer, percentages were determined by comparing the number of blue-stained cells (retaining trypan blue) with the number of total cells (both cells positive for trypan blue and unstained cells). The mean percentage of permeabilized cells was obtained from three independent experiments for each concentration of digitonin.

### **Cell survival Assay**

Permeabilized cell aliquots ( $1 \times 10^5$  cells) were suspended in 100  $\mu$ L of acetate buffer, then divided in two ( $0.5 \times 10^5$  individually) for visual depiction of trypan blue uptake and assessment of cell survival. For cell survival, aliquots (50 $\mu$ L) were maintained in suspension for 1hr at room temperature, then replated into individual wells of 24-well cell culture plates containing complete DMEM supplemented with 2mM of CaCl<sub>2</sub> (which

inhibits digitonin activity). After 2-4 hours, CaCl<sub>2</sub> supplemented media was removed, then cells were cultured in normal DMEM. A total of 24 hours following treatment, cells were photographed random field near the center of each well using a NikonD100, connected to a Meiji Techno 5400 Inverted Phase Contrast Microscope.

### **In-plate Digitonin Treatments**

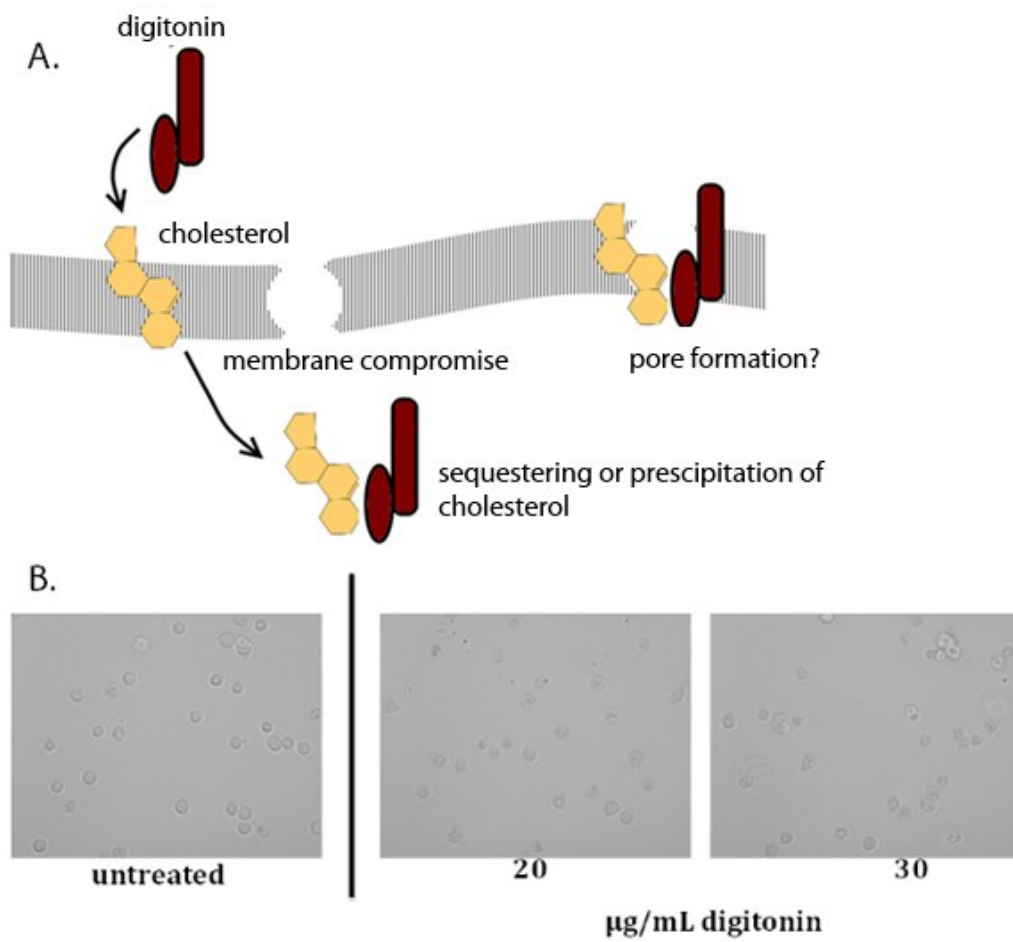
Equal amounts of 3T3 cells ( $\sim 2.5 \times 10^5$ ) were seeded into a 24 well prior to treatment. Cells subjected to treatment were at 80% confluence. First, media was removed from cultured cells by gentle aspiration. Then, for each in-plate wash step, buffers were added for 5 minutes, and then removed from adherent cells. Cells were washed twice with 1mL PBS, and twice with 1mL acetate transport buffer. For permeabilization, cells were treated with 200 $\mu$ L of digitonin diluted in acetate buffer, at concentrations of 0, 10, 20, 30, 40, or 50  $\mu$ g/mL. Digitonin was removed, and cells were washed with 1mL acetate buffer. For trypan blue staining, cells were incubated for 2 minutes with 1mL of diluted trypan blue (0.4% trypan blue in acetate buffer). Diluted trypan blue was aspirated for removal. Cells were than washed twice with acetate buffer, and maintained in acetate buffer for observation under the microscope.

### **Propidium Iodide Treatment**

Prepared cells were first treated with either 100 $\mu$ L of 0 or 30  $\mu$ g/mL digitonin in acetate transport buffer for 1-2 minutes. Then 900 $\mu$ L of acetate buffer was added to permeabilized cells to dilute digitonin activity. Diluted calls and digitonin were

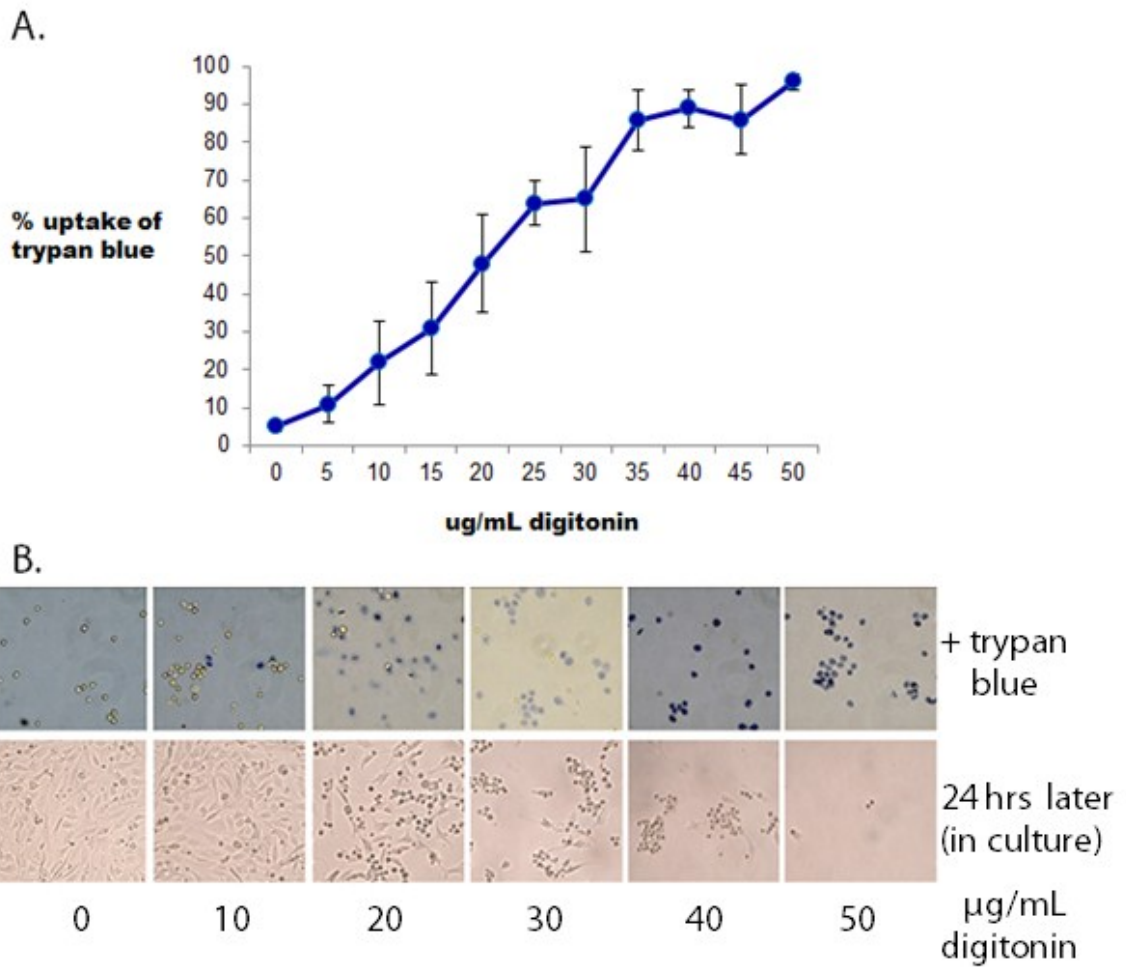
centrifuged at 300 x g for 10 minutes. Following centrifugation cells were first incubated with 100 $\mu$ L of 1  $\mu$ g/mL of Hoechst 33342 in acetate transport buffer for 15 minutes at 4°C. Then cells were centrifuged at 300 x g for 10 minutes. Finally, pelleted cells were suspended in 100 $\mu$ L of 0.5  $\mu$ g/mL of propidium iodide in acetate transport buffer for 3 minutes, 25  $\mu$ L of treated cells were pipetted to the center of a glass slide, and a cover was gently placed on top.

**Results/Figures:**

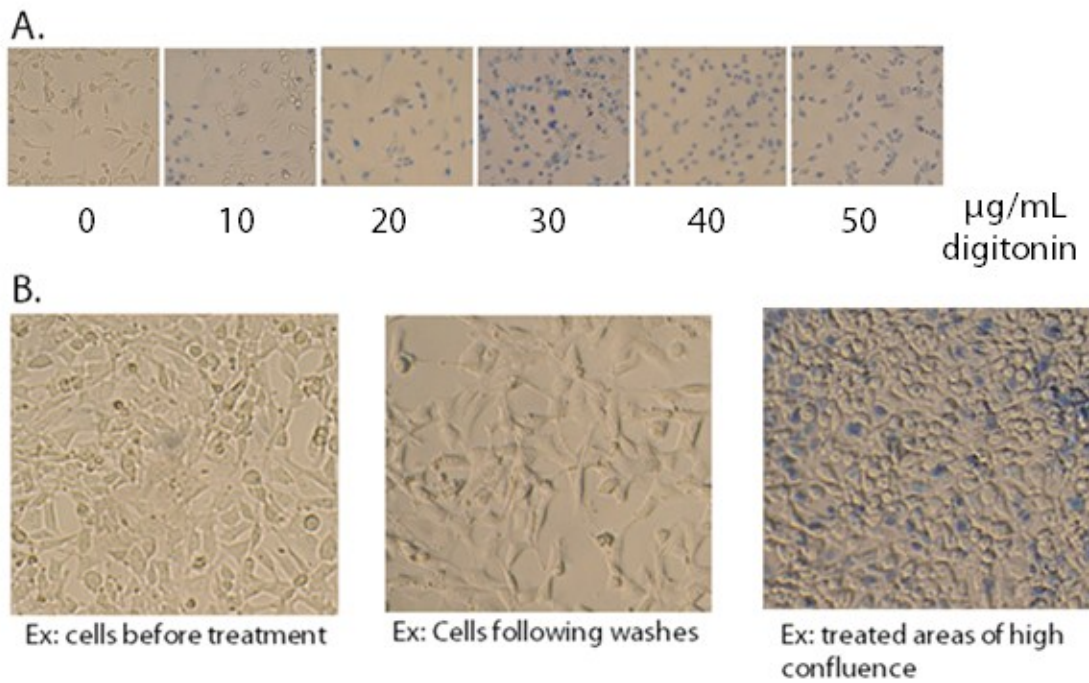


**Figure 2.1** The effects of digitonin on mouse 3T3 cells. **A.** Depiction of permeabilization mechanism(s) with digitonin. **B.** Observable changes in 3T3 cell morphology, appearance following brief treatment with digitonin.

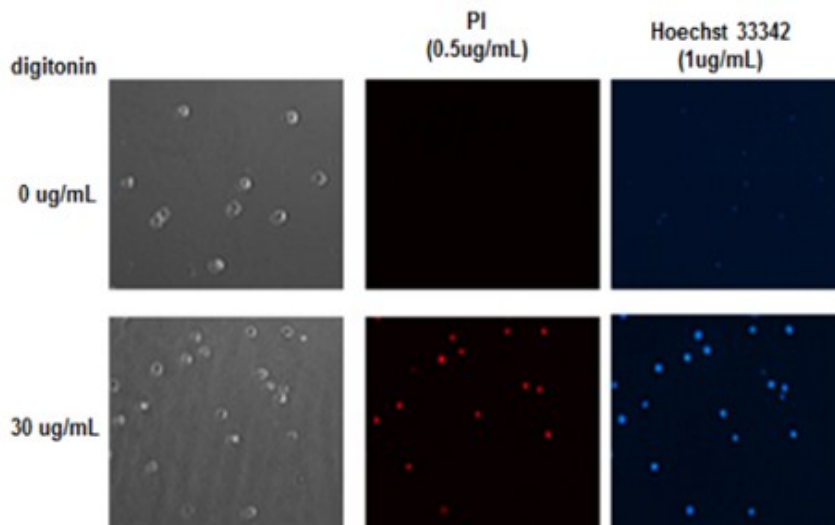




**Figure 2.2** The effects of digitonin concentration ( $\mu\text{g/mL}$ ) on trypan blue uptake (%) in 3T3 cells, and their subsequent survival after 24hrs. **A.** Percentage of cells staining positive for trypan blue following treatment with digitonin at various concentrations accessed on a hemocytometer. The average number of cells positive for trypan blue staining was determined for three or more replicate experiments. Error bars represent one standard deviation. **B.** A random field of trypan blue-stained cells on glass slides, following brief treatment with digitonin and staining. Parallel, surviving cells after 24 hours following brief exposure digitonin, dilution of activity, and incubation for two hours.



**Figure 2.3** Uptake of trypan blue by digitonin treated 3T3 cells in cell culture plates. **A.** 3T3 cells were washed, and then treated with an increasing concentration of digitonin. Subsequently cells were exposed to 50/50 trypan blue stain in PBS, and then visualized under a phase-contrast microscope. **B.** Prior to treatment, 3T3 cells appear healthy and confluent in culture. Some cells are lost after necessary washing steps. Cells are resistant to trypan blue uptake at high confluence, such as exterior areas around the plate.



**Figure 2.4** Propidium Iodide staining of digitonin treated cells. Digitonin treated cells were exposed to Hoechst 33342 and propidium iodide, and then visualized by fluorescence microscopy. Treated, permeabilized cells appeared bright red upon excitation, all cells stained positive for Hoechst 33342 (treated or untreated) however permeabilized cells appeared brighter.

### **Morphological Changes during Digitonin Treatment**

To determine if morphological changes can be used as an observable sign of cell permeabilization, treated cells were analyzed using a light microscope. Cells were treated with 0, 20, or 30  $\mu\text{g}/\text{mL}$  of digitonin, and were then pipetted onto glass slides for observation. In control cells, (**Figure 2.1B left**) treated only with acetate buffer, a “halo” surrounds the membrane highlighting the cells spherical, 3-dimensional shapes. Cells treated with digitonin lack the “halo” near their outer edge coinciding with a slightly darker interior (**Figure 2.1B right**). Therefore observable, morphological are visible following permeabilization.

### **Trypan Blue Exclusion Assay (Suspension Treatment)**

To assess the digitonin-mediated permeabilization of mouse 3T3 cells, cells were treated with increasing amounts of digitonin (0-50  $\mu\text{g}/\text{mL}$ ) and then stained with trypan blue. In untreated cells, a low percentage of cells stained positive for trypan blue (<10%) (**Figure 2.2A**). In cells treated with increasing concentrations of digitonin a near proportional uptake of trypan blue was observed (**Figure 2.2A**). Significant trypan blue staining was observed following treatment with 20-30  $\mu\text{g}/\text{mL}$  of digitonin. In cells treated with 20  $\mu\text{g}/\text{mL}$  nearly 50% of cells were positive and in cells treated with 30  $\mu\text{g}/\text{mL}$  nearly 65% of cells were positive for trypan blue (**Figure 2.2B**). At treatment concentrations beyond 30  $\mu\text{g}/\text{mL}$  greater than 80% of cells were positive for trypan blue and nearly 100% of cells were stained following treatment with 50  $\mu\text{g}/\text{mL}$  of digitonin.

### **Visible Assessment and Cell Survival**

To determine cell survival following permeabilization, mouse 3T3 cells were first treated with digitonin, then either 1) stained with trypan blue (**Figure 2.2B top**) or 2) incubated in acetate buffer for two hours, then plated in a cell culture dish for observation after 24 hours (**Figure 2.2B**). Few cells treated in acetate transport buffer alone or 10  $\mu\text{g}/\text{mL}$  of digitonin were permeant to trypan blue, and the cells remained viable after treatment (**Figure 2.2B**). Following treatment with 20-30  $\mu\text{g}/\text{mL}$  of digitonin, a significant number of cells were permeable to trypan blue, yet small portions of cells either did not attach or appeared rounded and loosely attached to cell culture plates (**Figure 2.2B**). At treatments of 40-50 $\mu\text{g}/\text{mL}$  digitonin, few cells attached. The surviving cells appeared visibly smaller

and loosely attached to the culture plate (**Figure 2.2B**). A treatment concentration between 20-30  $\mu\text{g}/\text{mL}$  of digitonin permeabilizes a significant number of cells (**Figure 2.2**) with a portion of cells surviving treatments.

### **In-plate Digitonin Treatments**

To assess in-plate digitonin treatments, adherent mouse 3T3 cells were treated with an increasing concentration of digitonin (0-50  $\mu\text{g}/\text{mL}$ ), then observed for permeability to trypan blue (**Figure 2.3**). A washing procedure, similar to cell suspension treatments, was adapted to treat cells attached to the wells of a cell culture plate. Despite the gentle application of buffers (PBS and acetate transport buffer), population loss was observed in cells treated with buffer alone (**Figure 2.3B**). Similar to suspension treatments (**Figure 2.2**), in-plate treatment with increasing concentrations of digitonin resulted in a proportional uptake of trypan blue (**Figure 2.3A**). At concentrations between 20 $\mu\text{g}/\text{mL}$  and 50 $\mu\text{g}/\text{mL}$ , cells loss is apparent in wells following digitonin treatment. Additionally, at high-treatment concentrations, cell attachment points are disturbed especially with the application of 50  $\mu\text{g}/\text{mL}$  of digitonin (**Figure 2.3A**). While photographs capture a random field at the center of treated wells, cells at different locations in the well may respond differently to digitonin treatments (**Figure 2.3B**).

## **Propidium Iodide Staining**

As an alternative to trypan blue, both Hoechst 33342 staining and PI were applied to cells treated with either (0  $\mu\text{g}/\text{mL}$ ), a treatment control, and cells treated with 30  $\mu\text{g}/\text{mL}$  digitonin, a concentration effective for the permeabilization of mouse 3T3 cells (**Figure 2.4**). Regardless of treatment condition, 3T3 cells displayed fluorescent signal for Hoechst 33342 staining (**Figure 2.4 right**). Cells treated with digitonin readily stained positive for PI while untreated control cells did not retain PI (**Figure 2.4 center**). Despite staining, variability in signal intensity was observed not only in PI staining, but also Hoechst 33342 staining.

## **Discussion:**

Although commonly employed, mechanisms of digitonin-mediated cell permeabilization have not been fully elucidated (**Figure 2.1A**). Proposed mechanisms suggest pore formation through an association with cholesterol, either directly through membrane affiliation or indirectly through precipitation and/or sequestration.<sup>31,32,33,34</sup> The effects of digitonin treatment can be detrimental for cell function and viability. The results of this chapter lay a foundation for an extract-based cell reprogramming system. Here I describe the optimization of cell permeabilization by digitonin using 3T3 cells as somatic recipient, with the additional consideration for cell survival following treatment.

As an initial approach to digitonin-mediated permeabilization, I elected to use conditions, which would ensure cell permeabilization through treatment duration (5min)

**(Chapter 1)**. In the experiments outlined here, I adjusted my treatment conditions to minimize cell exposure to digitonin (1-2 minutes) while attempting to maximize cell viability through the addition of protease inhibitors. As basic indicator, the appearance of cultured cells can indicate overall cell health. Morphologically, nonviable cells appear apoptotic (shrunken, budding) or necrotic (blebbing).<sup>26</sup> Optimization of cell viability is crucial to successful reprogramming following permeabilization.<sup>6</sup> By observing changes in cell morphology during treatment, treatment steps and factors destroying cells can be identified. In my experience, cell integrity is best preserved in acetate buffer (not HBSS or PBS) lacking particulates, through the use wide-bore pipette tips, and in the presence of protease inhibitors. At early stages detrimental effects can be confirmed using trypan blue exclusion; however at later stages morphological changes play a greater role for assessing cell viability due to the limitations of viability assays.

Aside from identifying nonviable cells, morphological changes can also reveal cell permeabilization and aid in monitoring the success of digitonin treatments (**Figure 2.1B**). A typical, “halo” is observable around the 3-dimensional shape of healthy cells. In cells treated with digitonin, the distinctive “halo” is lost coinciding with a slightly darker appearance of the cells. Such changes are distinctly different from nonviable cells, but harder to distinguish among untreated cells, except at digitonin concentrations permeabilizing a high percentage of cells (**Figure 2.1B**). Together with indicators of cell death, this observable morphological difference is useful for individual sample evaluation alongside treatment controls. Scanning electron micrographs have previously revealed

membrane perturbation and visible pore formation in digitonin treated chromaffin cells.<sup>4</sup> Its possible that such changes culminate in the visible difference observed in digitonin treated cells (**Figure 2.1B**).

Depending upon treatment conditions, previously established systems have revealed a range of digitonin concentrations effective for cell permeabilization.<sup>6,21,35</sup> However, since individual preparations of digitonin can vary and permeabilization differs among cell types, effective treatment concentrations must be determined experimentally. Therefore, I prepared digitonin from a large stock solution to ensure consistent permeabilization in subsequent experiments (**Chapter 3, 4**). As measured by trypan blue exclusion, digitonin concentration had a near-linear relationship with membrane permeability (**Figure 2A**). In addition to permeabilization, I considered cell survival following treatment at various concentrations. Cells treated with 0 or 10  $\mu\text{g}/\text{mL}$  of digitonin appear healthy 24 hours following treatment (**Figure 2.2B**). However, at 10  $\mu\text{g}/\text{mL}$  only about 20% of cells are permeable to trypan blue (**Figure 2.2A**). Cell attachment appears affected in cells treated with 20-50  $\mu\text{g}/\text{mL}$  of digitonin (**Figure 2.2B**) indicating a portion of cells no longer survived following treatment. Although treatment with 40 or 50  $\mu\text{g}/\text{mL}$  of digitonin effectively permeabilized cells, at these treatment concentration effects on cell attachment appeared detrimental to survival (**Figure 2.2**). Because 20 or 30  $\mu\text{g}/\text{mL}$  digitonin effectively permeabilized cells, and a significant portion of cells survive following treatment at these concentrations, they may represent optimal treatment concentrations for digitonin-mediated cell reprogramming (**Figure**



**2.2).** Despite reattachment, some digitonin treated mouse 3T3 cells exhibited atypical morphology in culture. 3T3 cells normally are elongated with defined attachment points; while in cell populations treated with 20-50  $\mu\text{g}/\text{mL}$  of digitonin rounded cells remain attached to the cell culture plate despite media changes. While many die, at least some appear to recover and survive (grow) in culture. At 50  $\mu\text{g}/\text{mL}$ , cells adhere in large groups and are nonviable based on morphology (shrunken appearance, adherent clusters) and by failure to reattach in culture 24 hours later (**Figure 2.2B**).

Generally, cell permeabilization prior to extract treatment is performed in cell suspensions rather than with adherent cells. To determine if “in-plate” permeabilization (and eventually extract treatment) of adherent cells was possible, I attempted to adapt suspension treatment protocols to adherent cells. An immediate concern during treatment was the loss of cells during wash steps (**Figure 2.3B**). Loss of cells might interfere with analysis following extract treatment. To monitor the feasibility of in-plate treatments, cells were first washed and treated with digitonin, then incubated with trypan blue. Similar to suspension treatments, attached cells treated with digitonin stained positive for trypan blue (**Figure 2.3A**). Although treated cells often appeared uniform, regions where cells were at a particularly high-confluence (such as the outer edges of wells), were more resistant to digitonin-mediated permeabilization (**Figure 2.3B right**). It has also been reported that large-molecule uptake is impeded in attached cells treated with SLO.<sup>2</sup> Therefore, cell confluence and attachment in culture may limit the surface area accessible by permeabilizing agents. Because washing steps adversely affected cell attachment and

“in-plate” digitonin permeabilization is both inconsistent and difficult to measure, I focused on treating cells in suspension for permeabilization and extract treatments.

The assessment of cell viability through the exclusion of small molecular probes remains controversial. Therefore, as an alternative to trypan-blue, I tested PI as an indicator of cell permeabilization. PI readily entered and stained cells treated with 30 µg/mL of digitonin (**Figure 2.4**) similarly trypan blue was retained in 3T3 cells treated at the same concentration (**Figure 2.2**). Although permeabilized cells stained positive for PI, the addition of a counterstain (Hoechst 33342), and the need to use a fluorescent microscope for visualization complicated assessment of cell permeability. Therefore, trypan blue distinguished itself for rapid and consistent staining suitable for both determining digitonin treatment concentrations and observing cell permeability prior to and during extract treatments.<sup>36</sup>

In this Chapter, I considered the effects of digitonin on both cell permeabilization and cell survival following treatment. Through adjustments to treatment conditions to emphasize cell survival, I was able to identify concentrations of digitonin which effectively permeabilized mouse 3T3 cells without adversely affecting cell survival in culture. While trypan blue can be used to examine small molecule penetration, it cannot reveal the penetration of large proteins such as those contained in cell extracts. Therefore in **Chapter 3**, I tested whether the established permeabilization assay supports large molecular penetration and retention.

## References:

1. Collas, P. Reprogramming Somatic Cells in Extract of Undifferentiated Carcinoma Cells. *www.collaslab.com* 1–11
2. Agarwal, S. Cellular reprogramming. *Methods in enzymology* **420**, 265–83 (2006).
3. Walev, I. *et al.* Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3185–90 (2001).
4. Grant, N. J., Aunis, D. & Bader, M. F. Morphology and secretory activity of digitonin- and alpha-toxin-permeabilized chromaffin cells. *Neuroscience* **23**, 1143–55 (1987).
5. Schulz, I. Permeabilizing cells: some methods and applications for the study of intracellular processes. *Methods in enzymology* **192**, 280–300 (1990).
6. Miyamoto, K. *et al.* Reversible membrane permeabilization of mammalian cells treated with digitonin and its use for inducing nuclear reprogramming by *Xenopus* egg extracts. *Cloning and stem cells* **10**, 535–42 (2008).
7. Melan, M. a. Overview of cell fixation and permeabilization. *Methods in molecular biology (Clifton, N.J.)* **34**, 55–66 (1994).
8. Fischer, A. H., Jacobson, K. A., Rose, J. & Zeller, R. Fixation and permeabilization of cells and tissues. *CSH protocols* **2008**, pdb.top36 (2008).
9. Moore, M. S. & Schwoebel, E. D. Nuclear import in digitonin-permeabilized cells. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]* **Chapter 11**, Unit 11.7 (2001).
10. Fiskum, G. Intracellular levels and distribution of Ca<sup>2+</sup> in digitonin-permeabilized cells. *Cell calcium* **6**, 25–37 (1985).
11. Wagner, S., Chiosea, S., Ivshina, M. & Nickerson, J. a. In vitro FRAP reveals the ATP-dependent nuclear mobilization of the exon junction complex protein SRm160. *The Journal of cell biology* **164**, 843–50 (2004).
12. Ramsby, M. & Makowski, G. Differential Detergent Fractionation of Eukaryotic Cells. *Cold Spring Harbor Protocols* **2011**, prot5592–prot5592 (2011).

13. Ramsby, M. L., Makowski, G. S. & Khairallah, E. A. Differential detergent fractionation of isolated hepatocytes: biochemical, immunochemical and two-dimensional gel electrophoresis characterization of cytoskeletal and noncytoskeletal compartments. *Electrophoresis* **15**, 265–77 (1994).
14. Lobner, D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis. *Journal of neuroscience methods* **96**, 147–52 (2000).
15. Giordano, a *et al.* tBid induces alterations of mitochondrial fatty acid oxidation flux by malonyl-CoA-independent inhibition of carnitine palmitoyltransferase-1. *Cell death and differentiation* **12**, 603–13 (2005).
16. Riss, T. L. & Moravec, R. A. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay and drug development technologies* **2**, 51–62 (2004).
17. Krampe, B. & Al-Rubeai, M. Cell death in mammalian cell culture: molecular mechanisms and cell line engineering strategies. *Cytotechnology* **62**, 175–88 (2010).
18. Watanabe, H. Removal of the outer membrane from brain mitochondria. *Journal of biochemistry* **69**, 275–81 (1971).
19. Benga, G., Hodarnau, A. & Tilinca, R. Fractionation of human liver mitochondria: enzymic and morphological characterization of the inner and outer membranes as compared to rat liver mitochondria. *Journal of Cell ...* **429**, 417–429 (1979).
20. Ishisaka, R. *et al.* Activation of caspase-3-like protease by digitonin-treated lysosomes. *FEBS letters* **435**, 233–6 (1998).
21. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Current biology : CB* **14**, 1475–80 (2004).
22. Taranger, C. K. *et al.* Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells. **16**, 5719–5735 (2005).
23. Bru, T. *et al.* Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. *Experimental cell research* **314**, 2634–42 (2008).

24. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by *Xenopus laevis* egg extracts. *Molecular reproduction and development* **74**, 1268–77 (2007).
25. Kehlenbach, R. H. & Paschal, B. M. Analysis of Nuclear Protein Import and Export in Digitonin-Permeabilized Cells. (2006).
26. Gewies, A. Introduction to Apoptosis. *ApoReview* 1–26 (2003).
27. Altman, S. a, Randers, L. & Rao, G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnology progress* **9**, 671–4 (1999).
28. Sittampalam, G., Gal-Edd, N., Arkin, M. & Auld, D. Cell Viability Assays. (2013). at <<http://www.ncbi.nlm.nih.gov/books/NBK144065/>>
29. Baker, D. J. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232–6 (2011).
30. Belloc, F. *et al.* A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry* **17**, 59–65 (1994).
31. Nishikawa, M., Nojima, S., Akiyama, T., Sankawa, U. & Inoue, K. Interaction of digitonin and its analogs with membrane cholesterol. *Journal of biochemistry* **96**, 1231–9 (1984).
32. Eilenberg, H., Klinger, E., Przedeki, F. & Shechter, I. Inactivation and activation of various membranal enzymes of the cholesterol biosynthetic pathway by digitonin. *Journal of lipid research* **30**, 1127–35 (1989).
33. Adam, S. a, Marr, R. S. & Gerace, L. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *The Journal of cell biology* **111**, 807–16 (1990).
34. Schulz, I. Permeabilizing Cells: Some Methods and Applications for the Study of Intracellular Processes. **192**, 280–300 (1990).
35. Allegrucci, C. *et al.* Epigenetic reprogramming of breast cancer cells with oocyte extracts. *Molecular cancer* **10**, 7 (2011).

36. Strober, W. Trypan blue exclusion test of cell viability. *Current protocols in immunology / edited by John E. Coligan ... [et al.] Appendix 3*, Appendix 3B (2001).

### **Chapter 3: Large Molecular Uptake and Retention**

#### **Abstract:**

The most crucial step during extract-based reprogramming is the uptake and retention of proteins responsible for successful reprogramming of somatic cells. Therefore proper treatment conditions and assessment of large molecule uptake and retention are mandated prior to extract treatments. To determine permeabilization conditions established in **Chapter 1** support uptake and retention of large molecules such as proteins I investigated the uptake and retention of molecular probes [FITC conjugated to 10 kDa dextran (FITC-10kDa-dextran), FITC conjugated to 70 kDa dextran (FITC-70kDa-dextran), and Texas Red conjugated to 70kDa dextran (Texas Red-70kDa-dextran)] into two somatic cells lines: BJ neonatal fibroblasts (a pilot treatment line) and mouse 3T3 cells (my primary treatment line). The proper observation of large molecular weight dyes required experimental refinements for both treatment and detection. In BJ neonatal fibroblasts FITC-10kDa-dextran is clearly visible throughout digitonin treated cells, while FITC-70kDa-dextran required high dye concentrations and fixation prior to visualization. Texas Red-70kDa-dextran dye was chosen for increased brightness over FITC probes, and was visibly retained in digitonin treated mouse 3T3 cells even at low probe concentrations. Texas Red-70kDa-dextran was also retained in mouse 3T3 cells over time. The results show that the established permeabilization conditions facilitate the uptake and retention of large molecules, suggesting that the established permeabilization assay can promote the uptake and retention of protein. Together with the described

permeabilization of somatic cells (**Chapter 2**), the uptake and retention of large molecules establishes a foundation for the development of routine extract-based cell reprogramming system (**Chapter 4**).

### **Introduction:**

The extract-based reprogramming strategy utilizes reversible cell permeabilization for the inclusion and retention of proteins and protein complexes during extract treatment.<sup>1,2,3</sup> While vital stains such as trypan blue<sup>4</sup> and propidium iodide<sup>3</sup> can be used to evaluate membrane integrity during and after permeabilization (**Chapter 2**), their inclusion within the cell only indicates that the treatment renders the cytoplasmic membrane permeable to small molecules such as peptides, oligonucleotides, and ions.<sup>5,6</sup> To evaluate the uptake of exogenous proteins, suitable probes are required which monitor both penetration and retention of small and large molecular weight molecules within treated cells.<sup>1</sup> Dextrans of different molecular weight are suitable probes for monitoring cell permeabilization by digitonin.

Dextrans are complex sugars composed of glucose monomers which comprise multiple branches of the overall polysaccharide.<sup>7</sup> Typically, dextrans are purified to emphasize an average molecular weight between 1-2,000KDa.<sup>8</sup> As molecular tracers, dextrans are well characterized and prominently used for their neutrality, water solubility, and low toxicity in living systems.<sup>8,9</sup> Arrays of dextran conjugates are available for various purposes, and most permeabilization related studies have utilized fluorescently-labeled dextrans.<sup>1,2,10,11</sup> In addition dextrans have been used to study permeability-related



mechanisms in cellular processes such as endo-cytosis,<sup>12,13</sup> and vascular permeability.<sup>14</sup> While some approaches emphasize the penetration of probes across a barrier, other techniques require their retention within a cell.<sup>12,15</sup>

Various fluorescently labeled dextrans are commercially available or can be generated *in vitro*.<sup>8,16</sup> In all cases fluorescent moieties are covalently attached to dextran molecules. The conjugation efficiency dictates how efficiently these conjugates can be detected in the context of cultured cells.<sup>8,17</sup> When prepared properly, fluorescently labeled dextrans are highly pure and free of unbound fluorescent moieties. Because of their small size, the contamination of labeled dextran preparations with unbound fluorescent moieties, which can penetrate non-permeabilized cells, can result in false positives in cell permeabilization assays.<sup>17,18</sup> Labeling efficiency is also directly affected by dextran size (and complexity).<sup>19,20</sup> Low molecular weight dextrans retain more fluorescent moieties per glucose subunit than larger dextrans.<sup>19,20</sup> Since type of fluorescent moiety, efficiency of dye conjugation (defined as number of fluorescent moieties incorporated per dextran molecule), and stability all affect probe brightness and subsequent visualization, selection of a molecular probe requires careful consideration.<sup>9</sup>

A wide variety of probes have been used for extract-based cell reprogramming studies including: 10KDa, 40KDa, 70KDa dextrans conjugated to either FITC, Oregon Green, or Texas Red.<sup>2,11,21,22,3</sup> Cell permeabilization through either SLO or digitonin (at low concentrations) creates pores in the plasma membrane supporting the entry of molecules as large as 200kDa,<sup>6,23</sup> but one study suggest effectors range between 30-

100kDa.<sup>10</sup> Therefore, larger probes should be tested for extract-based reprogramming studies.

Digitonin mediated cell permeabilization, allows small and large molecular weight proteins (and protein complexes) access to the cytosol of somatic cells.<sup>3</sup> While various reprogramming activities have been reported following brief extract treatments (30 min),<sup>24,25</sup> some reports suggest a longer incubation times are required for reprogramming events (1-2 hrs).<sup>3,2,25</sup> However, it remains unknown if the activities of various protein extracts directly reprogram cells during the short treatment process (0.5-2 hrs), or after treatment.<sup>1,4</sup> Proteins are retained in somatic cells following treatment, and some activities have been described in cultured cells long after treatment.<sup>3,24,26</sup> Therefore, permeability assays should not only monitor uptake of large molecules, but also their retention over time after extract treatment. Here, I describe my experimental approach to assess uptake and retention of florescent probes into permeabilized somatic cells (**Figure 3.1A, 1B**). Here I demonstrate the uptake and retention of FITC Dextran conjugates (10kDa, 70kDa) in treated BJ neonatal fibroblasts, and Texas Red-70kDa-dextran in treated mouse 3T3 fibroblasts. The experiments reveal that the established permeabilization assay (**Chapter 2**) supports the uptake and retention of small and large molecules and provide the basis for the development of a protein extract based cell reprogramming system (**Chapter 4**).

## Materials and Methods

### Cell Preparation

Human BJ neonatal fibroblasts or mouse 3T3 cells were cultured under standard conditions (see **Chapter 1: Somatic Cell Culture**). Cells used for experiments were grown to 50-80% confluence, and then harvested by disassociation in 1mL of trypsin (Invitrogen) until detachment was observed (typically 3 minutes). To suspend cells and inactivate trypsin, 9 mL of **complete DMEM** was added to cell culture dishes, and then cell suspensions were transferred to 15 mL collection tubes. For collection, cells were first pelleted at 300 x g for 5 minutes at 4°C and the media-containing supernatant was discarded. In subsequent steps, cells as pellets or in suspension were maintained on ice or at 4°C. Collected cells were first washed twice in PBS (Phosphate Buffered Saline without calcium or magnesium). Each wash consisted of suspension then sedimentation at 300 x g for 5 minutes at 4°C. Pelleted cells were resuspended in freshly prepared and filtered **acetate transport buffer** containing 20 mM HEPES, 110 mM potassium acetate, 5mM sodium acetate, 2mM magnesium acetate, and 1mM EGTA, 0.1mM DTT, 0.1mM PMSF, and protease inhibitors (Roche).<sup>3</sup> In preparation for treatment cells were counted under on a hemocytometer (for individual aliquots). Following the initial wash in **acetate transport buffer**, cells were resuspended in acetate transport buffer at a concentration of 100,000 cells per milliliter, and dispersed among 1.5mL reaction tubes. Aliquoted cells were then centrifuged for 10 minutes at 300 x g, then remaining acetate buffer was removed ensuring cell pellets were left undisturbed. Individual aliquots of pelleted cells

were then processed for experimentation; typically cells were treated or resuspended at concentrations of 1,000 cells per  $\mu\text{L}$  (see **Florescent Probe Incorporation**).

### **Florescent Probe Incorporation**

Digitonin stock solutions (**Chapter 2: Digitonin Preparation**), were thawed by boiling  $95^{\circ}\text{C}$  for 5 minutes just prior to use. Then, working solutions were prepared by diluting digitonin in ice-cold acetate transport buffer. Aliquots of cells were then gently resuspended, in digitonin solutions at a treatment concentration of 1,000 cells per  $\mu\text{L}$  (typically  $1 \times 10^5$  total cells) using wide-bore or cut-off  $200\mu\text{L}$  pipette tips.<sup>27</sup> For digitonin treatments, resuspended cells were maintained on ice for 1-2 minutes.

Following treatment,  $900\mu\text{L}$  of **acetate transport buffer** was added to dilute digitonin (decreasing its activity). Then cells were centrifuged at  $300 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  in a barrel rotor (Sorvall 6300-168). After centrifugation, digitonin-containing **acetate transport buffer** solutions were gently removed (with special care not to disturb cell pellets) prior to florescent probe treatments. In the case of FITC probes, buffer and digitonin treated BJ neonatal fibroblasts were resuspended in  $100\mu\text{L}$  of  $1\text{mg/mL}$  of FITC-10kDa-dextran or  $2\text{mg/mL}$  of FITC-70kDa-dextran graciously provided by the laboratory of Guillermo Aguilar. Control and treated mouse 3T3 cells were resuspended  $100\mu\text{L}$  of  $50\mu\text{g/mL}$  of Texas Red-70kDa-dextran. During treatment with florescent probes cells were maintained in a  $37^{\circ}\text{C}$  water bath. Every 10 minutes, cells were gently resuspended by gentle tapping and inversion of  $1.5\text{mL}$  reaction tubes. Following treatment with florescent probes, cells were collected by centrifugation, and the

supernatant containing florescent conjugates was removed. Cells were resuspended in **complete DMEM** supplemented with 2mM of CaCl<sub>2</sub>. Resuspended cells were then pipetted onto gelatin-coated coverslip, which were placed within the wells of 12-well plates. After 2-4 hours, **complete DMEM** supplemented with CaCl<sub>2</sub> was replaced with **complete DMEM**. 24 hours after florescent probe treatment, cells were examined using a florescent microscope (see **Observation of Florescent Conjugates**).

### **Observation of Florescent Conjugates (24hrs)**

First, media was removed from wells containing treated cells overlaid onto coverslips (see Florescent Probe Incorporation above), then cells were overlaid with 1 mL of **complete DMEM** containing 1 µg/mL of Hoechst 33345. Cells were allowed to recover for 1 hour in complete DMEM. To remove unbound florescent probe, cells were first washed with a non-specific blocker containing PBS supplemented with 20% FBS and 1mg/mL of filtered BSA. Cells were then washed three times through the gentle application of PBS for 5 minutes at room temperature. Finally, remaining PBS was removed and cells were cultured in **microscope media**: DMEM (Sigma) without sodium bicarbonate or phenol red, supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamate (Mediatech), 1mM sodium pyruvate (Sigma), and 0.1 U/ml penicillin, 0.1 µg/ml streptomycin and 20mM HEPES pH 7.4. Just prior to examination, 25 µL drops of **microscope media** was placed on glass slides, then the coverslips (with attached cells) were removed from wells using a needle and tweezers, flipped, and placed upside-down onto the drop of media on the glass slide. Finally, cells were observed and photographed using an Eclipse Ti microscope (Nikon, UCR stem cell core).

### **Cell Fixation**

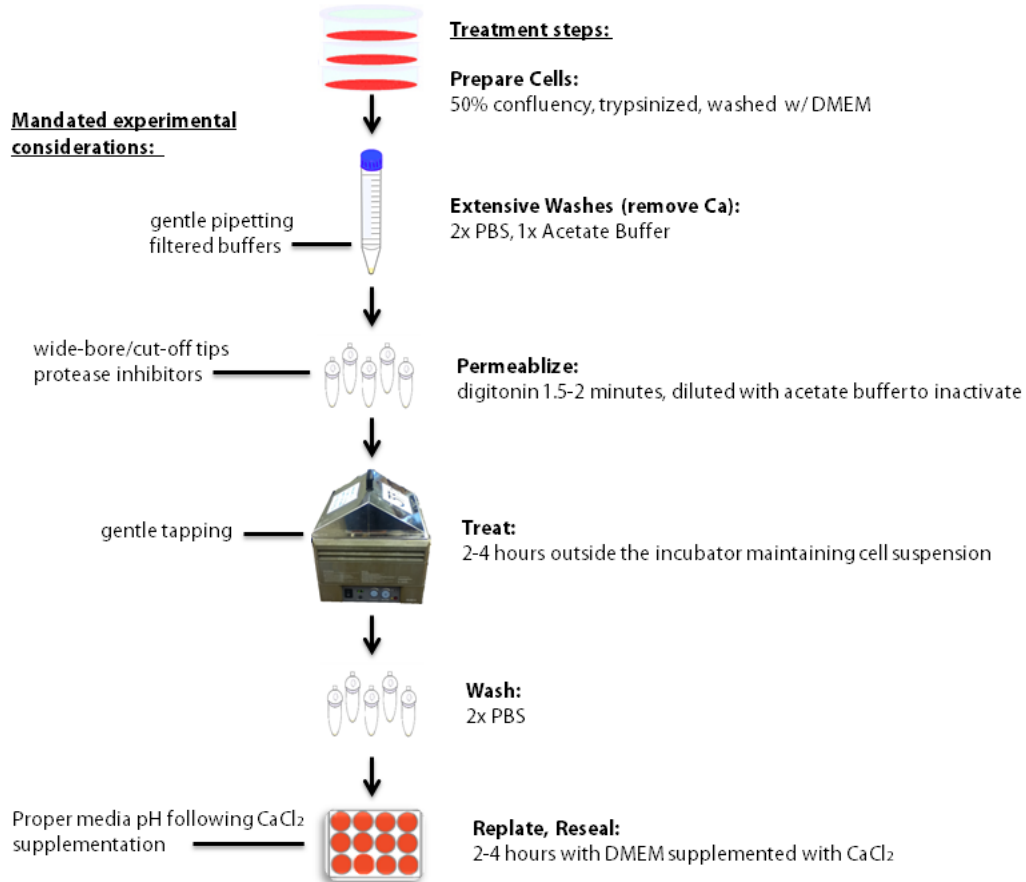
Cells were prepared as described in **Observation of Florescent Conjugates (24hrs)**, with some modifications. Following Hoechst 33345 staining, the application of blocking buffer, and subsequent PBS washes, cells were fixed with 3.7% formaldehyde for 15 minutes, and then washed four additional times with PBS. Then adherent cells and coverslips were overlaid with **acetate transport buffer**. Just prior to examination, 25  $\mu\text{L}$  drops of **acetate transport buffer** was placed on glass slides, then coverslips (with attached cells), were removed from wells using a needle and placed up-side down onto drops of microscope media on glass slides. Finally, cells were observed and photographed using an Ellipse Ti florescent microscope (Nikon).

### **Retention of Probes**

Three aliquots of mouse 3T3 cells were prepared as described in Cell Preparation and treated with florescent probes as described in Florescent Probe Incorporation with some modifications. Cells were permeabilized and treated at a concentration of 1,000 cells per microliter, however instead of using individual aliquots containing  $1 \times 10^5$  cells, they contained  $3 \times 10^5$  cells. Then, buffer or digitonin treated cells were incubated in 300  $\mu\text{L}$  of acetate transport buffer containing 50  $\mu\text{g}/\text{mL}$  Texas Red-70kDa-dextran (a final concentration of 1,000 cells per microliter). Then, cells were resuspended in complete DMEM supplemented with 2mM of  $\text{CaCl}_2$ , and individual aliquots were divided and seeded at  $1 \times 10^5$  total cells for each time point into individual wells containing gelatin coated slides.

## Results/Figures:

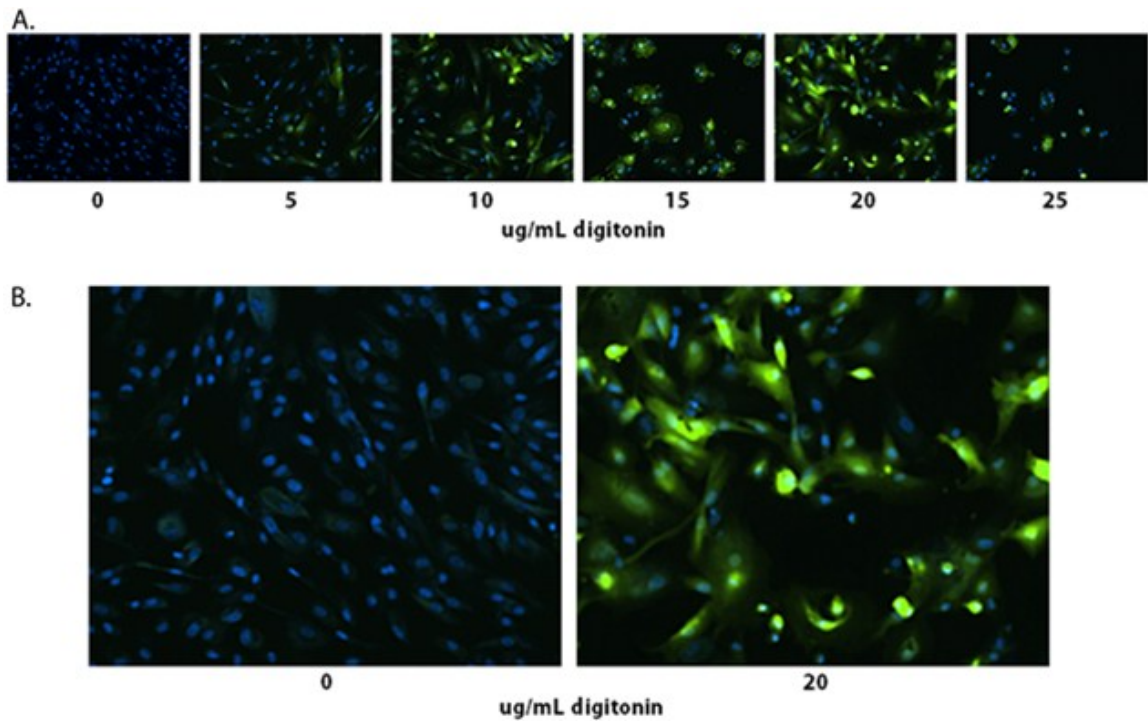
### A. Experimental Approach



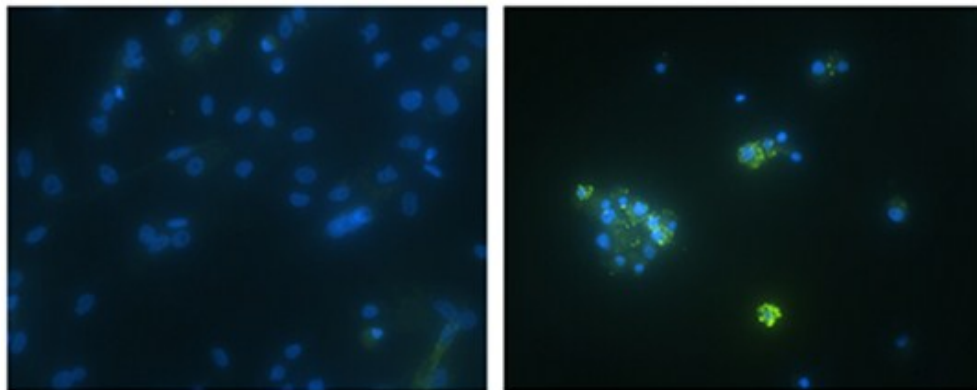
### B. Observation



**Figure 3.1:** Overall procedure for cell staining and visualization of fluorescent conjugates in treated cells. **A.** Schematic representation of the overall treatment process, with emphasis on permeability assessment (**Chapter 2**) and large molecule retention steps. **B.** Steps required for the observation of fluorescent probes (FITC, Texas Red dextrans) used in this study.

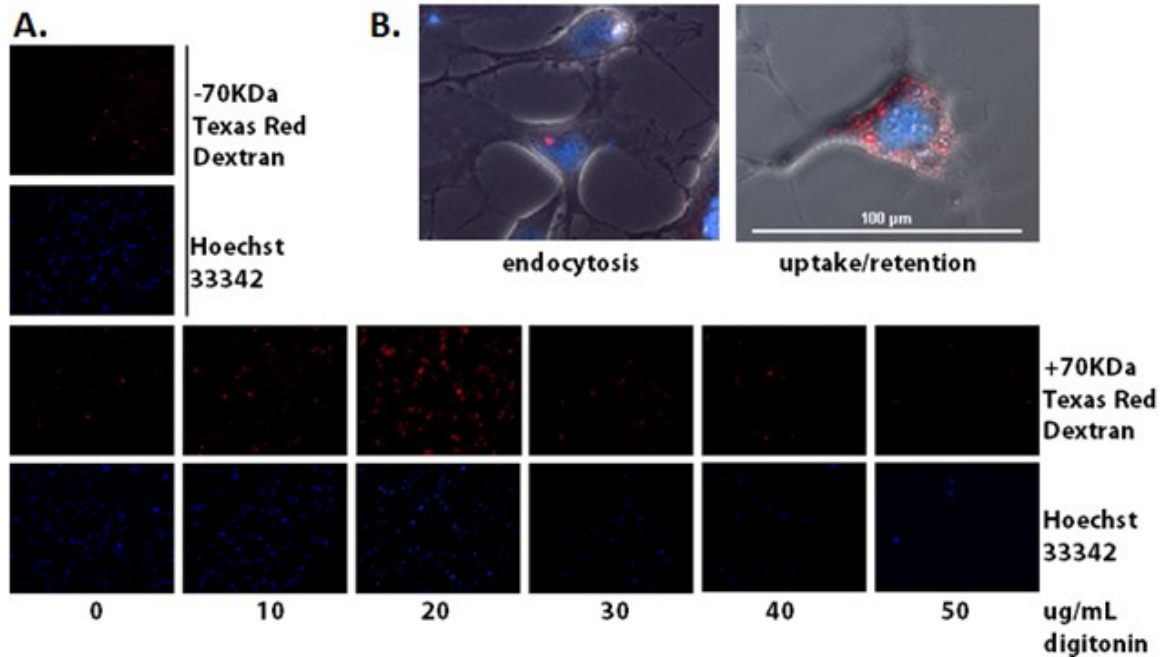


**Figure 3.2** FITC-10kDa-dextran retention in BJ neonatal fibroblasts. **A.** Prepared BJ neonatal fibroblast cells were treated with increasing concentrations of digitonin, then incubated with FITC-10kDa-dextran, and resealed with  $\text{CaCl}_2$  supplemented media. 24hrs later, live cells were visualized by using florescent microscopy

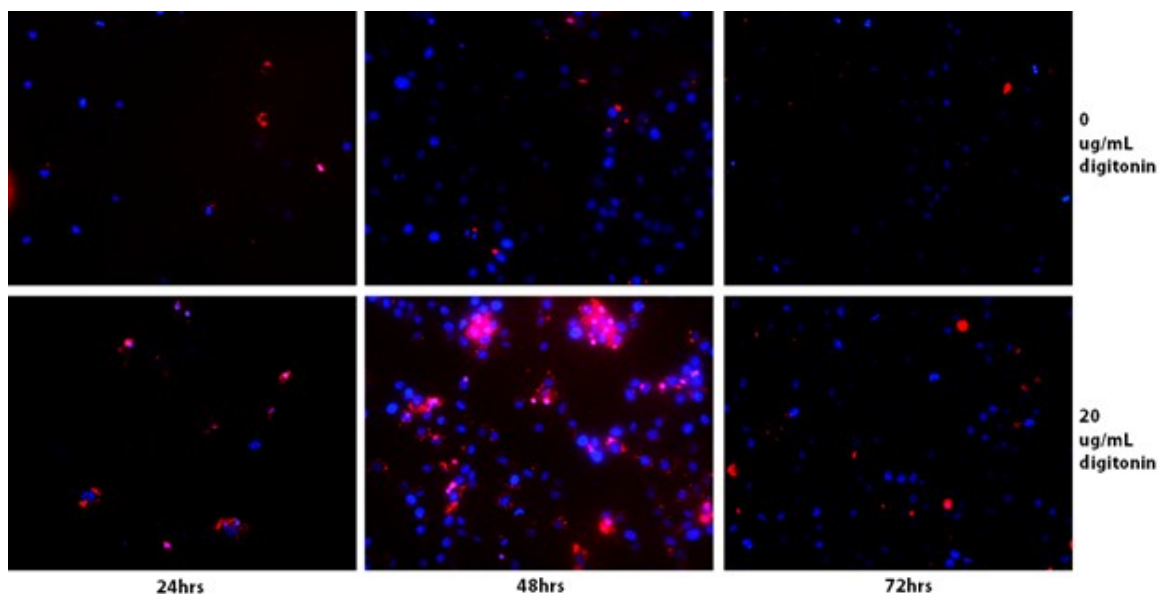


**Figure 3.3** FITC-70kDa-dextran retention in BJ neonatal fibroblasts. Cell nuclear staining with Hoechst 33342 and observation of FITC-70kDa-dextran in fixed BJ neonatal fibroblasts untreated (left) and digitonin treated (right) BJ neonatal fibroblasts.





**Figure 3.4** Texas Red-70kDa-dextran retention by mouse 3T3 fibroblasts. Both treated and untreated cells were labeled with Hoechst 33342 to denote location. **A.** Prepared mouse 3T3 cells were treated with increasing concentrations of digitonin, then incubated with Texas Red-70kDa-dextran, and resealed with  $\text{CaCl}_2$  supplemented media. 24hrs later, live cells were visualized using florescent microscopy (10x). **B.** Small amounts of dye retained in untreated cells (left), larger amounts of dye retained at 20 $\mu\text{g/mL}$  of digitonin (right).



**Figure 3.5** Texas Red-70kDa-dextran retention by 3T3 cells overtime. Two batches of prepared cells were treated with either 0  $\mu\text{g}/\text{mL}$  or 20  $\mu\text{g}/\text{mL}$  of digitonin, and then incubated with 50  $\mu\text{g}/\text{mL}$  of Texas Red-70kDa-dextran for 2 hours. Three samples from each batch of cells were plated in 12 well plates over glass coverslips: untreated (top) and treated (bottom) then visualized at 24, 48, and 72 hours. Both treated and untreated cells were labeled with Hoechst 33342 just prior to visualization.

### **Retention of FITC-10kDa-dextran in BJ neonatal fibroblasts**

To optimize the retention of florescent conjugates in permeabilized cells, human BJ neonatal fibroblasts were treated with various concentrations of digitonin (0-25  $\mu\text{g}/\mu\text{L}$ ), incubated with FITC-10kDa-dextran (1  $\text{mg}/\text{mL}$ ), and then observed through florescent microscopy. FITC-10kDa-dextran was chosen because generally low molecular weight dextran probes are generally brighter than high molecular weight dextran probes (see **Introduction**). Cells were observed 24 hrs after incubation in florescent probes, a time point chosen to demonstrate long-term retention. As expected, human BJ neonatal

fibroblasts treated with **acetate transport buffer** (0  $\mu\text{g}/\text{mL}$  of digitonin), did not retain FITC-10kDa-dextran (**Figure 3.2A**). In contrast, intracellular FITC-10kDa-dextran was observed in cells treated with digitonin (5-25  $\mu\text{g}/\text{mL}$ ) (**Figure 3.2A**). When permeabilized with 5  $\mu\text{g}/\text{mL}$  digitonin, few cells contained FITC-10kDa-dextran, however once increased to 10 $\mu\text{g}/\text{mL}$  digitonin retention increased dramatically (**Figure 3.2A**). The retention of FITC-10kDa-dextran was most obvious in cells permeabilized with 20  $\mu\text{g}/\text{mL}$  of digitonin (**Figure 3.2A**). While some cells treated with 25  $\mu\text{g}/\text{mL}$  retained FITC-10kDa-dextran, cells treated with this concentration displayed unusual cell morphology. Typically BJ neonatal fibroblasts are elongated with defined attachment points; however these cells appeared rounded or “egg shaped”.

### **Retention of FITC-70kDa-dextran by BJ neonatal fibroblasts**

To assess whether treatment conditions identified using FITC-10kDa-dextran also facilitate the uptake and retention of larger molecules, human BJ neonatal fibroblasts were first permeabilized with digitonin and then incubated in FITC-70kDa-dextran. As with FITC-10kDa-dextran (1mg/mL), a high probe concentration was required to observe intracellular retention of FITC-70kDa-dextran (2mg/mL). Because retention of FITC-70kDa-dextran is difficult to observe in BJ neonatal fibroblasts, I tested cell fixation (**see fixation treatment**) to improve probe visualization while also reducing background. Following fixation, the retention of FITC-70kDa-dextran was observed in treated cells (**Figure 3.3 right**), with little background in the control cells (**Figure 3.3 left**). Of note, the detected signal within cells appeared grainy in fixed cells **Figure 3.3 Initial**

experiments revealed potential problem for using BJ neonatal fibroblasts as somatic cell recipient. Because BJ neonatal fibroblasts grow slowly and for a limited number of passages, cellular material was a limiting factor for experimentation. Therefore, in subsequent experiments I elected to use mouse 3T3 cells as a recipient for both florescent probes and treatment with extracts.

### **Retention of Texas Red-70kDa-dextran by 3T3 cells**

Because high concentrations of probe were necessary to visualize intracellular FITC-70kDa-dextran in permeabilized cells, I decided to use Texas Red-70kDa-dextran for subsequent experiments, because lower effective concentrations have been reported.<sup>27</sup> To optimize large molecule retention, 3T3 cells were first treated with various concentrations of digitonin (0-50  $\mu\text{g}/\text{mL}$ ) then incubated with Texas Red-70kDa-dextran (50  $\mu\text{g}/\text{mL}$ ). In untreated cells (without digitonin or fluorescent conjugate), no florescent signal was detected [aside from red spots (likely dust) which upon focusing are below the coverslip plane] **Figure 3.4A**. In cultures treated with **acetate transport buffer** (0  $\mu\text{g}/\text{mL}$  digitonin) and incubated for 2 hours in Texas Red-70kDa-dextran, very few cells retained florescent probe after 24 hours in culture. The few unpermeabilized cells containing intracellular signal, may have retained the fluorescent probe by an alternative mechanism such as endocytosis (**Figure 3.4B**). In 3T3 cells treated with 10, 20, or 30  $\mu\text{g}/\text{mL}$  of digitonin, Red-70kDa-dextran is visible within a number of surviving cells, but was most obvious in cells permeabilized with 20  $\mu\text{g}/\text{mL}$  of digitonin (**Figure 3.4A**). While digitonin treatment with 30  $\mu\text{g}/\text{mL}$  affected cell attachment (**Figure 3.4A**), surviving cells

retained Red-70kDa-dextran. In 3T3 cells treated with 40 or 50  $\mu\text{g}/\text{mL}$  of digitonin, only ~10% of cells survived after 24 h and the survivors contained weak fluorescent signal (**Figure 3.4A**).

### **Retention Texas Red-70kDa-dextran over time in 3T3 cells**

Since not only the uptake but also the retention of large molecules (within extract) plays an important role in extract-based reprogramming, I decided to assess fluorescent probe retention. Mouse 3T3 cells were permeabilized with 20  $\mu\text{g}/\text{mL}$  of digitonin, incubated with Texas Red-70kDa-dextran probe, and intracellular dye retention was monitored in resealed cells 24, 48, and 72 hours following treatment (**Figure 3.4**). Control cells did not retain Texas Red-70kDa-dextran after 24, 48, and 72 hours (**Figure 3.5 top**). In cells treated with 20  $\mu\text{g}/\text{mL}$  of digitonin, Texas Red-70kDa-dextran was clearly retained in the majority of cells after 24 hours (**Figure 3.5 bottom**). After 48hrs, permeabilized mouse 3T3 cells also retained Texas Red-70kDa-dextran. However while Texas Red-70kDa-dextran was observed in individual cells 24 hours after treatment, cells retaining Texas Red-70kDa-dextran were clustered together after 48 hrs, (**Figure 3.5**). Texas Red-70kDa-dextran was no longer detectable in cells after being cultured for 72hrs. Therefore, digitonin-treated cells retain Texas Red-70kDa-dextran for at least two days (48 hrs) post treatment.

## Discussion:

The detection of fluorescent probes within permeabilized (and subsequently resealed) cells is a multi-step process that requires optimization at several levels (**Figure 3.1A**). Large-molecule uptake and retention is influenced by three factors: separation of cells during incubation by gentle tapping, proper pH of the resealing media (basic media causes detachment), and concentration of fluorescent conjugates. Aside from probe retention, the observation of fluorescently labeled dextran probes within living cells requires special considerations such as: media to support cell viability and the application of a non-specific blocker (to remove excess probe) (**Figure 3.1B**).<sup>28</sup>

The inclusion and retention of protein within somatic cells is paramount for extract-based reprogramming.<sup>1,3,25,27</sup> While digitonin-mediated permeabilization can be measured using trypan blue (**Chapter 1,2**), trypan blue is drastically smaller (~980Da)<sup>29</sup> than the size capacity of digitonin-mediated pores (200 kDa),<sup>6</sup> Therefore, molecular probes larger than trypan blue are required to determine if digitonin-mediated cell permeabilization can facilitate the uptake and retention of larger molecules, such as protein from oocyte/egg or cellular sources.

As a first step, I used a neutral probe resembling small proteins and peptides, FITC-10kDa-dextran, to develop my experimental approach to treatment and subsequent observations. Smaller probes aid in visualization because higher dye conjugation, as a result of additional fluorescent moieties, improves signal intensity.<sup>19,20</sup> Using FITC-10kDa-dextran, I identified crucial steps required for uptake of molecular conjugates.

First, in order for cells to survive permeabilization, careful attention is required for cell handling, permeabilization mandates wide-bore tips to minimize shearing forces (**Figure 3.1A**).<sup>27</sup> Because cells also stick to one another during treatment, steps are required to maintain cells in suspension during fluorescent probe treatments (**Figure 3.1A**). If probes cannot sufficiently access digitonin-mediated pores, then the probe cannot penetrate permeabilized cells. To reduce unspecific fluorescent background signals, the gentle washing of treated cells and the use of block buffer removes excessive probe binding nonspecifically on the outside of cells or plated among cells (**Figure 3.1B**), thereby aiding in the observation of internalized probe signal.

Very little FITC-10kDa-dextran was observed in BJ neonatal fibroblasts treated with **acetate transport buffer** (0  $\mu\text{g}/\text{mL}$  of digitonin); confirming that even small molecular probes (and proteins/peptides) require permeabilization for sufficient uptake and retention in somatic cells. FITC-10kDa-dextran was retained in cells treated with 5-25  $\mu\text{g}/\text{mL}$  digitonin; however each digitonin concentration affected probe retention differently. While BJ neonatal fibroblasts treated with 5-15 $\mu\text{g}/\text{mL}$  of digitonin retained FITC-10kDa-dextran, 20  $\mu\text{g}/\text{mL}$  digitonin was the most effective concentration for probe retention following permeabilization. As with trypan blue ( $\sim 980\text{Da}$ )<sup>29</sup>, 10 kDa probes are sufficient to monitor uptake of small molecules, however 10kDa probes cannot recapitulate large molecule uptake required for extract-based cell reprogramming . Therefore using conditions derived from FITC-10kDa-dextran, I tested whether permeabilized BJ neonatal fibroblasts take-up and retain FITC-70kDa-dextran. Under the same conditions used for FITC-10kDa-dextran, BJ neonatal fibroblasts permeabilized

with 20  $\mu\text{g}/\text{mL}$  of digitonin retained FITC-70kDa-dextran (**Figure 3.3**). Cell fixation was necessary to observe FITC-70kDa-dextran retention, but also affected the signal distribution (**Figure 3.3**). Signal in cells treated with FITC-10kDa-dextran was evenly distributed, while in cells which were fixed following treatment with FITC-70kDa-dextran, the fluorescent signal appeared grainy. It appears possible that the lack of fixable cross-link groups on FITC-70kDa-dextran is responsible for the grainy distribution of the fluorescent dye in cells.<sup>30</sup> Therefore, fluorescent signal within fixed cells may originate from FITC-70kDa-dextran probe trapped within intracellular compartments. The intracellular retention of FITC-70kDa-dextran indicates digitonin-mediated permeabilization permits the uptake and retention of large molecules.

In my experiments, Texas Red-70kDa-dextran was the most effective probe to assess uptake and retention of molecular markers into digitonin treated 3T3 cells (**Figure 3.4**). Even at a low treatment concentration of 50  $\mu\text{g}/\text{mL}$ , Texas Red-70kDa-dextran is reliable and consistently observable in 3T3 cells permeabilized with digitonin. While some control cells also retained dye, the visual appearance of such cells resembles uptake of dye by endocytosis (**Figure 3.4B left**) as opposed to digitonin-dependent uptake and retention with large amounts of dye distributed throughout the cell (**Figure 3.4B right**). Although the digitonin concentration required for large molecule retention differs among studies both origin (company) and preparation can determine the effective treatment concentration. In my preparations, similar digitonin concentrations were effective for large molecule retention in both human BJ neonatal fibroblasts and mouse 3T3 cells. At 20  $\mu\text{g}/\text{mL}$  each cell line retained a significant amount of probe: FITC-10kDa-dextran and



FITC-70kDa-dextran in BJ neonatal fibroblasts (**Figure 3.2, 3.3**), and Texas Red-70kDa-dextran in mouse 3T3 cells (**Figure 4**). Digitonin concentrations greater than 30  $\mu\text{g}/\text{mL}$  adversely affected 3T3 cell attachment, these results corroborate with examinations of cell survival following digitonin treatment in **Chapter 2**.

Various extract treatment times have been described in published studies.<sup>1,2,25</sup> While role of extract treatment time remains unclear, it can be determined by a number of experimental variables. Extended incubation in extracts may adversely affect permeabilized cells causing cell death or inhibition of cell survival necessary for reprogramming. Incubation time is crucial for treatment optimization: maximizing uptake of protein, while limiting cell death. Following a 2 hr incubation period, permeabilized mouse 3T3 cells retained Texas Red-70kDa-dextran and survived following treatment.

Mouse 3T3 cells permeabilized with 20  $\mu\text{g}/\text{mL}$  of digitonin contained cells that retained Texas Red-70kDa-dextran and cells, which contained no intracellular probe. Fewer mouse 3T3 cells permeabilized with 30  $\mu\text{g}/\text{mL}$  reattached following treatment, however among surviving cells, most contained intracellular Red-70kDa-dextran (**Figure 3.4**). Therefore during extract treatments, I elected to permeabilize mouse 3T3 cells using two digitonin concentrations 20  $\mu\text{g}/\text{mL}$  and 30  $\mu\text{g}/\text{mL}$ . One emphasized overall cell survival following treatment (20  $\mu\text{g}/\text{mL}$ ), and the other while affecting cell attachment, emphasized survival of permeabilized cells (30  $\mu\text{g}/\text{mL}$ ).

Another aspect of treatment is large molecule retention by treated cells over time. Since effectors within extract are likely retained by cells following treatment, I elected to

assess retention of large molecules. After 24 hrs, recovered cells retained 10kDa and 70kDa FITC conjugated dextrans (in BJ neonatal fibroblasts) and Texas Red-70kDa-dextran (mouse 3T3 cells). Although longer retention times have been reported,<sup>10</sup> in the case of 3T3 cells, Texas Red-70kDa-dextran is retained after 48hrs and appears distributed in dividing cells (**Figure 3.5**). Therefore, effectors within extracts can be retained within cells for at least 24-48hrs.

Multiple publications document the use of FITC and Texas Red labeled dextrans for permeabilization studies, including cellular reprogramming.<sup>11,14,23</sup> The treatment of somatic, recipient cells with florescent probes revealed necessary steps required to maximize protein uptake within somatic cells (**Figure 3.1A**). Experiments in **Chapter 2**, established a range of digitonin treatment concentrations, which were used for the testing the uptake and retention of large molecular weight probes described in this chapter. In mouse 3T3 cells, permeabilization assays using 20 µg/mL and 30 µg/mL digitonin mediated uptake of Texas Red-70kDa-dextran, indicating that the established permeabilization assay can support the uptake of large molecules such as proteins into cells. Moreover, cells treated with 20 µg/mL digitonin retained the fluorescent marker for 48hr and survived the permeabilization assay, revealing that the established permeabilization assays mediates retention of large molecules without compromising cell viability. The results establish that the established permeabilization assays can be used for the establishment of **extract-based cell reprogramming assays (Chapter 4)**.

## References:

1. Agarwal, S. Cellular reprogramming. *Methods in enzymology* **420**, 265–83 (2006).
2. Taranger, C. K. *et al.* Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells. **16**, 5719–5735 (2005).
3. Miyamoto, K. *et al.* Reversible membrane permeabilization of mammalian cells treated with digitonin and its use for inducing nuclear reprogramming by Xenopus egg extracts. *Cloning and stem cells* **10**, 535–42 (2008).
4. Bru, T. *et al.* Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. *Experimental cell research* **314**, 2634–42 (2008).
5. Zaharoff, D. a, Henshaw, J. W., Mossop, B. & Yuan, F. Mechanistic analysis of electroporation-induced cellular uptake of macromolecules. *Experimental biology and medicine (Maywood, N.J.)* **233**, 94–105 (2008).
6. Schulz, I. Permeabilizing cells: some methods and applications for the study of intracellular processes. *Methods in enzymology* **192**, 280–300 (1990).
7. Bovey, F. A. Enzymatic polymerization. I. Molecular weight and branching during the formation of dextran. *Journal of Polymer Science* **35**, 167–182 (1959).
8. Dextran Conjugates. *Invitrogen Product Information* (2006).
9. Mobbs, P., Becker, D., Williamson, R., Bate, M. & Warner, A. Techniques for dye injection and cell labelling. 361–387
10. Cho, H.-J. *et al.* Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* **116**, 386–95 (2010).
11. Allegrucci, C. *et al.* Epigenetic reprogramming of breast cancer cells with oocyte extracts. *Molecular cancer* **10**, 7 (2011).
12. Kawamura, N. *et al.* Delivery of endosomes to lysosomes via microautophagy in the visceral endoderm of mouse embryos. *Nature communications* **3**, 1071 (2012).

13. Incardona, J. P. *et al.* Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 12044–9 (2000).
14. Pink, D. B. S., Schulte, W., Parseghian, M. H., Zijlstra, A. & Lewis, J. D. Real-time visualization and quantitation of vascular permeability in vivo: implications for drug delivery. *PloS one* **7**, e33760 (2012).
15. Mosnier, L. O., Yang, X. V & Griffin, J. H. Activated protein C mutant with minimal anticoagulant activity, normal cytoprotective activity, and preservation of thrombin activable fibrinolysis inhibitor-dependent cytoprotective functions. *The Journal of biological chemistry* **282**, 33022–33 (2007).
16. Prigent-Richard, S. *et al.* Fluorescent and radiolabeling of polysaccharides: binding and internalization experiments on vascular cells. *Journal of biomedical materials research* **40**, 275–81 (1998).
17. Preston, R. a, Murphy, R. F. & Jones, E. W. Apparent endocytosis of fluorescein isothiocyanate-conjugated dextran by *Saccharomyces cerevisiae* reflects uptake of low molecular weight impurities, not dextran. *The Journal of cell biology* **105**, 1981–7 (1987).
18. Cole, L., Coleman, J., Evans, D. & Hawes, C. Internalisation of fluorescein isothiocyanate and fluorescein isothiocyanate- dextran by suspension-cultured plant cells. 721–730 (1989).
19. Molecular Probes dextran conjugates—Table 14.4. *The Molecular Probes® Handbook* at <<http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/tables/Molecular-Probes-selection-of-dextran-conjugates.html>>
20. Kodama, T., Doukas, A. G. & Hamblin, M. R. Shock wave-mediated molecular delivery into cells. *Biochimica et biophysica acta* **1542**, 186–94 (2002).
21. Xu, Y.-N. *et al.* ES cell extract-induced expression of pluripotent factors in somatic cells. *Anatomical record (Hoboken, N.J. : 2007)* **292**, 1229–34 (2009).
22. Han, J., Sachdev, P. S. & Sidhu, K. S. A combined epigenetic and non-genetic approach for reprogramming human somatic cells. *PloS one* **5**, e12297 (2010).
23. Walev, I. *et al.* Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3185–90 (2001).

24. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Current biology : CB* **14**, 1475–80 (2004).
25. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by *Xenopus laevis* egg extracts. *Molecular reproduction and development* **74**, 1268–77 (2007).
26. Singhal, N. *et al.* Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. *Cell* **141**, 943–55 (2010).
27. Collas, P. Reprogramming Somatic Cells in Extract of Undifferentiated Carcinoma Cells. *www.collaslab.com* 1–11
28. Handbook, I. *et al.* Chapter 19 Confocal Microscopy of Live Cells.
29. Thibodeau, a, Yao, X. & Forte, J. G. Acid secretion in alpha-toxin-permeabilized gastric glands. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **72**, 26–35 (1994).
30. Dorfman, J. & Macara, I. G. STRADalpha regulates LKB1 localization by blocking access to importin-alpha, and by association with Crm1 and exportin-7. *Molecular biology of the cell* **19**, 1614–26 (2008).

## **Chapter 4: Treatment of Somatic Cells with Salmon Roe Extracts**

### **Abstract**

Extract-based cell reprogramming is an intricate, multistep process. The experiments described in **Chapters 2 and 3** established experimental conditions for cell permeabilization and large molecule uptake and retention. These experiments establish the foundation for my efforts to study extract based cell reprogramming. Here, I describe efforts to reprogram somatic, mouse 3T3 cells with a protein extract obtained from salmon roe. Mouse 3T3 cells were treated with salmon roe extract using various conditions for extract preparation and treatment. Treated 3T3 cells exhibit morphological and molecular features associated with pluripotent cells, indicating that the salmon roe extract can at least partially reprogram somatic mouse cells toward a pluripotent state. These experiments provide evidence that salmon roe extract is a suitable pluripotent extract source for extract based cell reprogramming experiments.

### **Introduction:**

The advent of iPSCs has reinvigorated the field of somatic cell reprogramming. Through the forced expression of four factors, i.e. *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, somatic cells can be converted toward a state of induced pluripotency.<sup>1,2</sup> However, while iPSCs cells are remarkably similar to ESCs,<sup>1,2,3</sup> crucial differences are emerging, such as aberrant promoter methylation,<sup>4</sup> differentiation toward a preferred lineage,<sup>5</sup> and tumorigenesis.<sup>6</sup> Some iPSCs have also been shown to retain gene expression patterns associated with their somatic cell origin.<sup>6,7</sup> These problems may contribute to the

immunorejection of differentiated iPSCs by their corresponding donor.<sup>5,6,7,8</sup> Therefore, further study is needed to reevaluate iPSC-based reprogramming and factors selected for the iPSC approach. One route is the exploration of reprogramming protein and activities originating sources such as oocytes/eggs and even cultured cells.<sup>4,5,6,7,8</sup>

The genes selected for iPSC reprogramming (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) were chosen for their prominent role in ESCs, but it remains unclear whether iPSC factors play any role in other forms of reprogramming such as SCNT.<sup>1,9</sup> SCNT can utilize oocyte and eggs to convert somatic cell nuclei toward a totipotent state in the form of an early embryo (zygote, morula).<sup>10,11</sup> Interestingly, Oct4, Sox2, Klf4, and c-Myc proteins are not detected in sufficient quantity to induce iPSC-based reprogramming within oocytes or zygotes.<sup>12</sup> Even ESCs may not express iPSC factors in sufficient quantity to induce somatic cell reprogramming through an iPSC-based mechanism.<sup>12</sup> Therefore it remains unclear what role, if any, iPSC factors play in SCNT, cell-fusion, or extract-based cell reprogramming.

The factors and mechanisms involved in somatic cell programming by SCNT and cell-cell fusion remain unknown. Theoretically, actors involved in somatic cell reprogramming can be identified by using various approaches such as an RNAi-based screen or biochemical approaches.<sup>13</sup> However, all of these experimental strategies require large amounts of cell materials. Because of technical and material limitations, mammalian SCNT and cell-cell fusion assays are not well suited for the dissection of reprogramming. Extract-based cell reprogramming fulfills all requirements for a

biochemical dissection of reprogramming, if a system becomes available which supports large-scale somatic cell reprogramming in a cost-effective fashion. The results in this chapter describe the development of an extract-based cell reprogramming system which fills this void and -in the future- can be used to identify factors involved in somatic cell reprogramming.

Somatic cell reprogramming often refers to the conversion of a somatic cell toward a stem-cell like state,<sup>14</sup> and can also refer to the induction of specific molecular events or cellular changes associated with the pluripotent state.<sup>15</sup> The induction of such activities can be achieved through the treatment of permeabilized mammalian somatic cells with protein extracts from a pluripotent source.<sup>16</sup> Interestingly, the reprogramming potential of oocytes is evolutionarily conserved and extract-based reprogramming has revealed a number of cellular changes and activities associated with cellular reprogramming (**Table 4.1**).<sup>15,17,18,19</sup> Interspecies extract donors are capable of inducing somatic cell reprogramming activities in permeabilized mammalian cell recipients.<sup>20,21</sup> Amphibian oocytes and eggs can provide far more material than their mammalian counterparts, as mammalian oocytes are approximately 10 times smaller than those of amphibians.<sup>19</sup> However, amphibian oocytes and eggs are difficult to obtain without a dedicated animal growth facility. Therefore, I sought to identify an alternative extract source, which could be utilized for extract-based reprogramming experiments.

Each component of the extract-based reprogramming system requires careful manipulation. Somatic cells requires effective permeabilization (**Chapter 2**) and both the



penetration and retention of large molecules within somatic cells (**Chapter 3**). However, another important aspect is the source and quality of protein extract, which is paramount for the outcome of reprogramming experiments. A number of variables affect the outcome of extract-based reprogramming experiments including: extract preparation, toxicity,<sup>22</sup> and protein activity. Because cultured cells and in particular permeabilized cells are sensitive to their environment, extract quality composition plays an important role in cell survival following extract treatments. For example, during treatments with *X. laevis* oocyte extracts, porcine fibroblasts respond differently depending upon treatment temperature. At the native egg temperature (23°C), a spliced Oct4 transcript is observed after two hours of treatment, while at 37°C primarily unspliced Oct4 transcript is observed after 30 minutes.<sup>17,23</sup> Therefore, extract treatment temperatures should reflect either donor extract source or somatic cell recipients. Additionally, to observe reprogramming in somatic cells, treated cells must survive extract treatment. Preparations of whole-cell extract can adversely affect somatic cells, for reasons which are poorly understood; therefore extracts should be tested for toxic effects.<sup>22</sup>

## **Materials and Methods**

### **Cell Preparation**

Mouse 3T3 cells were cultured under standard conditions (see **Chapter 1: Somatic Cell Culture**). Cells used for experiments were grown to 50-80% confluence, and harvested by disassociation in 1 mL of trypsin (Invitrogen) until detachment was observed (typically 3 minutes). To suspend cells and inactivate trypsin, 9 mL of **complete DMEM** was

added to cell culture dishes, and then cell suspensions were transferred to 15 mL collection tubes. Cells were pelleted at 300 x g for 5 minutes at 4°C and the media-containing supernatant was discarded. In subsequent steps, cells as pellets or in suspension were maintained on ice or at 4°C wherever possible. Collected cells were first washed twice in PBS (Phosphate Buffered Saline without calcium or magnesium); each wash consisted of suspension then sedimentation at 300 x g for 5 minutes at 4°C. Then, pelleted cells were resuspended in freshly prepared and filtered **acetate transport buffer** [20 mM HEPES, 110 mM potassium acetate, 5mM sodium acetate, 2mM magnesium acetate, and 1mM EGTA, 0.1mM DTT, 0.1mM PMSF, and protease inhibitors (Roche)]. In preparation for treatment cells were counted under on a hemocytometer (for individual aliquots). Following the initial wash in **acetate transport buffer**, cells were resuspended in acetate transport buffer at a concentration of 100,000 cells per milliliter, and dispersed among 1.5mL reaction tubes. Aliquoted cells were then centrifuged for 10 minutes at 300 x g, and remaining acetate buffer was removed. Individual aliquots of pelleted cells were then processed for experimentation; typically cells were treated or resuspended at concentrations of 1,000 cells per uL (see **Extract Treatment or Extract Toxicity Assay**).

### **Extract Preparation**

To address the cost and material concerns associated with available extract donors, I decided to test extracts prepared from king salmon (*Oncorhynchus tshawytscha*) roe as an alternative extract donor for extract-based reprogramming experiments. Frozen Salmon Roe was purchased from Sunrise Bait (Oregon, USA). For my experiments I selected

healthy, intact oocytes, which were identified by their characteristic orange-yellow color and location at the exterior of the overall egg mass. Notably smaller and less developed red oocytes, located at the center of the egg mass were surrounded by vascularization, and avoided wherever possible. The selected oocytes/eggs were flash frozen in 50mL tubes and resuspended in an equal volume of 0.9% NaCl, PBS, or *X. laevis* egg extraction buffer (100 mM KCl, 10 mM HEPES pH 7.4, 1 mM MgCl<sub>2</sub>, 50 mM sucrose). (**Table 4.2**).<sup>15</sup> Homogenization buffers were selected for different purposes: 0.9% NaCl was utilized in a previous publication to generate fish egg extracts (**extracts 1, 4**),<sup>24</sup> PBS may aide in cell survival during treatment (**extract 2**), and *X. laevis* oocyte/egg extraction buffer is commonly employed for generating *X. laevis* oocyte and egg extracts (**extract 4**). Cells were lysed by one of two methods: using a Yamato homogenizer (**extracts 1, 2, and 3**) or mechanically by smashing frozen eggs with a hammer (**extract 4**) at 4°C. Lysates were than centrifuged at 4,000 rpm at 4°C to remove insoluble debris. The supernatant was cleared by pouring the extract through cheesecloth. Cleared extract was centrifuged two times at 13,000 rpm for 30 minutes at 4°C. After each centrifugation step the upper lipid layer and pellet were discarded, while retaining clarified extract. Clarified extract was then filtered in a step-wise fashion through 5 µm, 0.45 µm, and 0.22 µm filters. Extract was than flash frozen in liquid nitrogen and stored at -80°C prior to use. Significant precipitate formed in extracts prepared in PBS and *X. laevis* extraction buffer suggesting that protein denatured or precipitated in these buffers. In contrast, extracts prepared in 0.9% NaCl showed very little precipitation; therefore extracts prepared with 0.9% NaCl were used for subsequent experiments. Protein from each extract preparation

was quantified by Bradford assay (**Table 4.2**) and analyzed by SDS-PAGE (**Figure 2**) to compare the protein content of the extracts and to determine the developmental stage of donor oocytes (see **Discussion below**). Extract used for treatment was supplemented with an ATP regenerating system at a final concentration of 1 mM ATP, 20 mM phosphocreatine and 20 U/ml creatine kinase and 1mM of GTP plus 100  $\mu$ M of NTPs (CTP, UTP). Extract treated cells are termed by the somatic type 3T3, and the corresponding donor extract preparation: salmon roe extract (SRE) 1,2,3,4.

### **Extract Toxicity Assay**

Mouse 3T3 cells were prepared as described in **Cell Preparation**. Each aliquot was resuspended in 100  $\mu$ L of **acetate transport buffer**, undiluted salmon roe extract, or salmon roe extract diluted 1:1000, 1:100, 1:10, 1:2 in **acetate transport buffer**. Cells were then incubated for 1 hour at 37°C, with gentle agitation. For microscopic observations, 50  $\mu$ L of resuspended cells (1,000 cells per  $\mu$ L) representative of each treatment condition were transferred into the wells of a 96-well cell culture plate. Then, morphological changes were assessed using a light microscope, Meiji Techno 5400 Inverted Phase Contrast Microscope and photographed using a NikonD100 camera.

### **Cell Treatments**

Mouse 3T3 cells prepared as described in **Cell Preparation**. Aliquots of cells were first resuspended at a concentration of 1,000 cells per  $\mu$ L (typically  $1 \times 10^5$  to  $2 \times 10^5$  total cells) in **acetate transport buffer** containing 0  $\mu$ g/mL, 20  $\mu$ g/mL, or 30  $\mu$ g/mL of digitonin and incubated for 1-2 minutes. Then 9 volumes of **acetate transport buffer**

were added to each aliquot to dilute digitonin activity. Cell suspensions were then centrifuged at 300 x g for 10 minutes. Aliquots of pelleted cells were then treated under a variety of conditions including extract dilutions of 1:2, 1:3, 1:10, 1:25, 1:50, and 1:100 at a variety of treatment temperatures, which included 4°C, 10°C, 23°C, and 37°C.

However, the experiments described here highlight two sets of treatment conditions: Set **1) Control cells:** 3T3 cells treated with 0 µg/mL of digitonin, and incubated in **acetate transport buffer** at 37°C. Experiment 3T3 cells, which were permeabilized with 30 µg/mL digitonin, and incubated in salmon roe extract 1 diluted 1:10 in **acetate transport buffer** (11.57 mg/mL) at 37°C. These cells were termed 3T3-SRE1 cells. Set **2) Control cells:** 3T3 cells initially treated with 0 µg/mL of digitonin, then were incubated in acetate transport buffer at 37°C. Experimental group: 3T3 cells permeabilized with digitonin and treated with SRE4 termed 3T3-SRE4 cells. Four different experimental approaches were used. 3T3 cells permeabilized with 20 µg/mL digitonin and incubated with salmon roe extract 4 diluted 1:3 (30.186 mg/mL) at either 10°C. b. 3T3 cells permeabilized with 20 µg/mL digitonin and incubated with salmon roe extract 4 diluted 1:3 (30.186 mg/mL) at either 37°C. c.) 3T3 cells permeabilized with 30 µg/mL digitonin and incubated with salmon roe extract 4 diluted 1:3 (30.186 mg/mL) at 10°C.d.) 3T3 cells permeabilized with 30 µg/mL digitonin and incubated with salmon roe extract 4 diluted 1:3 (30.186 mg/mL) at either 37°C. Regardless of condition all cells were incubated for 3 hours.

Following incubation in buffer alone or extracts each cell aliquot was centrifuged at 300 x g for 10 minutes at 4°C. Protein supernatants were discarded, and then cells were washed twice in **acetate transport buffer**. For each wash cells were gently resuspended

in **acetate transport buffer** using a wide-bore/cut-off 200  $\mu$ L pipette tip then centrifuged at 300 x g for 10 minutes at 4°C. Cells were resuspended in **complete Knockout DMEM** supplemented with 2mM CaCl<sub>2</sub> (see **Chapter 1**), and divided among 3-4 wells of a 24-well cell culture plate. After 24 hours, media was changed and all unattached cells were removed and attached cells were periodically observed for morphological changes.

### **Colony Formation and Marker Assessment**

Cells were examined daily following treatment using a light microscope. Initially, formation of ESC-like colonies with defined edges and 3 dimensional shapes as compared to 3T3 cells was used to assess the success of reprogramming assays (**Chapter 1, Figure 4, 5**). Colony formation directed additional marker assessment. In the case of 3T3-SRE1 cells, colonies were manually isolated after 3 weeks using a 1 mL pipette tip and subjected to immunostaining (see **SSEA1 marker staining**). 3T3-SRE4 cells were stained in-plate for alkaline phosphatase activity (see **Alkaline Phosphatase (AP staining)**).

### **SSEA1 Marker Staining**

Isolated 3T3-SRE1 colonies were plated onto gelatin coated coverslips within the wells of a 12-well cell culture plate. Colonies isolated from 3T3-SRE1 cells were incubated for 15 minutes in **complete KO DMEM** containing 1  $\mu$ g/mL of Hoechst 33342. Then cells were washed three times with PBS. For each wash PBS was gently applied to the cells for

5 minutes, and then removed using a 1mL pipette. Then, 3T3-SRE1 colonies were fixed with 3.7% formaldehyde for 10 minutes, and cells were washed three additional times with PBS. For all remaining steps, cell culture plates were maintained at 4°C, on a gentle rocker. First, cells were incubated in with 0.1% Triton-X-100 for 15 minutes. Then cells were incubated 1 hour in blocking solution containing 5% chicken serum in PBS. After blocking, cells were washed three times with PBS. Then cells were incubated in primary antibody, SSEA1 (UCR stem cell core) diluted 1:10 in PBS for 2 hours and then cells were washed three times with PBS. Secondary antibody Alexa Fluor 488 goat anti mouse (Invitrogen) diluted 1:500 in PBS was added for 1 hour at a concentration of 4 µg/mL. After that, cells were washed four more times with PBS, and once with HBSS. Finally, coverslips were pried out using a 22G needle, and using tweezers flipped onto 20 µL of HBSS on a glass slide. Antigen-antibody complexes were visualized and documented using a Ellipse Ti (Nikon, UCR stem cell core facility).

#### **Alkaline Phosphatase (AP staining)**

Both control cells and 3T3-SRE4 cells were fixed with 3.7% formaldehyde for 2 minutes. Following fixation, cells were washed three times through the gentle application and removal of 1mL PBS for 5 minutes. Cells were overlaid with AP Buffer (100 mM Tris pH 8.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>). Stocks of 5-Bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium chloride (each 50 mg/mL in dimethyl formamide), were diluted to final concentrations of 500µg/mL in AP buffer. Then cells were incubated in chemical substrates. Cells were than observed using a light microscope Meiji Techno

5400 Inverted Phase Contrast Microscope, and photographed using a NikonD100 camera after 30 minutes. Pictures emphasize colonies among a field of cells within the well of a 24-well plate. Control cells represent a random field of cells with no detectable AP staining. 293T and NT2 cells were used as negative and positive control, respectively.

### **Bradford Assay**

Bradford assay was performed as described.<sup>25</sup> To create a standard curve 0, 2, 4, 6, 8, and 10 µg/mL BSA were assayed. Extracts were diluted 1:100 or 1:1000 in water. 800 mL protein solution was mixed with 200µL of Bradford reagent samples were vortexed and incubated for 5 minutes. The absorbance (595nm) of each sample was measured using the Nanodrop spectrophotometer (Thermo Scientific), and protein concentrations were determined by extrapolation using the standard curve.

### **SDS-PAGE**

SDS-PAGE was performed as previously described.<sup>25</sup> Briefly, extract samples were boiled in 4x loading buffer and separated on 12.5 % polyacrylamide gels at 80 volts; once bands entered the resolving gel voltage was increased to 120 volts. Gels were stained for 30 minutes with coomassie blue staining solution (500 mL ddH<sub>2</sub>O, 100 mL glacial acetic acid, 400 mL methanol, 1g coomassie blue R250) and destained overnight in destainer (450 mL ddH<sub>2</sub>O, 100 mL glacial acetic acid, 450 mL methanol).



## **RT-PCR**

### **Isolation of RNA and reverse transcription**

Cells were either isolated by trypsinization (attached cells) or centrifugation (floating cells). Isolated cells were suspended in PBS and centrifuged at 300 x g to remove traces of DMEM. Cells were resuspended in 1mL of TRIZOL reagent (Invitrogen) for 5 minutes after which 200 $\mu$ L of chloroform was added to each reaction tube. Mixtures were then centrifuged at 13,000 x g for 10 minutes. The aqueous phase was isolated from each sample and mixed with 500 $\mu$ L isopropanol. RNA was pelleted by centrifugation at 13,000 RPMs for 10 minutes. Pellets were then washed with 1mL of 70% EtOH and centrifugation at 12,000 RPMs for 10 minutes. Pellets were allowed to dry for 10 minutes under a chemical hood. RNA was resuspended in 25  $\mu$ L DNase, RNase free H<sub>2</sub>O. To remove genomic DNA (gDNA) contamination; samples were treated with DNase I (Roche) for 1 hr. Then DNase was heat inactivated by boiling at 65°C for 15 minutes and RNA was purified using RNA Clean & Concentrator™-5 kit from (Zymo) according to manufactures instructions. cDNA was prepared using SuperScript III (Invitrogen), according to manufacturer's instructions using 0.25  $\mu$ g of total RNA for each sample.

## **PCR**

PCR reactions were performed using 2X GoTaq Green Master mix (Promega) with a total reaction volume of 25  $\mu$ L including: 0.5  $\mu$ L each of forward and reverse primers,

with 0.5  $\mu$ L of cDNA or gDNA (for primer validation). Products were amplified using the following program: 95 °C for 3 min, followed by 40 cycles of 95°C for 30s, 60°C for 30s and 72°C for 1 minute. GAPDH transcript was detected and used for standardization of cDNA pools. All samples were free of gDNA, and primers used were exon spanning where possible as verified using Genbank (NCBI) and In-Silico PCR (UCSC genome browser). Genbank confirmation:

>[NM\\_001252452.1](#) Mus musculus POU domain, class 5, transcription factor 1 (Pou5f1), transcript variant 2, mRNA

product length = 595

Forward primer 1 GCTGCTGAAGCAGAAGAGGA 20  
Template 138 ..... 157

Reverse primer 1 GACGGGAACAGAGGGAAAGG 20  
Template 732 ..... 713

>[NM\\_028016.2](#) Mus musculus Nanog homeobox (Nanog), mRNA

product length = 294

Forward primer 1 CCAGTCCCAAACAAAAGCTC 20  
Template 424 ..... 443

Reverse primer 1 GAATCAGACCATTGCTAGTCTTCA 24  
Template 717 ..... 694

>[NM\\_008084.2](#) Mus musculus glyceraldehyde-3-phosphate dehydrogenase (Gapdh), mRNA

product length = 379

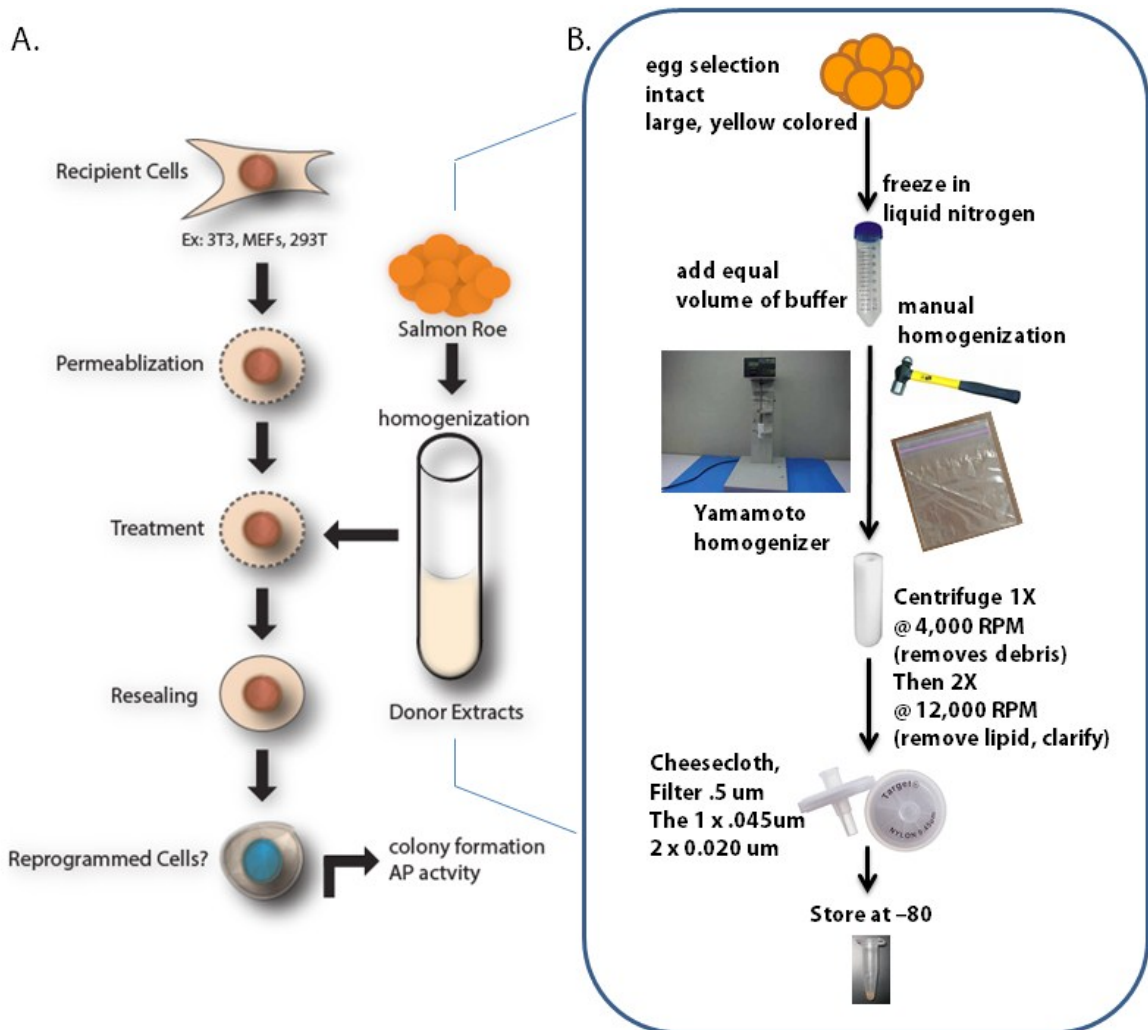
Forward primer 1 ACTCCACTCACGGCAAATTC 20  
Template 193 ..... 212

Reverse primer 1 GTCATGAGCCCTTCCACAAT 20  
Template 571 ..... 552

## Results/Figures

Extract Donor	Recipient Cell	morphology	Upregulated	Downregulated	AP activity	Nuclear Structure	Epigenetic (+)	Factor	Reference
X. laevis eggs	293T	cell clusters, spheres	<i>OCT4, GCAP</i>		expressed (GCAP)			BRG1	(Hansis et al., 2004)
X. laevis eggs	porcine fibroblasts	small colonies	<i>Oct4</i> unspliced @ 23C spliced @ 37C			incorporation of Lamin LIII	B4 incorporation, global loss of Ace-H3K9	B4, Lamin LIII	(Miyamoto et al., 2007)
X. laevis ovary/oocyte	293T					loss of LMNA/C			(Bru et al., 2008)
X. laevis eggs	bovine cumulus		<i>OCT4, SOX2, NANOG</i>			incorporation of Lamin LIII	B4 incorporation	TBP loss	(Miyamoto et al., 2008)
axolotl oocyte	MCF7, HCC1954		<i>NANOG</i>						(Allegrucci et al., 2011)
mESC CJ7	293T	colonies	<i>OCT4, NANOG</i>	<i>LMNA</i>					Ch. 1
46C, J1 (mESCs)	293T	colonies	<i>NANOG, OCT4, SOX2, KLF4</i>	<i>LMNA</i>		loss of Lamin A/C	loss of repressive marks on pluripotent promoters		(Bru et al., 2008)
salmon oocytes	3T3	colonies at 37°C colonies at 10°C	<i>Oct4</i>		activity in defined colonies, no activity in irregular colonies				Ch. 4

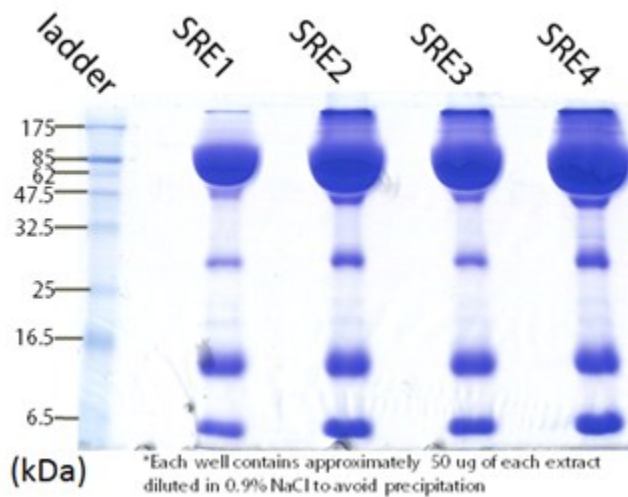
**Table 4.1** Interspecies extract-based reprogramming experiments. Various reprogramming activities have been identified through the use of amphibian extracts (green) and mammalian whole-cell extracts (blue) applied to somatic cells originating from different species. Here we describe activities induced by a novel extract originating from salmon roe (orange) and applied to mouse 3T3 cells.



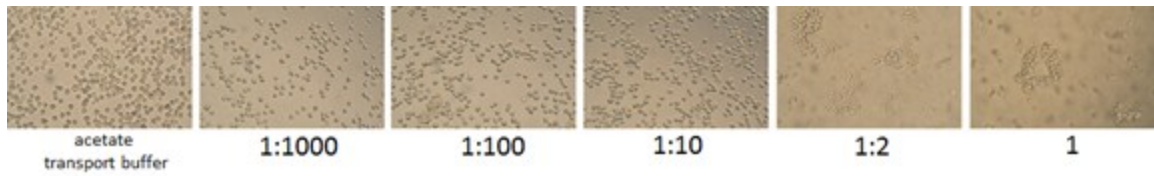
**Figure 4.1** – Treatment of mouse 3T3 cells by previously prepared salmon roe extract. **A.** Mouse 3T3 cells are prepared through extensive washing, permeabilized with digitonin, and then treated with salmon roe extract. Cells were subjected to various forms of analysis following resealing with calcium supplemented medium. **B.** King salmon roe was purchased from and extracted by a bait supplier, frozen extract was shipped and intact oocytes were selected bases of size and color. Then, oocytes were flash frozen and homogenized with an equal volume of various buffers. Extract was then subjected to high-speed centrifugation and filtering, prior to its storage in subsequent use in the extract-based reprogramming system.

Extract	Method of Extraction	Concentration (mg/mL)	Active Dilution	Active Treatment Concentration (mg/mL)	comments/problems
1 salmon roe (0.9% NaCl)	Yamamoto Homogenizer	115.700	1/10	11.57	
2 salmon roe (PBS)	Yamamoto Homogenizer	130.700	--	--	PBS increased precipitation
3 salmon roe ( <i>X. laevis</i> egg extraction buffer)	Yamamoto Homogenizer	108.460	--	--	Extraction buffer increased precipitation
4 salmon roe (0.9% NaCl)	manual	90.560	1/3	30.186	

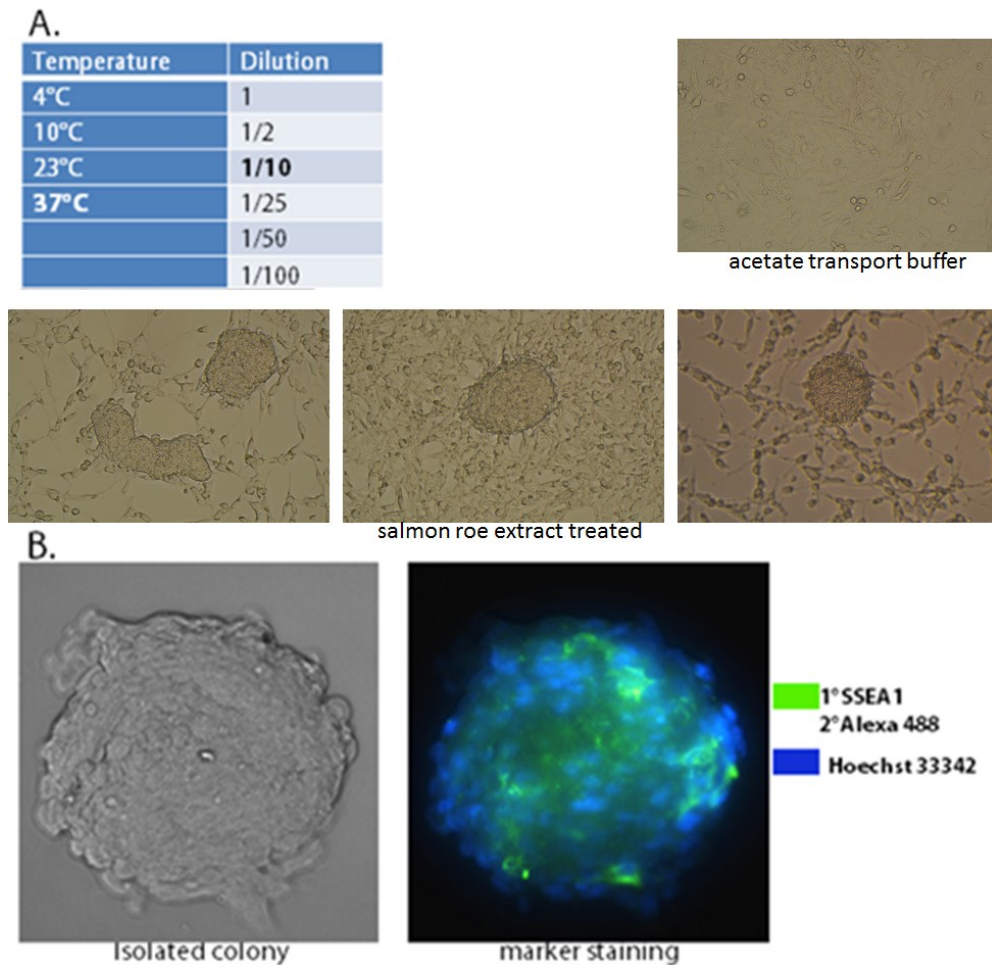
**Table 4.2** Various extracts used in this study. Salmon roe extract was prepared either through mechanical or manual homogenization in three solutions: 0.9% NaCl, PBS, and *Xenopus laevis* egg extraction buffer. Cells were first treated on the basis of dilution for the determination of active concentrations.



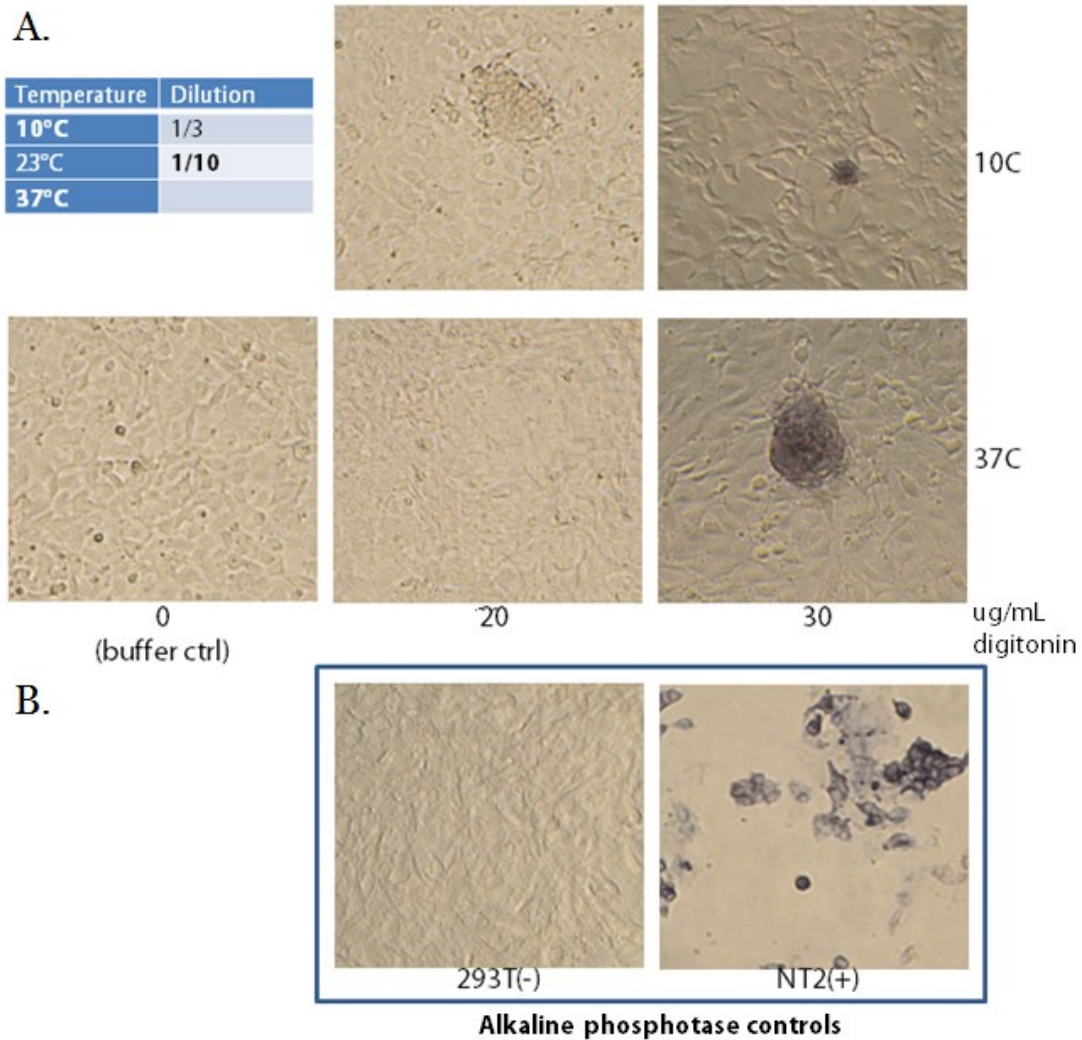
**Figure 4.2** SDS-PAGE of salmon roe extract protein. Diluted salmon roe extract was first normalized, and then loaded onto a 12.5% SDS-PAGE gel. Despite different approaches to extraction and various buffers, homogenized protein patterns are quite similar resembling late stage oocyte banding from zebrafish.<sup>26</sup>



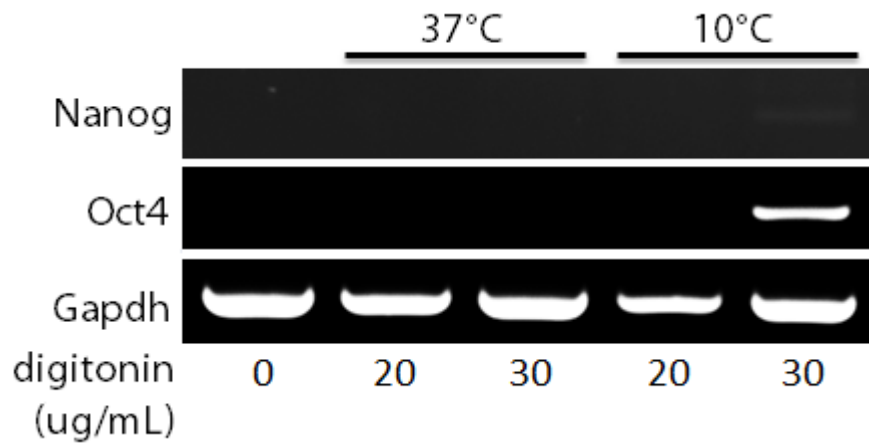
**Figure 4.3** – Extract toxicity assay. Observation of mouse 3T3 cells treated with various dilutions of salmon roe extract. Compromised morphology as an indication of extract toxicity following 3T3 cell treatment with various dilutions of salmon roe extract #1.



**Figure 4.4** Colony formation and SSEA1 marker detection in 3T3-SRE cells. Permeabilized mouse 3T3 cells were treated with salmon roe extract under various conditions (top left). After two weeks, defined colonies formed in cells permeabilized with 30  $\mu\text{g}/\text{mL}$  of digitonin and treated with  $\sim 1\text{mg}/\text{mL}$  of salmon roe extract 1 (middle). Colonies were then isolated and stained for SSEA1 surface marker expression (bottom).



**Figure 4.5** – Alkaline Phosphatase Activity following Salmon Roe extract treatment. **A.** Mouse 3T3 cells were permeabilized with 20 or 30  $\mu\text{g/mL}$  of digitonin and treated at 10, 23, 37 degrees Celsius. Four days after treatment, visible colonies formed and were stained for alkaline phosphatase activity. After 30 minutes colonies and random fields of cells were captured using a light microscope equipped with a camera. No cells exhibited colorimetric changes in control cells. **B.** Alongside alkaline phosphatase staining of treated cells, negative control 293T cells and a positive control NT2 cells were also stained.



**Figure 4.6** – Oct4 expression in 3T3-SRE4 cell populations. 3T3 cells permeabilized with 20 or 30 µg/mL of digitonin and were then incubated in salmon roe extract. Cells permeabilized with 30 µg/mL of digitonin and incubated in SRE4 at 10°C expressed *Oct4*, coinciding with the formation of small well-defined colonies. Despite colony formation in other treatment conditions, *Oct4* was not detected. *Oct4* was not detected in 3T3 control, buffer treated cells. *Gapdh* was detected in all samples.

### Extract Toxicity

It has been reported that depending on preparation, cellular extracts can be toxic to cells.<sup>22</sup> Therefore, I incubated mouse 3T3 cells in salmon roe extract and examined treated cells microscopically for visible signs of extract toxicity. 3T3 cells incubated with **acetate transport buffer** appeared morphologically intact (**Figure 4.3 left**). Similarly, 3T3 cells incubated with salmon roe extracts diluted in **acetate transport buffer** (1:1000, 1:100, 1:10), resembled cells treated with **acetate transport buffer** (**Figure 4.3**). In contrast, 3T3 cells treated with extract diluted 1:2 in **acetate transport buffer**, or undiluted extract were toxic to cells, as indicated by morphological changes such as cell shrinking and clustering (**Figure 4.3 right**).<sup>27</sup> Concentrated extracts are toxic, potentially



limiting cell survival during treatment; therefore I used diluted extracts for 3T3 cell treatments.

### **Extract Treatments and Colony Formation**

To optimize conditions for salmon roe extract treatments, I tested the effect of various protein concentrations and extract treatment temperatures (**Figure 4.4, 4.5 upper left**) on mouse 3T3 cells permeabilized with 20 or 30  $\mu\text{g}/\text{mL}$  of digitonin (conditions established in **Chapter 3**). Extract-treated cells were monitored for visible morphological changes following treatment. Consistent with results of permeabilization and retention assays (**Chapter 2, 3**) a portion of cells did not attach to the wells of cell culture plates following permeabilization and incubation with salmon roe extracts. However, surviving cells regained normal 3T3 cell morphology 24 hours following extract treatment. Colony formation was only observed after two weeks in 3T3 cells treated with 11.57 mg/mL of SRE1 extract (**Figure 4.4**), and after only four days in 3T3 cells treated with 30.19 mg/mL of SRE4 extract (**Figure 4.5**). Colony formation was also dependent upon treatment temperature. Permeabilized 3T3 cells treated with SRE1 extract at 37°C formed colonies, and cells treated with SRE4 at both 10°C and 37°C formed colonies. Interestingly, only cells permeabilized with 30  $\mu\text{g}/\text{mL}$  of digitonin, formed colonies with defined edges. Irregular colonies were observed in 3T3 cells permeabilized with 20  $\mu\text{g}/\text{mL}$  of digitonin and incubated at 10°C. Treatment of somatic 3T3 cells with salmon roe extract (10-30 mg/mL) can induce temperature-dependent (10, 37°C) colony formation, a morphological feature associated with ESCs and the pluripotent state.

### **SSEA1 staining**

To determine if the cells in the ESC-like colonies formed by treated 3T3 cells express surface antigens associated with pluripotency, I decided to monitor SSEA1 surface marker expression in isolated 3T3 colonies (4 weeks) through immunostaining. For this experiment, 3T3-SRE1 colonies were isolated from surrounding cells. Cultured 3T3-SRE1 colonies retained well-defined, 3-dimensional colony morphology (**Figure 4.4 bottom**). (**Figure 4.4 bottom**), small patches of SSEA1-specific immunostaining were observed on the colony periphery. The expression of SSEA1 in a colony of 3T3-SRE1 cells indicates that cells within the colony express a pluripotency marker. The result implies that at least a subset of cells within the colony has been reprogrammed towards the pluripotent state.

### **AP staining**

Because alkaline phosphatase (AP) has emerged as an early marker of reprogramming, I decided to assess AP activity in 3T3-SRE4 cells. To detect AP activity, I used a colorimetric assay that detects AP activity within cells. AP activity was assayed in treated cells, 293T cells (negative control), and NT2 cells (positive control) (**Figure 4.5B**). 3T3 cells, which were treated with buffer only, and 293T cells, exhibited no AP activity (**Figure 4.5**). Similarly, cells surrounding colonies in treated 3T3 cultures also displayed no detectable AP activity (**Figure 4.5A**). Surprisingly, irregular colonies formed in 3T3-SRE4 cells were also negative for AP activity (**Figure 4.5A top, center**). In contrast, AP activity was detectable in colonies with defined edges observed in cells treated with

extract at either 10°C or 37°C. 293T cells served as a negative control, and NT2 cells were used as a positive control. (**Figure 4.5**). The detection of AP activity indicates that the cells in the tested colonies express an enzyme, which serves as a marker for pluripotency. AP expression has been associated with the pluripotent state and early phases of the reprogramming process. The expression of AP in 3T3-SRE4 cells and the formation of colonies resembling the colonies formed by pluripotent cells suggest that 3T3-SRE4 cells have been reprogrammed towards the pluripotent cell state.

### **Pluripotent Gene Expression**

To determine if 3T3-SRE4 cells express additional pluripotency markers, the expression of pluripotent marker genes Oct4 and Nanog was examined by RT PCR (**Figure 4.6**). As expected, control cells (3T3 cells treated with **acetate transport buffer**) did not express Oct4 or Nanog. Similarly, 3T3 cells permeabilized with 20 µg/mL digitonin and treated with SRE4 did not express Oct4 or Nanog, regardless of treatment temperature. Surprisingly, Oct4 and Nanog were detected in the large colonies formed by 3T3-SRE4 cells. In 3T3 cells, which were permeabilized with 30 µg/mL of digitonin then treated with SRE4, are coinciding with the formation of small colonies. These results support that 3T3-SRE4 cells are programmed toward a pluripotent state. They also suggest that treating cells with salmon roe extract at 10°C, benefits cell reprogramming as revealed by Oct4 marker expression (**Figure 4.6**).

## Discussion:

Somatic cell reprogramming has been achieved through a variety of means.<sup>1,16,28,29</sup> Recent approaches have focused on iPSC-based technology, in particular the delivery of four factors (or their derivatives) by an assortment of techniques and efforts to improve the safety and efficiency of iPSC derivation.<sup>30,31</sup> However, differences are emerging between ESCs and iPSC.<sup>4,5,6,7,8</sup> One approach to refine the iPSC technique is the identification of reprogramming mechanisms and factors in material with demonstrated reprogramming potential such as oocytes, eggs, embryos, and cultured cell sources.<sup>20,32</sup> Although a wide range of pluripotent sources can reprogram cells only material from oocyte/eggs have successfully been used in somatic cell reprogramming experiments.<sup>33</sup>

It has been established that oocytes, even from distantly related species are capable of reprogramming mammalian somatic cells (**Table 4.1**). Extract-based reprogramming is one approach toward the identification of fundamental and evolutionarily conserved reprogramming activities native to oocytes and eggs.<sup>33</sup> Despite drastically different developmental potential oocytes/eggs from amphibians can elicit pluripotent gene expression and morphological and structural changes in mammalian somatic recipients (**Table 4.1**). This suggests factors associated with reprogramming are evolutionarily conserved. The evolutionarily conserved nature of reprogramming is beneficial to extract-based reprogramming experiments, permitting studies using a variety of oocyte extract donors.

Despite the evolutionarily conserved nature of reprogramming activities, the developmental stage of oocyte extract donors can impact the results of extract-based

reprogramming experiments. During mammalian SCNT, MII oocytes are required for the successful generation of cloned animals.<sup>34</sup> However, while extracts derived from either porcine GV or MII stage oocytes can induce somatic cell reprogramming (in porcine fibroblasts), porcine GV extracts possess greater reprogramming potential as highlighted by the induction of *Nanog* expression.<sup>19</sup> In contrast, extracts derived from *Xenopus laevis* eggs (MII oocytes) have greater reprogramming potential than extracts derived from intact *Xenopus laevis* ovaries.<sup>15,20</sup> A common theme of oocyte extract sources is the use of developed, late-stage oocytes.<sup>19,17</sup> As a source, roe is unique because it contains oocytes at various stages of oogenesis.<sup>35,36</sup> Roe represents a median between an oocyte tissue and released eggs, because at late stages of oogenesis the oocyte can be deposited as an egg depending upon environmental conditions or mechanical force (squeezing).<sup>37,38</sup> In my experiments, I attempted to isolate morphologically, healthy late stage salmon oocytes located near the exterior of the egg mass. The size and appearance of selected oocytes resembles stages IV-V oocytes in zebra fish, with stage IV being metaphase II.<sup>39</sup> Aside from morphological similarities, the protein pattern of salmon roe extracts on SDS-PAGE resembles the pattern of protein extract generated from stage IV-V zebra fish oocytes (**Figure 4.2**).<sup>35,26</sup> My selection of large, morphologically healthy, and developed oocytes should emphasize later stages of oogenesis similar to *X. laevis* eggs (MII oocytes), which are commonly employed for extract reprogramming experiments.

The testing of novel extract donors requires attention to both extract preparation and the subsequent treatment of somatic cells with extracts. One concern is identifying conditions suitable for oocyte protein preparations, which conserve protein function and

thus the reprogramming potential of the protein extract.<sup>40</sup> To that end, I used different approaches for protein extraction with somatic cell treatments in mind (**Table 4.2**). PBS and *X. laevis* extraction buffers appeared to denature proteins as evident by the formation of protein precipitate. Precipitation indicates protein denaturation and results in loss of protein, both compromising the reprogramming potential of the extract. Therefore, I elected to use protein extracts with limited protein precipitation during extraction (salmon roe extracts 1 and 4). High concentrations of salmon roe extract were toxic to intact mouse 3T3 cells (**Figure 4.3**); therefore for identifying extract treatment conditions which favored the reprogramming and survival of 3T3 cells, I used diluted extract for treatments. Permeabilized 3T3 cells formed colonies following treatment with salmon roe extracts 1 and 4, at concentrations of 11 and 30 mg/mL respectively. These concentrations are consistent with previously reported effective concentrations for *Xenopus laevis* egg or ECC whole cell extracts, which also induced colony formation (among other reprogramming activities) in permeabilized mammalian recipient cells.<sup>20,19,16</sup>

Aside from extract concentration, the formation of colonies in extract treated cells was favored by two extract treatment temperatures (10°C and 37°C). Two types of colonies emerged following treatment with salmon oocyte extracts: 1) tightly compact cells organized into 3-dimensional structures with clearly defined edges (termed defined colonies) (**Figure 4.4 middle, Figure 4.5 bottom, and right panel**). 2) irregular-shaped clusters of cells with few or no distinguishable, defined edges (**Figure 4.5 top, center panel**). Various colony morphologies have been reported during the derivation of human

iPSCs.<sup>41,42</sup> Defined colonies are associated with fully reprogrammed cells, whereas irregular morphologies have been associated with partial reprogramming.<sup>41,42</sup> Therefore, differences in colony morphology may reflect different degrees of reprogramming in salmon oocyte extract treated cells. Initially, defined colonies, which were isolated from 3T3-SRE1 and 3T3-SRE4 cells, grew in cell culture. 3T3-SRE1 colonies grew rapidly for approximately 4 weeks.

To support that salmon roe extract can induce reprogramming in mouse 3T3 cells, I tested whether obtained cell colonies expressed the pluripotency markers SSEA1 and AP. SSEA1 is a surface marker specific to mESCs and AP is an enzyme limited to expression in pluripotent cells and specific organs (liver or bone).<sup>43,44,45</sup> Aside from serving as markers of pluripotent cells both SEA1 and AP have been identified during early stages of iPSC-based reprogramming and precede the expression of other pluripotent genes such as Oct4 and Nanog.<sup>43</sup>

3T3-SRE1 colonies were isolated for staining. SSEA1 was detected in at least one 3T3-SRE1 colony, a sign of somatic cell reprogramming (**Figure 4.4**). Despite well-defined colony morphology, only some portions of the colony stained positive for SSEA1 suggesting that certain cells of the colony express SSEA1 while others do not. Alternatively, it is also possible that the antibody staining was ineffective, because the antibodies were unable to penetrate 3T3-SRE1 colonies (**Figure 4.4 bottom**). In mouse colonies isolated from mouse iPSC, detection of SSEA1 can also vary with clonal

selection and passage number.<sup>46</sup> Therefore, while a positive indicator of reprogramming; SSEA1 detection may vary among reprogrammed colonies.

Additionally, I tested 3T3-SRE4 cells for AP activity. Defined colonies were observed after only four days of extract treatment, in cells permeabilized with 30 µg/mL digitonin and incubated with extract at both 10°C and 37°C (**Figure 4.5**). Defined colonies in either treatment condition were positive for AP activity. Notably, 3T3-SRE4 cells surrounding colonies were negative for AP activity (**Figure 4.5**). Additionally, AP activity was not observed in cells treated with acetate transport buffer alone or in poorly-defined colonies observed in 3T3-SRE4 cells (**Figure 4.5**). Therefore, AP activity appears specifically in well-defined colonies.

To determine if pluripotent gene expression was activated in 3T3-SRE4 cell populations express additional pluripotent marker genes, I assessed whether 3T3-SRE4 cells were evaluated express *Oct4* and *Nanog* expression. Interestingly, the expression of *Oct4* and *Nanog* was only observed in 3T3-SRE4 cells which had been permeabilized with 30 µg/mL of digitonin and incubated with extract 10°C (**Figure 4.6**). Despite the formation of large colonies in cells treated with 30 µg/mL of digitonin and incubated with extract at 37°C, neither *Oct4* and *Nanog* were detected (**Figure 4.6**). In 3T3-SRE4 cell populations which contained poorly defined colonies (**Figure 4.5**), neither *Oct4* nor *Nanog* were detected (**Figure 4.6**). These results suggest that induction of pluripotent gene expression in permeabilized mouse 3T3 cells permeabilized with 30 µg/mL of digitonin depends upon extract treatment temperature (**Figure 4.6**). It has been suggested



that permeabilized somatic cells respond differently to extract treatment, depending upon incubation temperature. While 10°C may emphasize salmon roe extract activity, the incubation of cells with extract at 37°C may favor somatic cell activities (together with extract effectors) Both treatment conditions resulted in defined colony formation and the induction of AP activity, while only cells treated at with extract at 10°C expressed pluripotent marker genes (**Figures 4.5, 4.6**). These results are similar to the temperature-dependent induction of *Oct4* expression in cells treated with *Xenopus laevis* egg extracts.<sup>17</sup>

The expression of *Oct4* expression in 3T3-SRE4 colonies is a significant molecular signature of somatic cell reprogramming.<sup>47, 48</sup> *Oct4* is considered central to the establishment and maintenance of pluripotency in mammalian cells and *Oct4* expression is often used to monitor the success of somatic cell reprogramming experiments.<sup>1,16,49,50</sup> *Oct4* expression has also been induced in mammalian cells either through interspecies SCNT,<sup>51,52</sup> or through the activity of *Xenopus laevis* egg extracts.<sup>20,18</sup> The induction of *Oct4* expression by protein originating from fish and amphibian oocytes suggests that induction of *Oct4* expression is an evolutionarily conserved event of somatic cell reprogramming . Aside from *Oct4*, trace amounts of *Nanog* were detected in extract reprogramming cells. Limited *Nanog* expression has been associated with partial cell reprogramming,<sup>53</sup> Thus, weak *Nanog* expression in 3T3SRE4 cell populations supports that cells within the population have been partially reprogrammed and that salmon roe extract can at least partially reprogram 3T3 cells toward the pluripotent state (discussed below).

During iPSC reprogramming, some cells also express SSEA1 and AP with limited or no pluripotent gene expression.<sup>54</sup> Such cells have been termed “pre-iPS cells” or partially reprogrammed cells.<sup>54,55</sup> Pre-iPSC colonies feature limited marker induction and do not proliferate in normal ESC culture conditions (LIF nonresponsive).<sup>54</sup> Colonies isolated from 3T3-SRE1 and 3T3-SRE4 treated cells initially expanded in culture but only for brief periods (3T3-SRE1 four weeks, 3T3-SRE4 one week). Similarly, partial reprogramming activities have been described in mammalian somatic cells treated with a variety of oocyte extracts.<sup>20,19</sup> Limited growth in culture coupled with AP activity, and limited pluripotent marker induction suggests that 3T3-SRE colonies are partially reprogrammed and resemble the pre-iPSC-state.

Across distantly related vertebrate species, factors associated with maintaining pluripotency such as *Oct4* and *Sox2* are phylogenetically conserved.<sup>56,57</sup> Interestingly, ancestral *Oct4* variants can substitute for mammalian *Oct4* in iPSC reprogramming experiments, despite differences in *Oct4* target binding sites across species.<sup>56,58</sup> The role of iPSC factors during SCNT remains unclear. Similarly, it is unknown if ancestral iPSC factors or their variants play a role in extract-based reprogramming experiments; however since iPSC factors are not detectable in mammalian oocytes or even zygotes other factors are likely responsible for both SCNT and interspecies extract-based reprogramming.<sup>12</sup> Additionally, epigenetic modifiers,<sup>59</sup> histone variants,<sup>60,61</sup> and chromatin remodeling complexes<sup>20,62</sup> are thought to have a role in the extract-based reprogramming process. The morphological and molecular changes induced by oocytes from a variety of species resemble the changes induced by salmon roe extract (**Table 4.1**) suggesting that salmon

oocytes and oocytes from other species share the same or a very similar set of factors capable of reprogramming somatic cells

Salmon roe extract can induce colony formation (**Figures 4.3, 4.4**) limited SSEA1 marker expression (**Figure 4.4**), alkaline phosphatase activity (**Figure 4.5**), and pluripotent gene expression (**Figure 4.6**) in somatic, mouse 3T3 cells. These activities correlate well with the results of similar studies using a variety of oocyte extract donors (**Table 4.1**), and iPSC reprogramming.<sup>43</sup> In contrast to other extract sources, large amounts of salmon roe extract can be generated at very little cost. Biochemical assays requiring significant amounts of starting material are necessary to identify the factors involved in cell reprogramming in protein extracts. Thus, salmon roe extract represents a suitable source for the biochemical identification of activities and factors involved in cell reprogramming.

## References:

1. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–76 (2006).
2. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–72 (2007).
3. Zhao, X. *et al.* iPS cells produce viable mice through tetraploid complementation. *Nature* **461**, 86–90 (2009).
4. Ohi, Y. *et al.* Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. *Nature cell biology* **13**, 541–9 (2011).
5. Bock, C. *et al.* Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell* **144**, 439–52 (2011).
6. Ohm, J. E. *et al.* Cancer-related epigenome changes associated with reprogramming to induced pluripotent stem cells. *Cancer research* **70**, 7662–73 (2010).
7. Kim, K. *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285–90 (2010).
8. Zhao, T., Zhang, Z.-N., Rong, Z. & Xu, Y. Immunogenicity of induced pluripotent stem cells. *Nature* **474**, 212–5 (2011).
9. Yamanaka, S. Pluripotency and nuclear reprogramming. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **363**, 2079–87 (2008).
10. Armstrong, L., Lako, M., Dean, W. & Stojkovic, M. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem cells (Dayton, Ohio)* **24**, 805–14 (2006).
11. Gurdon, J. B. Nuclear reprogramming in eggs. *Nature medicine* **15**, 1141–4 (2009).
12. Pfeiffer, M. J. *et al.* Proteomic analysis of mouse oocytes reveals 28 candidate factors of the “reprogrammome”. *Journal of proteome research* **10**, 2140–53 (2011).

13. Piccolo, F. M. *et al.* Using heterokaryons to understand pluripotency and reprogramming. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **366**, 2260–5 (2011).
14. Saric, T. & Hescheler, J. Stem cells and nuclear reprogramming. *Minimally invasive therapy & allied technologies : MITAT : official journal of the Society for Minimally Invasive Therapy* **17**, 64–78 (2008).
15. Bru, T. *et al.* Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. *Experimental cell research* **314**, 2634–42 (2008).
16. Taranger, C. K. *et al.* Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells. **16**, 5719–5735 (2005).
17. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by *Xenopus laevis* egg extracts. *Molecular reproduction and development* **74**, 1268–77 (2007).
18. Miyamoto, K. *et al.* Reversible membrane permeabilization of mammalian cells treated with digitonin and its use for inducing nuclear reprogramming by *Xenopus* egg extracts. *Cloning and stem cells* **10**, 535–42 (2008).
19. Miyamoto, K. *et al.* Cell-free extracts from mammalian oocytes partially induce nuclear reprogramming in somatic cells. *Biology of reproduction* **80**, 935–43 (2009).
20. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming of human somatic cells by *xenopus* egg extract requires BRG1. *Current biology : CB* **14**, 1475–80 (2004).
21. Allegrucci, C. *et al.* Epigenetic reprogramming of breast cancer cells with oocyte extracts. *Molecular cancer* **10**, 7 (2011).
22. Collas, P. Reprogramming Somatic Cells in Extract of Undifferentiated Carcinoma Cells. *www.collaslab.com* 1–11
23. Byrne, J. a *et al.* Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature* **450**, 497–502 (2007).
24. Zhu, X.-Q. *et al.* Transient in vitro epigenetic reprogramming of skin fibroblasts into multipotent cells. *Biomaterials* **31**, 2779–87 (2010).

25. Meyer, C. R. Laboratory Manual BCH 102.
26. Ziv, T. *et al.* Comparative proteomics of the developing fish (zebrafish and gilthead seabream) oocytes. *Comparative biochemistry and physiology. Part D, Genomics & proteomics* **3**, 12–35 (2008).
27. Gewies, A. Introduction to Apoptosis. *ApoReview* 1–26 (2003).
28. Gurdon, J. B. & Melton, D. a. Nuclear reprogramming in cells. *Science (New York, N.Y.)* **322**, 1811–5 (2008).
29. Pereira, C. F. *et al.* Heterokaryon-based reprogramming of human B lymphocytes for pluripotency requires Oct4 but not Sox2. *PLoS genetics* **4**, e1000170 (2008).
30. Lai, M. I. *et al.* Advancements in reprogramming strategies for the generation of induced pluripotent stem cells. *Journal of assisted reproduction and genetics* **28**, 291–301 (2011).
31. Hussein, S. M. I. & Nagy, A. a. Progress made in the reprogramming field: new factors, new strategies and a new outlook. *Current opinion in genetics & development* **22**, 435–43 (2012).
32. Häkelién, A.-M. *et al.* Long-term in vitro, cell-type-specific genome-wide reprogramming of gene expression. *Experimental cell research* **309**, 32–47 (2005).
33. Gurdon, J. B. & Wilmut, I. Nuclear transfer to eggs and oocytes. *Cold Spring Harbor perspectives in biology* **3**, (2011).
34. Campbell, K. H. S. *et al.* Somatic cell nuclear transfer: Past, present and future perspectives. *Theriogenology* **68S**, S214–31 (2007).
35. Lubzens, E., Young, G., Bobe, J. & Cerdà, J. Oogenesis in teleosts: how eggs are formed. *General and comparative endocrinology* **165**, 367–89 (2010).
36. Campbell, B. *et al.* Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory p. *Biology of reproduction* **75**, 34–44 (2006).
37. Ma, H., Hostuttler, M., Wei, H., Rexroad, C. E. & Yao, J. Characterization of the rainbow trout egg microRNA transcriptome. *PloS one* **7**, e39649 (2012).

38. Tokumoto, T., Yamaguchi, T., Ii, S. & Tokumoto, M. In vivo induction of oocyte maturation and ovulation in zebrafish. *PLoS one* **6**, e25206 (2011).
39. Bally-Cuif, L., Schatz, W. J. & Ho, R. K. Characterization of the zebrafish Orb/CPEB-related RNA binding protein and localization of maternal components in the zebrafish oocyte. *Mechanisms of development* **77**, 31–47 (1998).
40. Agarwal, S. Cellular reprogramming. *Methods in enzymology* **420**, 265–83 (2006).
41. Chan, E. M. *et al.* Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. *Nature biotechnology* **27**, 1033–7 (2009).
42. Park, I.-H. & Daley, G. Q. *Human iPS cell derivation/reprogramming. Current protocols in stem cell biology Chapter 4*, Unit 4A.1 (2009).
43. Brambrink, T. *et al.* Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell stem cell* **2**, 151–9 (2008).
44. Cui, L. *et al.* Spatial distribution and initial changes of SSEA-1 and other cell adhesion-related molecules on mouse embryonic stem cells before and during differentiation. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **52**, 1447–57 (2004).
45. Goldstein, D. J., Rogers, C. E. & Harris, H. Expression of alkaline phosphatase loci in mammalian tissues. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 2857–60 (1980).
46. Pfannkuche, K., Fatima, A., Gupta, M. K., Dieterich, R. & Hescheler, J. Initial colony morphology-based selection for iPS cells derived from adult fibroblasts is substantially improved by temporary UTF1-based selection. *PLoS one* **5**, e9580 (2010).
47. Greder, L. V *et al.* Analysis of endogenous Oct4 activation during induced pluripotent stem cell reprogramming using an inducible Oct4 lineage label. *Stem cells (Dayton, Ohio)* **30**, 2596–601 (2012).
48. Babaie, Y. *et al.* Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. *Stem cells (Dayton, Ohio)* **25**, 500–10 (2007).
49. Jullien, J., Pasque, V., Halley-Stott, R. P., Miyamoto, K. & Gurdon, J. B. Mechanisms of nuclear reprogramming by eggs and oocytes: a deterministic process? *Nature reviews. Molecular cell biology* **12**, 453–9 (2011).

50. Cowan, C. a, Atienza, J., Melton, D. a & Eggan, K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science (New York, N.Y.)* **309**, 1369–73 (2005).
51. Li, F. *et al.* Activation of human embryonic gene expression in cytoplasmic hybrid embryos constructed between bovine oocytes and human fibroblasts. *Cloning and stem cells* **10**, 297–305 (2008).
52. Koziol, M. J., Garrett, N. & Gurdon, J. B. Tpt1 activates transcription of oct4 and nanog in transplanted somatic nuclei. *Current biology : CB* **17**, 801–7 (2007).
53. Silva, J. *et al.* Nanog is the gateway to the pluripotent ground state. *Cell* **138**, 722–37 (2009).
54. Silva, J. *et al.* Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS biology* **6**, e253 (2008).
55. Van Oosten, A. L., Costa, Y., Smith, A. & Silva, J. C. R. JAK/STAT3 signalling is sufficient and dominant over antagonistic cues for the establishment of naive pluripotency. *Nature communications* **3**, 817 (2012).
56. Fuellen, G. & Struckmann, S. Evolution of gene regulation of pluripotency--the case for wiki tracks at genome browsers. *Biology direct* **5**, 67 (2010).
57. Fernandez-Tresguerres, B. *et al.* Evolution of the mammalian embryonic pluripotency gene regulatory network. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 19955–60 (2010).
58. Tapia, N. *et al.* Reprogramming to pluripotency is an ancient trait of vertebrate Oct4 and Pou2 proteins. *Nature communications* **3**, 1279 (2012).
59. Han, J., Sachdev, P. S. & Sidhu, K. S. A combined epigenetic and non-genetic approach for reprogramming human somatic cells. *PloS one* **5**, e12297 (2010).
60. Pasque, V., Gillich, A., Garrett, N. & Gurdon, J. B. Histone variant macroH2A confers resistance to nuclear reprogramming. *The EMBO journal* **30**, 2373–87 (2011).
61. Dimitrov, S., Dasso, M. C. & Wolffe, a P. Remodeling sperm chromatin in *Xenopus laevis* egg extracts: the role of core histone phosphorylation and linker histone B4 in chromatin assembly. *The Journal of cell biology* **126**, 591–601 (1994).



62. Singhal, N. *et al.* Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. *Cell* **141**, 943–55 (2010).

## **Conclusion:**

The reprogramming of somatic cells toward a state of pluripotency has been achieved through somatic cell nuclear transfer (SCNT),<sup>1</sup> pluripotent-somatic cell fusion,<sup>2,3</sup> and the forced expression of transcription factors (i.e. Oct4, Sox2, Klf4, c-Myc)<sup>4,5</sup> Extract-based reprogramming is a method developed to study reprogramming mechanisms and factors contained within cellular sources.<sup>6,7</sup> To date, extract-based reprogramming has affirmed the reprogramming potential of pluripotent stem cells and embryonic carcinoma cells originating from mouse and human.<sup>7,8,9,10</sup> In addition the oocytes and eggs from a variety of species have the capacity to reprogram mammalian somatic cells.<sup>6,11,12,13</sup> Here, I have described the development of a standardized extract-based reprogramming system through focus on specific control points: recipient and donor selection (**Chapter 1**), cell permeabilization (**Chapter 2**), and large molecule penetration and retention (**Chapter 3**). Then, I utilized this system to study the reprogramming potential of a protein source for extract-based reprogramming (**Chapter 4**).

Extract-based reprogramming has been achieved through a variety of extract sources and source selection is pivotal for the establishment of a successful reprogramming assay (**Introduction, Table 0.1**). As a source, cultured cells mESC CJ7 and ECC NTERA-2 cl.D1 cells were tested for reprogramming potential (**Chapter 1, Figures 1.1, 1.2**). Although able to induce gene expression and morphological changes (**Chapter 1, Figures 1.3, 1.4, 1.5, 1.6**), they revealed the material and cost limitation of cultured pluripotent cells as extract donors for cell reprogramming experiments. As a recipient, 293T cells performed well experimentally. However, these cells were dropped

as recipient cells, as reports surfaced that , these cells can express pluripotent genes in response to culture conditions.<sup>14</sup>

Efficient, survivable cell permeabilization is paramount for the success of extract-based reprogramming.<sup>12,15</sup> Cell survival following treatment can be influenced by cell culture media pH and protease inhibition therefore each should be considered during cell permeabilization. (**Chapter 2, Figure 2.1**). Then, I identified appropriate concentrations of digitonin, which permeabilized cell membrane of 3T3 cells with little impact on cell survival (**Chapter 2, Figure 2.2**). I also tested alternate means of treatment (in-plate vs. suspension) (**Chapter 2, Figure 2.3**) and permeability assessment (trypan blue and propidium iodide) (**Chapter 2, Figure 2.4**) resulting in the use trypan blue for permeabilization assessments.

Extract-based reprogramming also requires experimental conditions for monitoring the uptake and retention of large molecular weight proteins and protein complexes.<sup>15,16</sup> I decided to monitor the uptake and retention of fluorescently-labeled large-molecular weight dextrans in BJ neonatal fibroblasts (a pilot line) and mouse 3T3 cells. Following experimental adjustments to treatment and visualization (**Chapter 3 Figure 3.1**), 10kDa FITC dextran was detected in digitonin treated BJ neonatal fibroblasts (**Chapter 3, Figure 3.2**), whereas a larger, 70kDa FITC-dextran was difficult to detect without fixation (**Chapter 3, Figure 3.3**). Therefore I elected to use Texas-red-labeled 70kDa dextran as a probe in our main experimental line 3T3. Between 20-30µg/mL digitonin facilitated retention of 70kDa Texas Red dextran (**Chapter 3, Figure 3.4**). To investigate retention of 70kDa Texas Red dextran I assessed fluorescence at various time points after treatment (**Chapter 3, Figure 3.5**). Significant amounts of

labeled large molecular weight dextran were retained for at least 48 hours suggesting that extract protein can be retained by somatic cells long after treatment.

The limitations of cell cultured pluripotent lines (**Chapter.1**), led to our search for a plentiful extract source for reprogramming experiments. As a source, salmon roe can be processed to select for optimal, late stage oocytes (**Chapter 4, Figure 4.1**).<sup>17,18</sup> We then devised a purification scheme for the homogenization of salmon roe oocytes, which did not cause protein precipitation and was suitable for later application in permeabilized, cultured cells (**Chapter 4, Figure 4.1, Table 3**). At high concentrations, salmon roe extract (SRE) was toxic to treated cells (**Chapter 4, Figure 2**); therefore various extracts, dilutions and treatment conditions were tested (**Chapter 4, Figures 4.3, 4.4**). Two extracts revealed reprogramming potential: SRE1 and SRE4 extracts both elicited colony formation in 3T3 cells (**Chapter 4, Figures 4.3 4.4**). While colonies formed in 3T3 cells treated with SRE1 revealed limited expression of the mESC marker SSEA1 (**Chapter 4 Figures 4.3**), 3T3-SRE4 colonies displayed obvious alkaline phosphatase activity in colonies, a pluripotent cell marker (**Chapter 4 Figure 4.4**). The formation of colonies, expression of SSEA1, Oct4, and alkaline phosphatase are consistent with the morphological and marker repertoire of partially reprogrammed or pre-iPSCs.<sup>19,20</sup> These experiments demonstrate the conserved reprogramming potential of oocyte sources.

## References:

1. Gurdon, J. B. & Byrne, J. a. The first half-century of nuclear transplantation. *Bioscience reports* **24**, 545–57 (2003).
2. Kimura, H., Tada, M., Nakatsuji, N. & Tada, T. Histone Code Modifications on Pluripotential Nuclei of Reprogrammed Somatic Cells Histone Code Modifications on Pluripotential Nuclei of Reprogrammed Somatic Cells. (2004). doi:10.1128/MCB.24.13.5710
3. Tada, T. Toti-/pluripotential stem cells and epigenetic modifications. *Neurodegenerative diseases* **3**, 32–7 (2006).
4. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–76 (2006).
5. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–72 (2007).
6. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Current biology : CB* **14**, 1475–80 (2004).
7. Håkelién, A.-M. *et al.* Long-term in vitro, cell-type-specific genome-wide reprogramming of gene expression. *Experimental cell research* **309**, 32–47 (2005).
8. Cho, H.-J. *et al.* Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* **116**, 386–95 (2010).
9. Han, J., Sachdev, P. S. & Sidhu, K. S. A combined epigenetic and non-genetic approach for reprogramming human somatic cells. *PloS one* **5**, e12297 (2010).
10. Singhal, N. *et al.* Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. *Cell* **141**, 943–55 (2010).
11. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by *Xenopus laevis* egg extracts. *Molecular reproduction and development* **74**, 1268–77 (2007).
12. Miyamoto, K. *et al.* Reversible membrane permeabilization of mammalian cells treated with digitonin and its use for inducing nuclear reprogramming by *Xenopus* egg extracts. *Cloning and stem cells* **10**, 535–42 (2008).

13. Miyamoto, K. *et al.* Cell-free extracts from mammalian oocytes partially induce nuclear reprogramming in somatic cells. *Biology of reproduction* **80**, 935–43 (2009).
14. Debeb, B. G. *et al.* Characterizing cancer cells with cancer stem cell-like features in 293T human embryonic kidney cells. *Molecular cancer* **9**, 180 (2010).
15. Collas, P. Reprogramming Somatic Cells in Extract of Undifferentiated Carcinoma Cells. 1–11
16. Agarwal, S. Cellular reprogramming. *Methods in enzymology* **420**, 265–83 (2006).
17. Ziv, T. *et al.* Comparative proteomics of the developing fish (zebrafish and gilthead seabream) oocytes. *Comparative biochemistry and physiology. Part D, Genomics & proteomics* **3**, 12–35 (2008).
18. Bally-Cuif, L., Schatz, W. J. & Ho, R. K. Characterization of the zebrafish Orb/CPEB-related RNA binding protein and localization of maternal components in the zebrafish oocyte. *Mechanisms of development* **77**, 31–47 (1998).
19. Brambrink, T. *et al.* Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell stem cell* **2**, 151–9 (2008).
20. Silva, J. *et al.* Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS biology* **6**, e253 (2008).