2015-01-12

Transdifferentiation of Fibroblast into Chondrocytes

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Transdifferentiation of Fibroblasts into Chondrocytes

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

INTERDISCIPLINARY DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOMEDICAL ENGINEERING

CALGARY, ALBERTA

DECEMBER 2014

Abstract

Great strides have been made in generating autologous induced pluripotent stem cells (iPSCs) via the process of cellular reprogramming. A key step in cellular reprogramming is the opening of chromatin. In newer transdifferentiation schemes, instead of reverting cells all the way to the embryonic state, cells can instead be redirected to specific cell types. One approach recently applied to the generation of cardiac and neural tissue involves partially reprogramming fibroblasts by blocking the JAK-STAT pathway followed by the introduction of specific differentiation factors. We have used a similar approach to transdifferentiate mouse embryo fibroblasts into chondrocytes. Following one day of exposure to the transcription factors Oct4, Sox2, Klf4 and c-Myc, (OSKM factors) and 10 days of exposure to a Jak inhibitor, resulting cells are dissociated and used to form micromass cultures for chondrogenic differentiation in the presence of TGF-b1 , and BMP-2. Cells formed aggregates, which differentiated into chondrocytes within 15 days, eventually formed hyaline and hypertrophic cartilage tissue based upon Alcian Blue, safranin-o and hematoxylin and eosin (H&E) staining. RT-PCR was used to establish a timeline of chondrocyte/cartilage gene expression. We also observed the expression of Col10 and Mmp13, which suggested the development of bone. Jak inhibitor made in all cases transdifferentiation into cartilage more efficient by avoiding bone formation.

Acknowledgements

I would like to thank my supervisor Dr. Derrick Rancourt, for his guidance and scientific contribution, and more importantly as one of the best mentors I ever had. I could never thank him enough for his help and encouragement in my career development. His great vision and positive attitude contributed enormously in finishing up this project. I would also like to thank the other members of my advisory committee; Dr. Roman Krawetz and Dr. Mark Ungrin, for their very appreciated advice and help over the course of my masters, especially at the end of this one.

I would like to give special thanks to Charlie Yu Ming, post-doctorate member of the Rancourt lab. Since the very first day I met Charlie, he showed a very positive and encouraging attitude. He always demonstrated interest, was invested in looking at my progress and constantly tried to keep me focus on what I needed to do. There are no words to describe how grateful am I for his help; he made a great if not the greatest impact in my personal growth.

Also I like to thank all of the Dr. Rancourt's lab members, past, present and future especially a Shiying Liu, Dr. Guoliang Meng, Eileen Rattner, Brad Day, Poh Lee, Teresa Scheidl-Yee, Mehdi Shafa, Ben Zhang, Yulia Shulga and Noor Alsaad, whom made a significant impact on my masters degree.

Finally, I would like to give special thanks to my parents Arturo Cota and Perla Elizondo whose support and encouragement are never ending. As well to all my friends especially to my best friend Linda Tamez, that ever since we started our undergraduate education together, she has always been around supporting me in every single way. I will be waiting for you when I decide to do a PhD. Thanks for moving to Calgary!

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List of Symbols, Abbreviations and Nomenclature

BSA	Bovine serum albumin
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
OSKM	Oct4, Sox2, Klf4 and c-Myc
LIF	Leukemia inhibitory factor
MEFs	Mouse embryonic fibroblast
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
qRT-PCR	Quantitative real time-polymerase chain reaction
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
Dnmt1	DNA methyltransferase 1

Units of Measurement

bp	Base pair
D	day
g	gram
hr	hour
M	moles/L
mg	milligram
Min	Minute
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometer
rpm	Revolutions per minute
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar

Epigraph

Nothing in life is to be feared, it is only to be understood.

Marie Curie

CHAPTER 1

Overview

The combination of four pluripotency transcription factors, known as Yamanaka factors, reprogram dermal cells into a pluripotent cell that resembles an embryonic stem cell (ESC) named induced pluripotent stem cell (iPSC) (Takahashi et al., 2006). This approach could avoid the ethical concern from ESCs for unlimited chondrocyte production for cartilage repair. However cellular reprogramming followed by chondrocyte differentiation, is an inefficient procedure for obtaining chondrocytes for clinical application.

An alternative to using iPSCs is the direct reprogramming of terminally differentiated cells into chondrocytes via a process called transdifferentiation. Previous transdifferentiation attempts have used different cocktails of lineage specific transcription factors to obtain chondrocytes. In order to use transdifferentiation in the clinic, we need to understand the molecular and epigenetic events that occur during the process. A promising transdifferentiation approach that fulfills those needs where the molecular and epigenetic events are well understood is one that uses the Yamanaka (OSKM) factors. The reprogramming process to iPSCs has been thoroughly studied since its discovery (2006). Today the events are well known.

This Thesis presents the transdifferentiation of mouse embryo fibroblasts (MEFs) into chondrocytes by partially reprogramming cells with the OSKM factors in the absence of LIF and the presence of JAK inhibitor followed by cytokine-induced differentiation. It

was observed that the formation of mature chondrocytes did not originate from intermediate iPSCs and that the process occurred in half the time compared to the iPSCs alternative. We suggest that application of a JAK inhibitor makes cartilage transdifferentiation more efficient because it blocks the cells from going to an iPSCs state, thus there was not apparent tumour formation. This protocol also avoids bone formation.

In order to provide enough background information for this work, the introduction will discuss four topics: 1) osteoarthritis, 2) chondrogenesis and cartilage tissue, 3) regenerative medicine, and 4) transdifferentiation.

Osteoarthritis

Osteoarthritis (OA) is the leading cause of disability in Canada, affecting one in ten adults per year during their lifetime. It is the most common form of arthritis, with symptoms of joint pain having different degrees of functional limitation. OA is defined as a common complex disorder with multiple risk factors. These risk factors are divided in two general categories that lead to OA. One is the local environment pathway include obesity, altered joint loading, abnormal anatomy and bone remodeling trauma. The other is with systemic factors, including: inflammation, immune responses, aging, sepsis, and genetic factors. Both pathways give rise to joint matrix destruction, an aberrant repair response and mechanical failure.

In general, the prevalence of OA increases with age (Osteoarthritis, national clinical guideline for care and management in adults, 2008). It has a considerable impact on health services and is expected to become the fourth leading cause of disability by the year of 2020, due to increasing life expectancy and aging demographics (Woolf and Pfleger 2003).

OA results from the degeneration of articular cartilage (hyaline cartilage), which leads to the formation of fibrocartilage. Fibrocartilage has different biomechanical properties compared to the hyaline cartilage (present in healthy joints) and does not provide protection to subchondral bone following degeneration (Ochi M. et al., 2001). There are some therapies using chondrocytes obtained from existing cartilage tissue (Detterline A.J. et al., 2008). The most common one is microfracture chondroplasty, which uses penetration of the subchondral bone to deliver non-chondrocyte endogenous progenitor cells from the bone marrow into the defected area. Another is autogenous osteochondral transplantation/mosaicplasty, which transfers autologous osteochondral grafts to the

affected area. Lastly, there is autologous chondrocyte implantation performed, after isolating chondrocytes from a piece of cartilage of a small non-load bearing area of the knee joint. These cells are then expanded *in vitro* and finally transplanted into the chondral defect (Myers K.R. et al., 2013).

Since there are few treatments for OA, the main options to alleviate the symptoms are physical therapy, weight loss, non-steroidal anti-inflammatory drugs (NSAIDs), injections of hyaluronic acid (HA) and finally total joint replacement at end-stage OA (Myers K.R. et al., 2013).

Chondrogenesis and Cartilage Tissue

Cartilage differentiation processes that lead to skeletal formation are chondrogenesis and endochondral ossification. These procedures form cartilage structures as a scaffold for bone formation and for other organs that need elasticity, support and shock absorption. During development, there is not a very definitive line to separate both processes. The only difference is that for bone formation chondrocytes proliferate with later apoptosis of these cells (Zuscik et al., 2008), while for articular cartilage chondrocytes are quiescent (do not divide as often (Pacifici et al., 2005).

Vertebrate skeleton formation starts with the differentiation of mesenchymal cells (Maria Barna and Lee Niswander 2007). In the first step prechondrogenic mesenchymal cells merge together, forming a cartilage anlagen (Figure 1a). At the molecular level, bone morphogenic proteins (BMPs) are present for the condensation of these mesenchymal cells

together with the expression of gene SRY-box 9 (*Sox9*), which is the primary transcription factor determinant in chondrogenesis (Akiyama, H. et al., 2002). Also some ECM markers are expressed at this stage, such as: IIa splice form of type II collagen (Col2a1), N-cadherin (Ncad), N-cam and tenascin C (Tnc) (Zuscik et al., 2008 and Goldring, 2012). At the same stage but transitioning into chondrocytes, cells start producing ECM rich in aggrecan (Agc) and IIb splice form of type II collagen Col2a1(IIb) (Zuscik et al., 2008 and Goldring, 2012).

This chondrocyte differentiation forms the template or anlagen for bone development. Groups of chondrocytes start the process of hypertrophic differentiation where they enlarge, mineralize and finally die by apoptosis. The residual cartilage matrix left by the hypertrophic chondrocytes, serves as a scaffold for mineral deposition and bone formation by osteoclasts and osteoblasts (Gerber H.P et al., 1999) and (Maria Barna and Lee Niswander 2007). During bone formation there are 1) hypertrophic chondrocytes (on cartilage at the end of the developing bone) and 2) more terminally differentiated hypertrophic chondrocytes (formed in the center of the developing cartilage)(figure 1aii). Both proliferate and undergo steps of maturation while changing to a flatter morphology. These flat columnar chondrocytes are the most proliferative and express high levels of Fgfr3, Nkx3.2 and Ptc1 (Zuscik et al., 2008). After these events, these cells undergo hypertrophy and withdraw from the cell cycle. They then enlarge and form a mineralized matrix that enhances the expression of type X collagen (Col10a1) and runt related transcription factor 2 (Runx2), involved in osteoblast differentiation (Zuscik et al., 2008). Finally only the most terminally differentiated hypertrophic chondrocytes express the

matrix-degrading enzyme (Mmp13). This enzyme degrades the cartilage matrix in order to complete bone formation (Figure1a) (Zuscik et al., 2008).

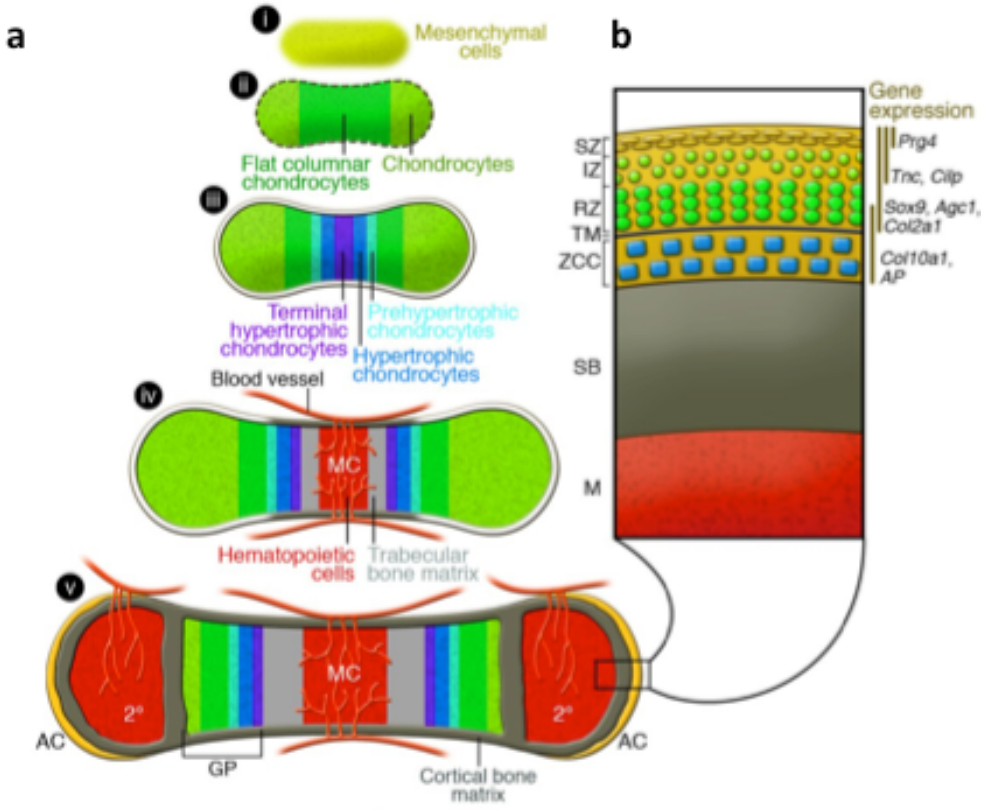

Figure 1. Schematics of cellular events during chondrogenesis and articular cartilage development. This is the cartilage differentiation process for skeletal formation in vertebrates. a) Representation of the development of long bone starting with mesenchymal cell condensation and later chondrocytes take different pathways b) articular cartilage formation at the joint (Zuscik et al., 2008).

Cartilage Tissue

Cartilage is an organized flexible connective tissue that contains a substance that forms an ECM. This is formed by sulfated glycosaminoglycan (GAGs), which is attached to proteins as part of proteoglycans. ECM provides resistance and compression (Barna M. et al., 2007). ECM is also formed by the sparse collective chondrocytes within the tissue. Cartilage is an avascular tissue where chondrocytes are not able to divide considerably, and by consequence are unable to repair cartilage upon degeneration.

There are three different types of cartilage tissue in vertebrates: elastic, fibrocartilage and hyaline (Figure 2) (Saladin K.S. 1998). Elastic cartilage is found in the pharyngotympanic tubes, epiglottis and ear lobes, since these are the areas that require elastic properties (Figure 2a). Fibrocartilage is found in the pubic symphysis and intervertebral disks. This type of cartilage functions as a shock absorbing material (Figure 2b). Finally, hyaline cartilage, which is most abundant, is found in the nose, ears, trachea and bronchial tubes as supportive tissue. Additionally is found in the surface of synovial joints and in the growth plate of long bones where it functions to provide mobility (Figure 2a) (Zuscik et al., 2008) and (Goldring 2012).

In the mouse, development of articular cartilage begins during embryogenesis where articular chondrocytes differentiate from cells of the interzone in the joint. This cellularity is maintained in the adult skeleton in four different zones: the superficial zone (SZ) with 1-2 layers of flattened chondrocytes having the characteristic to express proteoglycan 4 (Prg4), Sox9, Col2a1 (IIb), aggrecan, Tnc and low levels of cartilage intermediate layer protein. In the intermediate zone (IZ), the chondrocyte's morphology is

more round: at the molecular level they do not express Prg4. Next, the radial zone (RZ) is characterized by the expression of Col10a1 and Alkaline phosphatase (AP), and finally the calcified cartilage zone. Importantly, chondrocyte maturation and endochondral bone formation is not restricted to just the development of the skeleton. Endochondral ossification is a natural part of bone healing following fracture. This process also occurs upon cartilage degeneration producing changes in the ECM structure. This in turn results in pain in joints and is what is known as OA.

The material involved has been removed because of copyright restrictions;
This image contained the different types of cartilage stained with H&E. The information can be found here:
http://www.mhhe.com/biosci/ap/histology_mh/hcart.jpg,
http://www.mhhe.com/biosci/ap/histology_mh/elcart.jpg
http://www.mhhe.com/biosci/ap/histology_mh/fibroc1.jpg

Figure 2. Examples of the structure of the three types of cartilage tissue in the body stained with H&E. Cartilage is a flexible shock absorbing connective tissue. There are 3 type of cartilage in the body: a) hyaline cartilage, very uniform tissue with dispersed chondrocytes in lacunae, b) Elastic cartilage is characteristic to have dark-staining elastic fibers, and c) Fibrocartilage has a more spongy structure, less uniform structure compared to hyaline cartilage (Rubbelke D. L. 1999).

Regenerative Medicine

Cell and tissue regeneration

The field of health sciences that aims to repair, replace or regenerate damaged tissues organs is called regenerative medicine. It developed from activities such as surgery, surgical implants and biomaterial scaffolds, bone marrow and organ transplantations (Chris Mason and Peter Dunnil, 2008). Regenerative medicine focuses on the study of cells because these are the primary building blocks for the formation of tissues and organs (Yalcinkaya 2013). The different type of cells used for tissue regeneration are: adult somatic cells, adult stem cells, embryonic stem cells (ESCs) and most recently pluripotent stem cells (iPSCs) (Chris Mason and Peter Dunnil, 2008).

The use of adult primary, tissue-specific cells has been seen as a very powerful and promising tool for tissue regeneration (Yalcinkaya 2013). A good example was shown in a study where hepatocytes were successfully manipulated for liver repair. Rat hepatocytes were seeded with polyglycolic acid (PGA) and injected in a syngeneic rat. A significant loss of cells post-transplant was observed at first because of a lack of cell stimulation, oxygen and nutrients. Later researchers obtained a more positive result (25 to 50% survival with a 6- to 7-fold increase of hepatocyte engraftment) by changing to a biomaterial that allowed vascularization. (Uyama S. et al., 1993). Nevertheless, there are some limitations for clinical application of adult somatic cells: 1) the source of cells is limited and most of

the time the process to obtain them is invasive for the patient, 2) many are difficult to expand due low density or to senescence.

Stem cells have been studied extensively for regenerative medicine because of their capacities. They are clonal entities that are undifferentiated, can self-renew and generate differentiated cells (Weissman et al., 2001; Smith, 2001). Thus, stem cells have been considered as a better alternative for cell therapy in tissue regeneration. Stem cells are present in embryos and adults and have distinctive differentiation developmental potential since they come from distinct developmental stages (Zhang Y. et al., 2012). According to their potency, stem cells are divided into totipotent, pluripotent and multipotent categories.

Multipotent stem cells are the adult stem cells that are recruited from specific niches within an organ (Figure 3). (Zhang Y., et al., 2012). These can only differentiate into cells that come from the same cell lineages (Figure 3). Their role is to maintain tissue homeostasis (Zhang Y., et al., 2012). These cells have been thought to be easier to use for clinical application due to the fact that their differentiation is restricted to specific lineages, making the possibility for tumor formation more limited. Bone marrow derived stem cells are often used for regeneration procedures. For example Shao-Liang Chen and colleagues (2004) studied the effect of autologous bone marrow stem cells (BMSCs) when they were given to patients who underwent primary percutaneous coronary intervention. Sixty-nine selected patients that had acute myocardial infarction received intracoronary injections of BMSCs (8-10 x 10^9 cells per patient) and were compared to those who only received a saline solution. Patients that had received BMSCs still had viable cells after 3 months of transplantation, and showed increased cardiac functional indexes (Chen S.L. et al., 2004).

Despite the positive results using MSCs, there are some drawbacks that limit their potential for clinical applications. They occur at a very low frequency and their growth depends on the density of cells cultured. Cell expansion takes more than two weeks and if the expansion takes longer they risk undergoing senescence and apoptosis (Chen S.L. et al., 2004). In addition to that, when cells undergo multiple passages, they can exhibit epigenetic changes, and give rise to different cell types.

Given the limitations of multipotent stem cells, alternative sources studied is naturally occurring pluripotent stem cells. These cells have the capacity to differentiate into all cell types of the embryo (endoderm, mesoderm and endoderm), but are not able to differentiate into extraembryonic tissues (Figure 3). There are two different types of pluripotent stem cells (PSCs). Embryonic stem cells, derived from the inner cell mass (ICM) of the preimplantation embryo, (Figure 3) (Evans and Kaufman, 1981, Thomson et al., 1998) and epiblast stem cells (EpiSCs) derived from the epiblast layer of the implanted embryo (Brons et al., 2007). These EpiSCs are closer to what it is known as a human ESC. Human ESCs and mouse EpiSCs share the same culture conditions, intolerance to single cell passaging and have a characteristic flattened colony morphology (Brons I.G. et al. 2009).

ESCs have a great potential for differentiation and are easy to expand and maintain, and hence are very attractive for tissue regeneration. Moreover, they are relatively easy to obtain than some types of multipotent stem cells. In a spinal cord injury study using rats, McDonald and collaborators (1999) demonstrated that mouse ESC-derived neural precursor cells could survive for 5 weeks and migrated 8 mm from the transplantation site. They differentiated into astrocytes, oligodendrocytes and neurons without any tumor

formation. These cells not only migrated, but enhanced the movement capacity of the rats as shown in an open field locomotion test (McDonald J. W. et al., 1999). In another study, retinal pigmented epithelium (RPE) cells were generated from ESCs. In clinical trials, RPE were transplanted in the eye of two patients with macular degeneration. The resulting RPE proliferated normally, did not form tumors, and did not present immune rejection (Schwartz S. D. et al., 2012).

However, there are still some technical challenges with using human ESCs in regenerative medicine. First, there is great concern with the immune rejection of transplanted cells. Even though there could be the application of immunosuppressive drugs to reduce the immune response, this cannot completely assure that the patient will be free of any infection. Second, the current process for deriving human ESCs requires the destruction of human embryos, which has raised significant ethical and legal concerns. Finally because ESC have the ability to form teratomas (tumors comprised of all three germ lineages), tumor formation following after transplantation currently limits their clinical application (Amariglio N., et al. 2009).

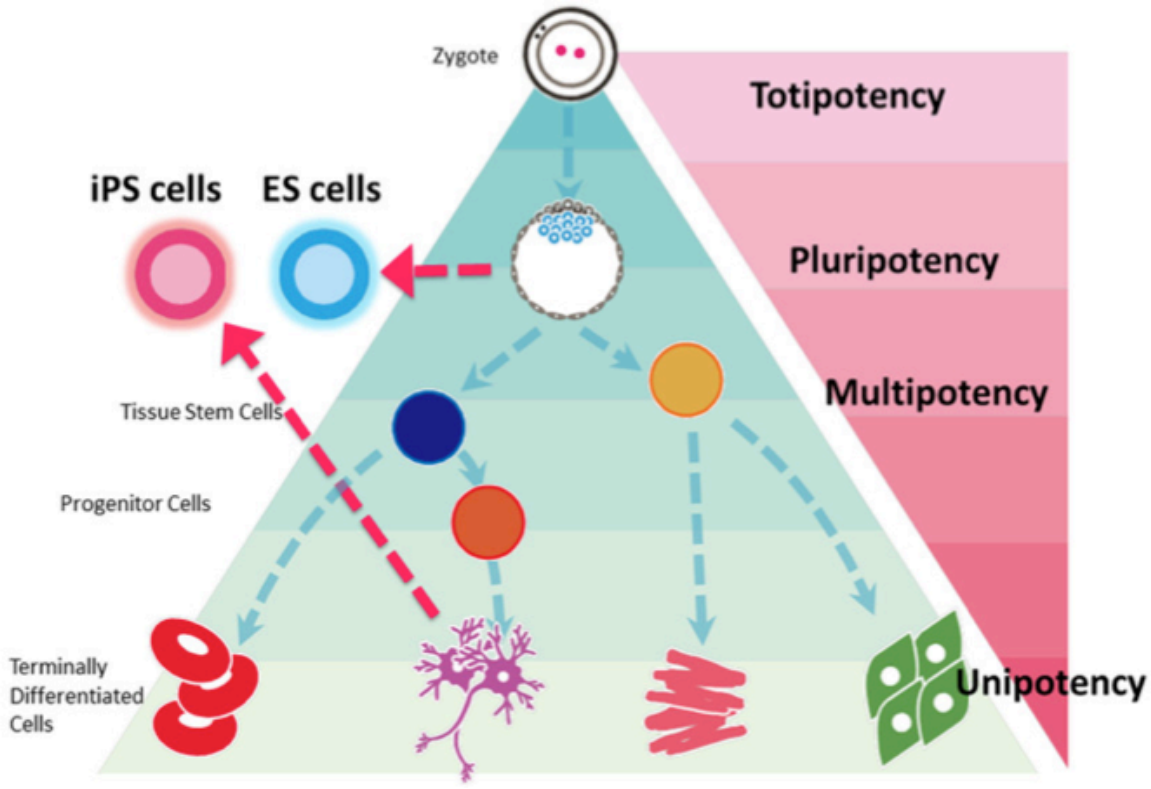

Figure 3. Schematics of stem cell potency hierarchy. Stem cells are classified depending on their differentiation potential. Totipotent cells have the capacity to differentiate into any type of cell and even generate an entire organism. Pluripotent stem cells (ESCs, iPSCs and EpiESCs) are able to differentiate into all cell types but not to extraembryonic tissue, finally multipotent cells are localized in a niche in each organ and are only able to differentiate into the same lineage they came from.

Taken from: http://hronrad.wordpress.com/2013/12/05/stem-cells/.

Induced pluripotent stem cells

Takahashi and Yamanaka (2006) first hypothesized that the transcription factors which play a role in maintaining ESC pluripotency could potentially turn somatic cells back into a pluripotent state. Starting with 24 candidate genes known to be involved in pluripotency and in a herculean combinatorial effort they reduced this original 24 factors down to four factors: Oct3/4, Klf4, Sox2, and c-Myc were able to reprogram mouse fibroblasts into an ESC-like state. Yamanaka and colleagues (2007) also reported the same results one year later for human cells when using this same combination of factors. Having pluripotent characteristics, these iPSCs are capable of giving rise to more than 200 cell types (Takahashi and Yamanaka, 2006). At the same, time another group in the United States, demonstrated the reprogramming of human fibroblasts to iPSCs using a different combination of factors: Oct4, Sox2, Nanog and Lin28 (Yu J., et al., 2007).

After the four-factor derivation of the iPSCs, much interest was focused on the process of somatic cell reprogramming. First studying the contribution that each of the core four transcription factors had during the reprogramming. Although eight years ago reprogramming was not completely understood, Scheper and Copray (2009) were the first to propose that reprogramming happens in two broad stages. In the first stage, Oct4 and Sox2 are working to repress genes associated with the host cell lineage and in consequence resetting the epigenome of the cell towards a permissive chromatin mode putting the cell in a plastic state. The second stage allowed the reprogramming factors to reactivate the endogenous autoregulatory loop that triggers the pluripotency transcriptional network

driven by the LIF-Jak-Stat pathway (Scheper W. and Copray S., 2009) (Figure 4) and (Figure 7).

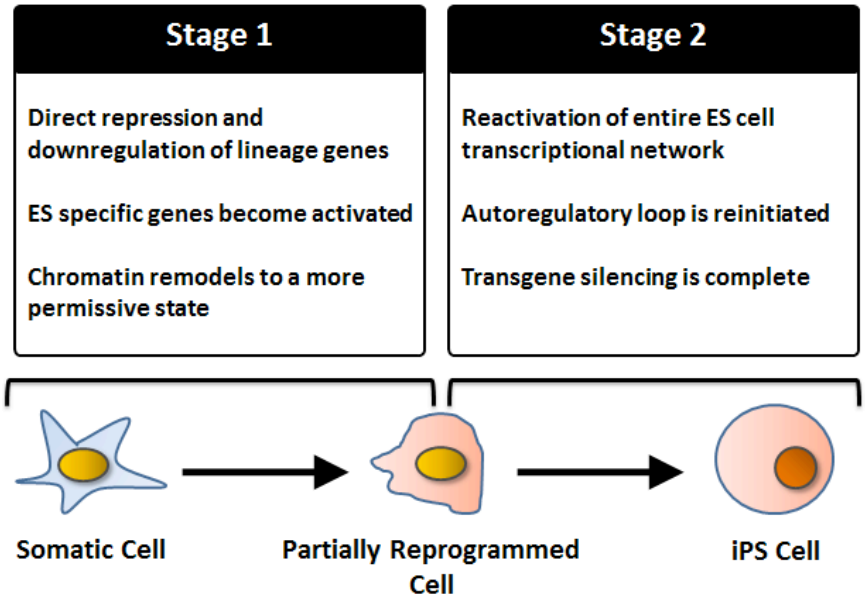

Figure 4. Two stage process for reprogramming of somatic cells. In the first stage, there is a downregulation of lineage specific genes and upregulation of pluripotency genes by Oct4 and Sox2 exogenous factors. Chromatin remodelling by the removal of repressive marks from the key pluripotency genes, and then the participation of c-Myc and Klf4 to proceed to the second stage, in which an autoregulatory loop maintains the pluripotency transcriptional network, independent of the transgene expression.

Many researchers started to ask questions about how these factors were interacting in order to modify existing epigenetic marks and return cells to a pluripotent state. In efforts to unveil that, reprogramming variations were established by substituting some of the factors such as Klf4 or c-Myc with other transcription factors (Nanog and Lin28 respectively) or with small molecules (valproic acid or Wnt ligand). These results, demonstrated that there are different pathways involved in this process of reprogramming, with epigenetic enzymes being activated in every case (Hochedlinger K. and Plath K., 2009).

Waddington defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (Waddington C., 1957). Later, he proposed a model based on potential energy surface (Waddington C., 1957). In this model, he suggested that cells committed to a specific lineage couldn't be recommitted to another lineage or change channels. However, with the recent milestone of iPSC generation, Yamanaka used the same analogy of channels from Waddington, suggesting that cells could be pushed back up the channel towards the pluripotent state (Figure 5). In addition to that, he suggested that during reprogramming cells could experience other events. They can be stopped by some epigenetic bumps, which define the limits of the supposed pathways, symbolizing regulation, and thus remain incompletely reprogrammed. In this situation cells return to their specific lineage or transition to another lineage. Another option is that instead of moving they can undergo apoptosis or cellular senescence. This model proposed by Yamanaka (2009) has been described as the stochastic model of iPSC generation (Figure 5) (Yamanaka S., 2009).

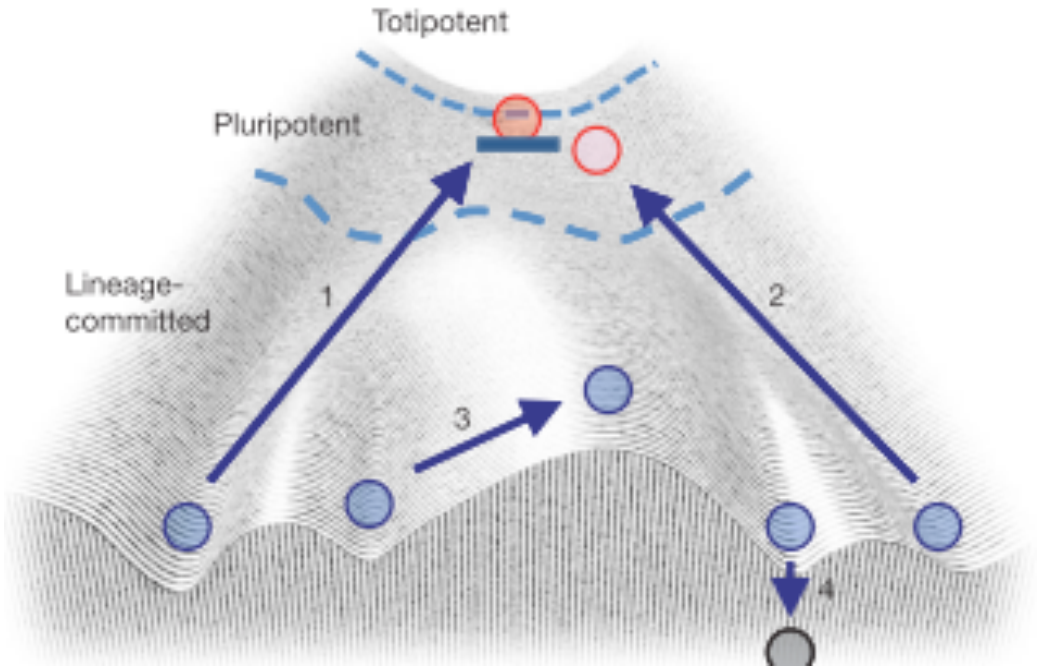

Figure 5. Stochastic model proposed by Yamanaka based on the Waddington model. In the stochastic model, it is proposed that cells are pushed up the slope in the valleys by reprogramming factors. Cells can undertake different paths when being reprogrammed. They could 1) overcome every epigenetic roadblock and reach pluripotency; 2) cells can reach an intermediate state in pluripotency, 3) cell can overcome some epigenetic blocks and proceed to a multipotent cell state committed to certain lineages; and finally 4) some cells can die in the process (Yamanaka, 2009).

Since the development of iPSCs, most researchers have focused their attention on the epigenetics changes that iPSCs acquire together with the chromatin dynamics during the reprogramming. The most used protocol for the production of iPSCs is the one that

involves the application of the four transcription factors previously described by Yamanaka (2006) (Takahashi K. and Yamanaka S., 2006). Thus initial studies have concentrated on how these four factors worked together to initiate the reprogramming cascade.

The state of ESC chromatin is known as "open", where the heterochromatin is disperse and dynamic, which at the same time reflects a hyperactive transcriptional state (Efroni S., et al., 2008). The molecular state for ESCs to maintain pluripotency requires an specific network of transcription factors with epigenetic proteins that are also interacting with the DNA. Due to the fact that iPSCs are like ESC, they have to sustain the same molecular structure. In addition, they have to overcome an epigenetic barrier (i.e. closed chromatin) during the reprogramming process. Hence, the reprogramming process involves a chain reaction of transcription factors interacting with chromatin modifier enzymes and other histone related enzymes.

It has been suggested that reprogramming starts with the exogenous transcription factors making epigenetic changes. These transcription factors either interact with the naked DNA, via histone modifiers or together with chromatin remodeling factors (Takahashi et al., 2007). Thus, there are identified time points where the OSKM factors start the reprogramming process. Certain transcription factors are able to interact with DNA or with a chromatin remodeling enzyme depending on what gene is activated. In brief, this molecular mechanism is determined by the locus, the type of transcription factor and the context (Takahashi et al., 2007).

Oct4 has been found to be indispensable for reprogramming. In some cases, the presence of Oct4 is sufficient for reprogramming as shown in neural stem cell reprogramming (Kim J.B. et al., 2009). Moreover, Oct4 also has a great effect in

reprogramming when combined with Sox2. Oct4 and Sox2 form a heterodimer that interacts with some promoters. In addition this heterodimer has been shown to interact with Nanog. Nanog is another transcription factor that is not part of the reprogramming cocktail presented by Yamanaka and colleagues (2007). However, it participates in the ESC regulatory circuitry together with Oct4 and Sox2 to maintain pluripotency (Boyer L.A. et al., 2005). When Oct4 and Sox2 interact with promoters in DNA, Nanog is also involved in the interaction (Boyer L.A. et al., 2005). Finally, it has been observed that Oct4 and Sox2 can activate transcription in a chromatin independent manner through interaction with other co activators (Levasseur D. N. et al., 2008 and Fong Y.W. et al., 2011). In this context it has been shown that in mouse ESCs, Oct4 and Nanog together interact with repression complexes. These complexes are often histone deacetylases such as Mta1. (Liang J. et al., 2008).

c-Myc is an important participant in recruiting multiple chromatin modifiers, such as histone acetyltransferases (GCN5, p300) and histone deacetylases (HDACs). An example is how c-Myc allows the augmentation of methylation in H3K4me3 site and the global acetylation (Lin C. H., et al., 2009). In the reprogramming process c-Myc, activates its target before other core pluripotency transcription factors are activated, facilitating the opening of the chromatin for other factors (Stadtfeld M., and Hochedlinger K., 2010 and Kim J., et al., 2010). An example of c-Myc's potential in opening chromatin is its association with Tip60-p400 a complex which acetylates and remodels nucleosomes, respectively. p400 is a member of the Swi2/Snt2 family which is well known among the ATPase chromatin remodelling enzymes, exchanging histones H2AZ-H2B within nucleosomes (Kim J., et al., 2010). It also functions to release RNA polymerase from a

paused state from only about one-third of the genes that are being actively transcribed. This activity might be enhancing the reprogramming of the cells (Rahl P. B., et al., 2010). At the same time, the transcription factor Klf4, activates the transcription of Sox2, which participates in the pluripotency cascade (Niwa H. et al., 2009).

The derivation of iPSCs using exogenous transcription factors, motivated researchers to pursue the same idea of reprogramming cells towards other lineages in a process called transdifferentiation. Transdifferentiation is the reprogramming of a somatic cell into another somatic cell. In the modified model of Waddington proposed by Yamanaka, that means that cells can be pushed up the slope through the valleys and redirected to another channel. The interest of this approach was to generate cells that could be use for tissue regeneration in a faster and more efficient manner.

Transdifferentiation

The proper definition for transdifferentiation is the conversion of a fully differentiated cell state directly into another different differentiated cell state without going to pluripotency and re-differentiating. Transdifferentiation is also referred to as lineage reprogramming or direct conversion (Figure 5) (Ma T., et al., 2013).

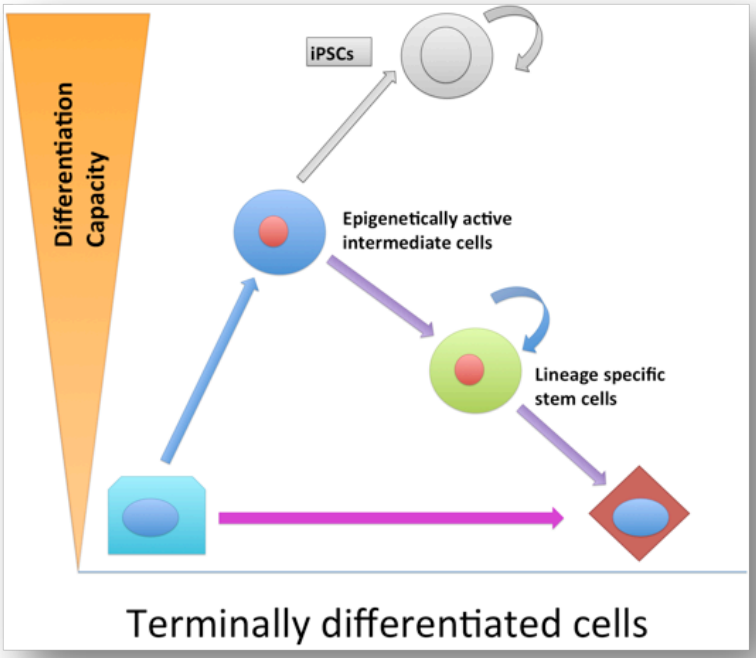

Figure 6. Schematics of the transdifferentiation strategy based on the OSKM factors. In this model, somatic cells (fibroblasts) are transduced with the OSKM factors to induce cellular plasticity, then exposed to differentiation conditions that promote lineage commitment into other cell types. Transdifferentiation of differentiated cells into another differentiated cell is faster than reprogramming cells to iPSCs and then differentiate them into a desired cells. Also a more efficient process. This one involves going directly to the desired cells without going to a full pluripotency state.

The accomplishment of transdifferentiation is demonstrated by fulfilling three criteria: 1) it should start with a defined cell type, 2) it requires validation and functional characterization of a resulting cell type, and 3) it should not pass through a pluripotency

intermediate. If pluripotency happened transiently, a by looking at a genetic mark is one way this could be demonstrated. For example the pluripotent state can be assessed by looking at the expression of pluripotency markers during the process. Also it could be observed by a marker gene (i.e. GFP) driven by one of the core pluripotency gene promoters such as *Nanog or Oct4*. If cells are marked, then that means that they are proceeding to pluripotency.

Transdifferentiation has given another alternative to the area of regenerative medicine. Using transdifferentiated cells rather than iPSCs has some advantages. The cells needed for tissue regeneration can be obtained faster by avoiding the iPSC state. Moreover by circumventing the iPSC state, the risk of teratoma formation is also mitigated (Guo J., et al., 2013).

Review of transdifferentiation/direct conversion experiments

After the discovery that somatic cells could be reprogrammed into iPSCs just by using four transcription factors, researchers thought that the same effect could be achieved by exposing a somatic cell to factors that will change them into another type of somatic cell. Research on exposing cells to defined factors started decades before iPSCs were developed. Taylor and Jones (1979) observed that by exposing a mouse fibroblast cell line (10T ½) to the demethylating agent 5-azacytidine, cells would generate clones with a skeletal muscle phenotype (Taylor and Jones, 1979). Later, they extracted genomic DNA from the formed skeletal muscle cells with the demethylating agent 5-azacytidine, and transfected it to untreated fibroblast (10T ½). They identified clones with the same skeletal muscle phenotype. With these experiments, the myogenic determination gene (*MyoD*) was

found to be responsible for the change of fate of fibroblastic cells (Lassar et al., 1986). Later Weintraub and collaborators (1989) demonstrated that if MyoD was expressed in nerve, fat and liver cells, it would convert them into skeletal muscle cells. Therefore, the effect of MyoD was the first evidence where a single gene was shown to act as a master switch, carrying on a program of differentiation (Tapscott S. J., 2005).

After the discovery of MyoD, there was a great interest in investigating how myogenic transcription factors interacted with MyoD. This was carried in order to elucidate the molecular process on differentiation (Tapscott S. J., 2005). MyoD's discovery indicated that the presence or overexpression of a key transcription factor was sufficient to change and override the endogenous gene expression pattern of a cell. These experiments however, demonstrated that changes could only be achieved within cells from the same germ layer, in this case mesoderm (Weintraub H. et al., 1989).

As MyoD was the skeletal muscle cell regulator, this prompted the idea that the presence of transcription factors that play the role of the regulator for other cells coming from the two other germ layers. Interest grew in exploring limits in somatic cells and in testing the plasticity of adult mammalian cells in terms of transdifferentiation. Researchers became interested in understanding the plasticity of MSCs. Their interest was seeded from observing the ability of MSCs to convert into different types of cells such as neurons (Mezey E. et al., 2000) (Brazelton T. R. et al., 2000), and cardiomyocytes (Jackson K.A. et al., 2001), (Mangi A.A. et al., 2003), (Matsuura K. et al., 2004), among other types of cells. Many of these studies showed that the resulting plasticity in bone marrow MSCs was not actually cell conversion, but happened because of cell fusion of these MSCs with host cells (Spees J.L. et al., 2003).

Understanding the effect of transcription factors that are introduced into somatic cell took some time to develop further after the discovery of MyoD. The main reason was interest in cell plasticity really skyrocketed with the discovery that different type of cells could be derived from bone marrow MSCs. The capacity of these cells was investigated *in vitro* and MSCs showed amazing results. An example was when MSCs were exposed to chemicals (transferrin, putrescine, insulin, progesterone, selenium, all-trans-retinoic acid and BDNF) and they changed their phenotype into what they thought were neural cells (Sanchez-Ramos J. et al., 2000). Unfortunately the discovery of cell fusion driving plasticity seeded doubt of many other *in vitro* experiments studying somatic cell conversion. The results of these MSC chemical conversion experiments were later analyzed by other research groups and found that the neural morphology established in these bone marrow MSCs occurred because of a rapid disruption of the actin cytoskeleton caused by the chemical (Neuhuber B. et al., 2004). Thus, for a period of time, transdifferentiation research was abandoned.

In 2006, Takahashi and Yamanaka's iPSCs work inspired somatic cell conversion, allowing the field of transdifferentiation to pursue endless possibilities. The idea was very simple: if four core pluripotency factors could change a somatic cell into a pluripotent cell, then for transdifferentiation there most be some core transcription factors allowing for the formation of different somatic cell types. As a result, many transdifferentiation protocols using transcription factors have arisen for cells such as neurons, cardiomyocytes, hepatocytes, chondrocytes, and so on.

Zhou and collaborators made a breakthrough in the area by showing that insulin-producing cells could be transdifferentiated *in vivo* (Zhou Q. et al., 2008). They identified 9

factors that led to beta cell phenotypes. Later, they used an adenoviral system to introduce the 9 factors into the pancreas of an immunodeficient mouse. They observed an increase in the number of beta cells. Remarkably, also with the introduction of these factors in a mouse model for type I diabetes, hyperglycemia in these mice was alleviated (Zhou Q. et al., 2008).

One of the most outstanding experiments for *in vitro* transdifferentiation using defined transcription factors, was demonstrated by Ieda et al., 2010. From a microarray analysis, they identified 13 potential cardiac reprogramming factors, and among them found that the combination of Gata4, Mef2c and Tbx5 were sufficient to reprogram mouse dermal fibroblast directly into a differentiated cardiomyocyte-like cell (Ieda M. et al., 2010). Similarly in 2010 Vierbuchen and colleagues used the same approach, but for the transdifferentiation of mouse fibroblasts to neurons. After, cloning nineteen transcription factors, they discovered Ascl1 as sufficient to induce immature neuronal features. However the addition of Brn2 and Myt11 was necessary to convert mouse fibroblasts into functional neurons (Vierbuchen T. et al., 2010).

Many groups followed this same method of screening for factors and their combination to change the fate of a cell. However, there are disadvantages for this type of transdifferentiation. First, the process of screening new master genes for each type of cell of interest is very time consuming. Second, this process gives rise to another differentiated cell, with very limited capacity to divide and expand. Finally, there are many different cocktails of transcription factors per cell types or even for a single type of cell. Each with diverse molecular and epigenetic paths. For transdifferentiation to be clinically applicable,

the molecular and epigenetic changes during transdifferentiation need to be well understood.

Transcription factors cocktails have also been used for chondrogenic transdifferentiation. Hiramatsu and collaborators (2013) were able to direct convert MEFs to chondrocytes by using two of the OSKM factors: Klf4 and c-Myc, together with a lineage specific factor Sox9 (mouse). They unfortunately found tumor formation upon implantation in nude mice. Continuing with this work, Long-Tam W. et al., 2014 produce iChon which are mouse dermal fibroblasts that have been direct converted into chondrocytes by using human transgenes Klf4, c-Myc and Sox9. With this last study, the wanted demonstrated novel endochondtral bone formation strategies by using chondrocytes obtained with direct reprogramming. Outani H. and colleagues (2013) were able to form hyaline cartilage by transdifferentiation in humans by using the same factors previously reported by Hiramatsu et al., (2013). This group was able to introduce human Klf4, c-Myc and Sox9 into human dermal fibroblast (HDF). This human cells formed cartilage and different from Hiramatsu's results this human transdifferentiated chondrocytes did not form tumors in nude mice (Outani et al., 2013).

A new transdifferentiation approach that uses the OSKM factors (originally used for iPSCs)(Yamanaka et al., 2007), has been proposed as a better alternative, because: 1) the process of reprogramming is now well understood, and 2) some of the OSKM factors are expressed in organisms that are able to pursue tissue-organ regeneration. The most complicated changes during reprogramming are the epigenetic, and these have been mostly unveiled. A good example of that is what Rais Y. and colleagues demonstrated in 2013.

They determined that Mbd3, a member of the Mbd3/NuRD (nucleosome remodeling and deacetylation) repressor complex, is highly involved in the process of pluripotency reprogramming. When Mbd3 is depleted, gives a reprogramming efficiency in iPSC of 100%. With this, researchers have demonstrated that they have gotten the control required to have deterministic reprogramming to iPSCs.

The OSKM factors were found to be mildly expressed in blastema during tissue-organ regeneration in zebra fish. That suggested that a partial reprogramming was necessary in this regeneration process (Christen B. et al., 2010). Thus, the presence of these pluripotency factors in a differentiated cell is thought to have the same effect of loosening the chromatin arrangement and placing the cell in a plastic position where it can be directed to become any type of cell (Meshorer E., et al., 2006).

Leukemia Inhibitor Factor

When differentiated cells are prone to become pluripotent *in vitro* by reprogramming with the OSKM factors, cells are also exposed to a media that provides the cues to drive pluripotency. In this case a very important driver (if not the most important) to pluripotency is the presence of leukemia inhibitor factor (LIF), throughout the reprogramming process.

LIF is part of the interleukin (IL)-6 family of cytokines (White U. A. and Stephens J. M., 2011) that includes IL-6, IL-11, IL-27, oncostatin M, ciliary neurotrophic factor,

cardiotrophin-1, novel neurotrophin-1/B cell stimulating factor-3 or cardiotrophin like cytokine, and neuropoietin. All of these cytokines are able to regulate different biological processes in the cell by using the gp130 signal transducer (White U. A. and Stephens J. M., 2011), and some such as neurotrophic factor (Peterson W. M. et al., 2000) and oncostatin M (Levy J. B. et al., 1996) act through the same gp130 transducer to activate the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway in mouse and human.

LIF in particular was first found to be secreted by feeder cells and to be essential for self-renewal and maintaining ESCs in a pluripotent state (Smith A. G. et al. 1988 and Williams R. L. et al., 1988). In ESCS, LIF targets different signaling pathways: JAK-STAT3, phosphatidylinositol 3-kinase (PI(3)K), also known as Akt and extra cellular-signal-regulated kinases (Erk). Because these pathways lead to the activation of the core circuitry of pluripotency transcription factors Oct3/4, Sox2 and Nanog, LIF was deemed to directly contribute to the core circuitry of pluripotency (Figure 6) (Niwa H. et al., 2009). LIF first reaches the receptor gp130 and sends a signal to JAK, which later phosphorylates Stat3 to activate Klf4 (Figure 6). Klf4 will later activate Sox2, which in sequence activates Oct3/4, thus promoting pluripotency.

Pluripotency can be maintained in the absence of LIF, provided that Klf4 is expressed artificially, as Klf4 keeps Oct3/4 activated through Sox2. As part of the pluripotency circuitry, the overexpression of Nanog was also found to support LIF-independent self renewal of mouse cells in the absence of Klf4 and Tbx3 (part of the PI(3)K pathway also activated by LIF (Niwa H. et al., 2009)). Thus, Klf4 and Tbx3 were found to be involved in connecting the signal from LIF to the core pluripotency circuitry.

However, Klf4 and Tbx3 are not necessary for pluripotency maintenance. Overexpressing Nanog while they were blocked tested this. (Niwa H. et al., 2009).

In the reprogramming process the LIF pathway is also very important for establishing pluripotency at the epigenetic level in mouse. From a report on the activity of Stat3, Tang and Collaborators (2012) observed that the enhancement of Stat3 could complete reprogramming of EpiSCs into full iPSCs in mouse. From that observation, they hypothesized that LIF/Jak-Stat3 signaling could promote direct reprogramming from terminally differentiated somatic cells. In their first study, MEFs transduced with OSKM and Stat3 in the presence of LIF, produced significantly more iPSC colonies compared to cells that were not transduced with Stat3. Upon blockage of PI3K and Erk pathways using a gp130 mutant receptor in order to provide constitutive Jak/Stat3 activation, they demonstrated that the JAK/Stat3 activity was needed for complete reprogramming. They compared transduced MEFs with OKSM factors in the presence of LIF and DMSO (control) to transduced MEFs with OKSM factors in the presence of LIF and JAK Inhibitor. Here with the last condition, they did not observe any colonies expressing endogenous Oct4. However when the PI3K pathway was blocked, expression of Oct4 decreased, but was not abrogated. Finally, they demonstrated that JAK/Stat3 activity for reprogramming is carried through an epigenetic process by the activation of DNA demethylation of the *Oct4* and *Nanog* genes. Thus, activated Stat3 downregulates DNA methyl transferase 1 (Dnmt1) in order for the pluripotency genes *Oct4* and *Nanog* to be demethylated. From their results, the Jak/Stat3 pathway is a prerequisite for the establishment of the pluripotency pathways in mouse (Tang Y. et al., 2012).

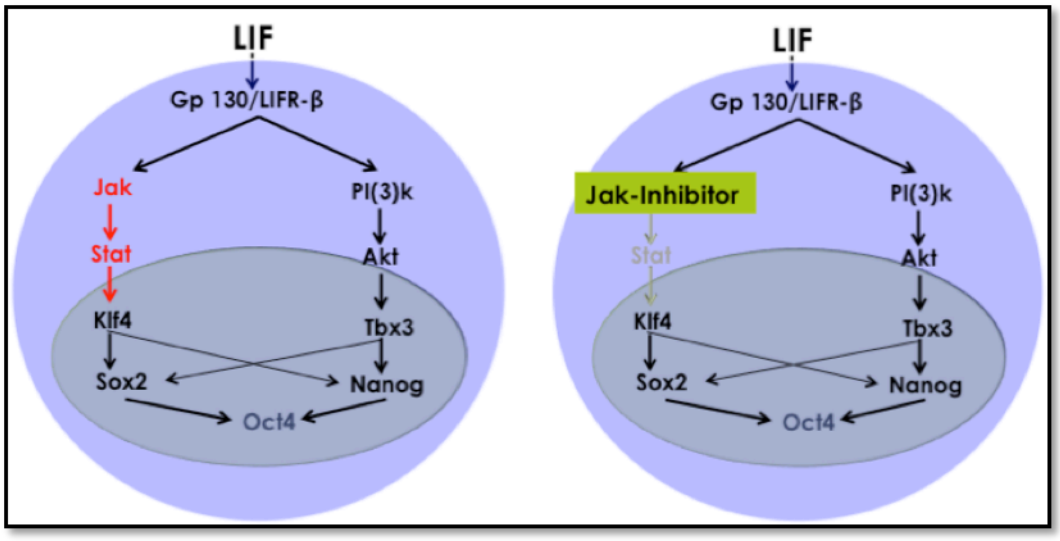

Figure 7. Schematics of the LIF/Jak-Stat3 pathway active and blocked by a Jak inhibitor. a) LIF binds to its Gp130/LIFR-β and activates the Jak-Stat3 pathway. Its activation leads to the direct stimulation of the pluripotency circuitry transcription factors in the nucleus for pluripotency reprogramming. b) When Jak-Stat and no other pathway is inactivated, the stimulation of the pluripotency circuitry decays and the reprogramming process is affected.

Recently, Efe *et al*., (2011) were able to directly differentiate mouse embryonic fibroblasts (MEFs) to cardiomyocytes by using the four pluripotency markers Oct4, Sox2, Klf4 and c-Myc. The idea behind using this method is that the factors will trigger the first events that occur when a cell is reprogrammed to pluripotency to become iPSCs. The approach wherein Efe and collaborators (2011) transdifferentiated MEFs into cardiomyocytes was as follows: 1) They exposed the cells to the four pluripotency factors in order for the cell to reach a plastic state, 2) At the same time, they put barriers to the

system in order to obtain a partial reprogramming state similar to what has been observed during the formation of blastema, and 3) finally, they exposed the cells to a differentiation media that provided the necessary growth factors to form cardiomyocytes.

Given the importance of LIF to promote and maintain pluripotency, they withdrew LIF from the media when cells were left to reach a plastic state. Because other cytokines can signal through gp130, the removal of LIF could not assure inactivation of pluripotency. Efe et al., 2011, inactivated the JAK/STAT pathway by using a JAK inhibitor (JI1). Activation of the pluripotency circuitry takes place through the activation of Stat3, which is part of the JAK-STAT pathway. However, it has also been reported that ESC-self renewal can be maintained upon the activation of Jak2 by interfering with the binding of heterochromatin factor HP1 with pluripotency genes (Griffits et al., 2011). Thus, in addition to the removal of LIF from the media, they also tried different approaches to enhance the reprogramming barrier. After examining the effect of both a PI3K inhibitor and a JAK inhibitor (JI), they found that the best results were obtained when using only JI confirming the importance of the JAK/STAT pathway in pluripotency and reprogramming (Figure 6). In addition to withdrawing LIF from the media during the first stage of the protocol, JI was used during this stage in order to further block the activation of the JAK/STAT pathway.

As a result of this transdifferentiation strategy, Efe et al. (2011) were able to obtain functional cardiomyocytes in a shorter period of time compared to differentiating iPSCs to cardiomyocytes. These results also demonstrated that they did not go through an iPSC intermediate cell in order to become cardiomyocytes. They even mentioned that the efficiency of beating colonies diminished if cells were grown in a media that supported

pluripotency. This transdifferentiation study has also been supported by the work of Janghwan Kim and colleagues in 2011, instead of forming cardiomyocytes, they formed neural progenitor cells using the same platform (i.e. OSKM factors-barrier to pluripotency-differentiation media).

Collectively these studies demonstrated that by using the same cocktail of reprogramming factors, they could control what type of cell they wanted to obtain. This approach has opened the possibility of transdifferentiate fibroblasts or other somatic cells into any progenitor cells that can be expanded and then further differentiated into a specific cell type of interest for clinical application.

Therapeutic implication of direct conversion

The importance of transdifferentiation or direct conversion is that it gives many possibilities to generate the type of cell needed that is otherwise difficult to obtain for regenerative medicine. There are two venues for the clinical application of transdifferentiation, 1) directly converting cells *in vivo* and 2) converting cells *in vitro* and then transplanting them *in vivo*. For direct conversion *in vivo,* some studies show promise for clinical application. One example was reprogramming of mouse cells into cardiomyocytes by the overexpressing of Gata4, Mef2c and Tbx5 in the heart of the mouse (Qian L. et al., 2012). Similarly, for the treatment of diabetes, transdifferentiation *in vivo* of pancreatic exocrine cells into beta cells through the forced expression of Ngn3, Pdx1 and MafA has been achieved (Zhou et al., 2008). The biggest drawback from this *in vivo* transdifferentiation event is transcription factor delivery and the effect of vector integration in the patient's genome (Hacein-Bey-Abina S. et al., 2003).

Going back to the work of Zhou and collaborators, they mentioned that transdifferentiation of exocrine cells into beta cells only occurred with certain types of cells (Zhou et al., 2008). Even though the delivery of the factors has to be improved, this technique presents a very efficient and highly effective method given that a patient's cells will be transdifferentiating in the microenvironment that is needed for the type of cell to be regenerated.

In vitro cell transdifferentiation followed by transplantation can result in a better approach for tissue regeneration, because it corrects causative mutations with healthy cells. Dopaminergic (DA) neurons were formed by transdifferentiating tail tip fibroblasts with ectopic expression of defined transcription factors and upon transplantation, these DA transdifferentiated neurons were able to alleviate symptoms in a mouse model of Parkinson's diseases (PD) (Kim J. et al., 2011). Similarly, mouse embryonic and lung fibroblasts transdifferentiated into induced oligodendrocyte progenitor cells (iOPCs) were able to ensheath host axons and generate compact myelin upon being transplanted into hypomyelinated mice (Najm F. J. et al., 2013). However the downside of this approach is that it has not been shown that these transdifferentiated cells can keep being generated without the necessity of an outside source, for example activation by transcription factors.

Transdifferentiation is a very promising cell technique that complements cellular reprogramming. However there are many unknown epigenetic changes in the cells, and thus yet very hard to reproduce. Further, it is hard to know whether transdifferentiated cells will maintain their identity *in vivo*. Nevertheless, the research here is very promising and soon an alternative for tissue regeneration with cells will be available for clinical application.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

Hypothesis

Direct conversion of mouse fibroblasts to chondrocytes is induced by OSKM partial reprogramming under a conducive condition (-LIF+JI), and later exposure to chondrogenic growth factors, without going to an iPSCs state.

Objectives

1. Develop a transdifferentiation protocol for generating cartilage
2. Characterize the transdifferentiated cartilage
3. Demonstrate that the generation of cartilage occurred via transdifferentiation
4. Characterize the kinetics of fibroblast to chondrocyte transdifferentiation

CHAPTER 3

MATERIALS AND METHODS

Mouse Embryonic Fibroblast (MEFs) Isolation

Fibroblasts are the most common type of cell of connective tissue in animals, and synthesize extracellular matrix and collage. In this case, MEFs are isolated from an embryo thus these is a mixture of mesenchymal cells with fibroblasts.

MEFs were isolated from E13.5 embryos. The head was first removed, followed by the vertebral column and internal organs. MEFs were dissociated in cold 0.05% trypsin (Gibco) overnight. Trypsin is an enzyme that catalyzes the hydrolysis of proteins in this case, proteins that allow the cells to be attached to the cell culture plate. The next day MEFs were placed in warm 0.25% trypsin for 10 min and then pipetted up and down for dissociation. Later trypsin was inactivated with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (Gibco), and 50 U of penicillin-streptomycin (Gibco). These dissociated MEFs were then plated and expanded.

Genetically modified MEFs were isolated from B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J mice similar to the procedure explained above. The transgene contains an Enhanced Green Fluorescent Protein gene under the control of the promoter and distal enhancer of the POU domain, class 5, transcription factor 1, (Pou5fl or Oct4).

Packaging of OKSM Retrovirions

Platinum-E (Plat-E) retroviral packaging cells (Cell Biolabs) were prepared for plasmid transfections by seeding 8×10^6 cells per 100 mm dish (one dish for each

reprogramming gene). Plat-E cells were maintained in Fibroblast-Platinum (FP) medium, which consisted of Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 50 U of penicillin-streptomycin. The next day, we introduced each pMXs retroviral plasmid DNA (Oct4, Sox2, Klf4, c-Myc) (Figure 8); and Ds-Red; Addgene plasmids 13366, 13367, 13370, 13375 and 22724 respectively) into Plat-E cells using X-tremeGENE 9 DNA transfection reagent (Roche) according to the manufacturer's recommendations. Briefly, 18 μl of X-tremeGENE 9 transfection reagent was added to 300 μl of OptiMEM in a 1.5-ml tube. Then, 8 μg of each retroviral vector was added into the prepared X-tremeGENE9-OptiMEM tube drop by drop and incubated for 15 min. Each vector–X-tremeGENE 9 complex was added drop-wise into the Plat-E cell–containing dishes and incubated overnight at 37 °C, 5% CO_2 The following day, the medium with 10 ml fresh FP medium was replaced. Forty-eight hours after transduction we collected virus-containing medium from each transfection by filtering through a 0.45 μm Acrodisc filter (Pall Life Sciences).

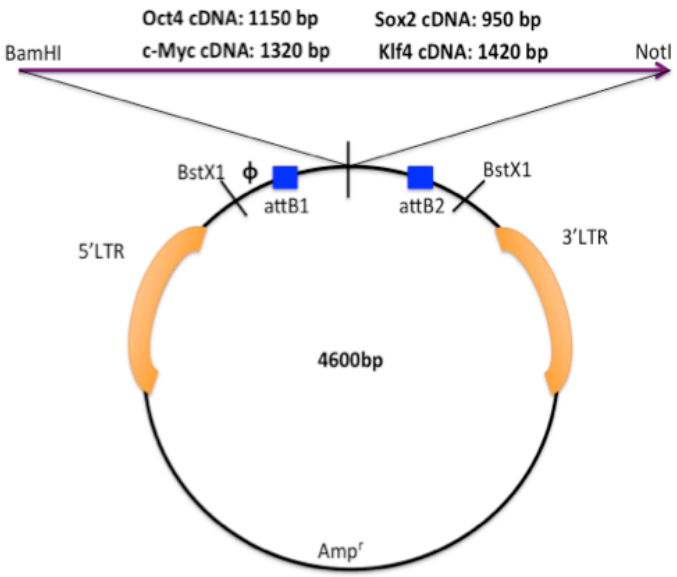

Figure 8. Schematics of the retroviral pMX vector with the OSKM transcription factor inserts. The characteristics of these vectors are that the transcription factor (either Oct4, Sox2, Klf4 or c-Myc) is flanked by lambda sites that are compatible to sites in the genome of the packaging cell line plat-E. This plat-E cell line contains the elements for the formation of retrovirus and when these are activated then the transcription factor excised from the pMX vector will be included in the viral particle genome.

OKSM Retroviral Transduction

MEFs procured from the Centre for Mouse Genomics were initially passaged in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 50 U of penicillin-streptomycin. Before viral transduction, cells were seeded onto six well-coated plates overnight at 37°C, 5% CO_2 at 3.5 x 10^4 cells per well in the previous media. The next day, equal parts of retroviral-conditioned medium supplemented with 0.5µl/ml polybrene (cationic polymer used to increase the efficiency of infection when cells are infected with a retrovirus system. It neutralizes the charge repulsion between virions and sialic acid on the cell surface) were mixed.

Cellular Reprogramming

After the addition of the virus (12-24 h) with polybrene, cells were washed with PBS and switched to Reprogramming Medium 1 (Knockout DMEM with 5% Knockout serum replacement, 15% ES cell-qualified FBS, 1% Glutamax, 1% nonessential amino acids, and 0.1 mM β-mercaptoethanol (all components from Invitrogen) for the first 6 days. After day 6, media was changed to Reprogramming Medium 2, (Knockout DMEM, 1% FBS, 14% KSR, 1% Glutamax, 1% nonessential amino acids and 0.1% mM mercaptoethanol). During the first 9 days, JI1 0.5 μM (Millipore) was added. Here conditions in the presence or absence of LIF (1000 U per ml) and/or JI1 were also tested. Fresh medium was added every 48 h throughout the experiment.

Chondrocyte Differentiation

On day 10, cells were dissociated using 0.1% trypsin-EDTA (Invitrogen). Dissociated pre-iPSCs were cultured at a high-density, 1.0×10^5 cells per 10 μl × 9 spots in 6 cm culture dish for 2 hours [8], [11]. After incubation, medium was added to each dish without dissociating the cell drops. For chondrocyte differentiation, we used differentiation media containing DMEM (Gibco), 1% non-essential amino acids (Invitrogen), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 1% ITS (Invitrogen), 1% FBS (Gibco), 10 ng/ml TGF-β1 (PeproTech), 10 ng/ml BMP-2 (PeproTech), and 50 μg/ml ascorbic acid (Sigma). Medium was changed every 2 days.

After 5 days, resulting pre-iPSC aggregates were separated from the dishes by pipetting and were transferred to suspension culture in 6 cm petri dishes containing differentiation media, DMEM, 1% non-essential amino acids, penicillin streptomycin, 0.1 mM 2-mercaptoethanol, 1% ITS, 1% FBS, 10 ng/ml BMP-2 and 50 μg/ml ascorbic acid (4).

RNA isolation

Total RNA was isolated from cell cultures at different stages in the protocol (day 0, 2, 6, 9, 12, 15 and 36) using a RNeasy Mini kit (Qiagen, 74106) according to the manufacturer's instructions with on-column DNase I digestion. RNA was measured using a NanoPhotometer P-Class (IMPLEN). Here 0.5-1 μg of total RNA was used as a template for cDNA synthesis with the High Capacity cDNA Archive Kit (Applied Biosystems, 4322171).

Quantitative PCR

Quantitative PCR (Q-PCR) was performed in a Step One Plus PCR System (Life Technologies) using a Fast SYBR green gene expression master mix (Applied Biosystems; CA, USA) with 50 nM primer concentration. The cycles used were those recommended for the Fast SYBR green reagent by Applied Biosystems. Briefly: 20 sec at 95°C as initial denaturation, followed by 3sec at 95°C, next 30 sec at 60°C for annealing and then 15 seconds of extension at 95°C for 40 cycles. Mouse pluripotency markers: Nanog, Rex-1 and chondrogenic markers: Sox9, ColII, ColX and Mmp13 gene expression was quantified during a time course of maintenance using the ΔΔCt method. Relative gene expression was normalized to Rsp29 and compared to day 0 Mouse embryonic fibroblast (MEFs) and to chondrocytes (cells that form cartilage tissue).

Flow Cytometry

MEFs and MEFs undergoing transdifferentiation were subjected to flow cytometry analysis under similar staining conditions. Both were dissociated with 0.25% trypsin for 5 min at 37 degrees. Cells were then washed two times with PBS (Gibco) using centrifugation 126X G for 5 minutes each time. MEFs undergoing transdifferentiation were later permeabilized with 0.5% Triton X-100 (Sigma T8532) in PBS for 10 min at room temperature. After washing twice with PBS, the cells were treated with a blocking buffer (1% BSA, 0.1% Triton X-100 in PBS for MEFs and 10% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS), for 1 hour. The primary antibody (anti-Oct4) was diluted in the blocking solution (1:100) and incubated for 1 hour at room temperature. Cells were washed with 2ml of PBS twice. Secondary antibodies were also diluted in the blocking

solutions (1:1000) and incubated with the cells for 1 hour at room temperature. For MEFs, conjugated antibodies (anti-H2kk and anti-Col2) were diluted in flow cytometer buffer (1:100) and incubated for 1 hour at room temperature. Finally the cells (MEFs and MEFs undergoing transdifferentiation) were washed with 2 ml of PBS twice and a final fixation was performed with 3.7% Paraformaldehyde (PFA) for 30-45 minutes at room temperature. Finally, PFA was diluted with PBS (Gibco) to a final volume of 5ml. These cells were later span down two times at 126 G for 5 minutes to remove all residues of PFA.

Mouse embryonic fibroblasts (MEFs) labeled for Col2 and H2kk (Fibroblasts marker) were used to mark cells their characterization on a Flow Cytometer Attune equipped with a 488nm and 633nm laser diode. In addition to those cells, transgenetically modified MEFs with a GFP gene insertion after the promoter of the transcription factor Oct4 were used to analyze the percentage of cells that were positive for GFP after transduction at different time points (Days 2, 6 and 9), by an Attune Flow Cytometer equipped with a 488nm laser. Additionally, the presence of exogenous Oct4 was detected after being labeled with the anti-Oct4 antibody was recorded under the same conditions.

Transplantation

Severe combined immune deficient (SCID) mice were ordered from Taconic company and housed in the animal facility of the Faculty of Medicine at the University of Calgary. Ms. Shiying Liu performed experiments as approved by the University of Calgary Health Sciences Animal Care Committee (Protocol AC11-0007). Cell aggregates were taken from static cultures after four weeks of transdifferentiation. Two mice were injected

with transdifferentiated cells in a total volume of 200µl PBS into a skin fold of the inner thigh. After 21 days, the mice were sacrificed and tissues were dissected and analyzed by histological procedures.

Histology, hematoxylin and eosin (H&E), alcian blue and safranin-o staining

Aggregates at day 36 and isolated tissue from the teratoma assays were fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. After dehydration by increasing concentrations of ethanol, the aggregates and tissue were embedded in paraffin. Later the aggregates and tissue were sectioned and mounted. Next, the paraffin was removed from the slide that contained the aggregates and the tissue by rehydrating with diminishing concentration of ethanol.

All the tissue sections were then stained with Hematoxylin and eosin (H&E) and examined for cartilage tissue by light microscopy. Half of the aggregate sections were stained with H&E and the other half with Alcian blue (Sigma-Aldrich). Histology analysis was performed blinded.

Statistical Analysis

Two-sided unpaired student's T test was used to compare sample groups. Asterisks depict the following α levels:

* $P < 0.050$
** $P < 0.010$
*** $P < 0.001$
n.s. $P > 0.050$, not significant

Tables

Table 1. Primer Sequence used in RT-qPCR

Gene	Sequence
Nanog	**FORWARD:** 5'-AAGCAGAAGATGCGGACTGT-3' **REVERSE:** 5'-GTGCTGAGCCCTTCTGAATC-3'
Rex-1	**FORWARD:** 5'-GGACTAAGAGCTGGGACACG-3' **REVERSE:** 5'-CACCTGCTTTTTGGTCAGTGG-3'
Sox9	**FORWARD:** 5'-AGCTCACCAGACCCTGAGAA-3' **REVERSE:** 5'-GATTCTCCAATCGTCCTCCA-3'
Col2	**FORWARD:** 5'-CCGTCATCGAGTACCGATCA-3' **REVERSE:** 5'-CAGGTCAGGTCAGCCATTCA-3'
Col10	**FORWARD:** 5'-TGCATTTGGAGGTAGGCTAAA-3' **REVERSE:** 5'-CCTCACATACCCACTGTTACTG-3'
Mmp13	**FORWARD:** 5'-CAGTTGACAGGCTCCGAGAA-3' **REVERSE:** 5'-CGTGTGCCAGAAGACCAGAA-3'
Rsp29	**FORWARD:** 5'-CGTCTGAAGGCAAGATGGGT-3' **REVERSE:** 5'-TATGTCCTTCGCGTACTGCC-3'

Table 2. Retroviral vectors used for packaging

Gene	**Addgene plasmids**
Oct4	13366
Sox2	13367
Klf4	13370
c-Myc	13375
Ds-Red	22724

CHAPTER 4

RESULTS

Recapitulation of Cardiomyocyte Transdifferentiation

iPSC technology has opened an area of opportunity to avoid ethical issues associated with ESCs, as also avoids immune rejection associated with regenerative medicine strategies. However, iPSCs are very slow to generate and to differentiate into specific cell types for tissue regeneration. In efforts to find an alternative to generate tissue in a more efficient manner using cellular reprogramming, a transdifferentiation strategy (Figure 6) using transcription factors was proposed to be faster and more efficient. OSKM pluripotency factors are well understood and known to bring cells to an epigenetically open state during the first stages of reprogramming. Thus, it was thought that these factors could be used to transdifferentiate a differentiated cells into another differentiated cell by letting them reach an open partial reprogrammed state. Following that, growth factors could be used to direct partially reprogrammed cells to the desired cell type.

In 2011, Efe and collaborators demonstrated for the first time that transdifferentiation of fibroblasts into cardiomyocytes could be achieved with a curtailed reprogramming process using OSKM factors in the absence of LIF and in the presence of a Jak inhibitor following later exposure to cardiac differentiation media. As a result, they obtained beating cardiac cells more efficiently and in a shorter period of time using a combination of cellular reprogramming followed by directed differentiation.

We chose to investigate if this transdifferentiation strategy could be applied to the generation of chondrocytes. However, we first investigated our ability to recapitulate the Efe et al. (2011) results.

The general procedure started with the production of viral particles. Later, MEFs were transduced with the viral particles that contained the OSKM factors. The next day the media with the virus was removed and for 9 days transdifferentiating MEFs were maintained in the absence of LIF and in the presence of a Jak Inhibitor (JI1) (according to the partial reprogramming process from Efe et al., 2011) (Figure 9a). At day 10 cells were exposed to media that supported differentiation of cardiomyocytes (according to Shafa et al., 2012) in order to determine whether the cells changed their phenotype into cardiomyocytes (Figure9a). Interestingly, 6 days after exposing the cells to cardiac differentiation media, beating cells were observed under the microscope (Figure9b), together with the presence of cardiac markers (Figure 9d and e). This transdifferentiation approach was one week faster than what Shafa et al., 2012 reported for the iPSC-cardiac differentiation process.

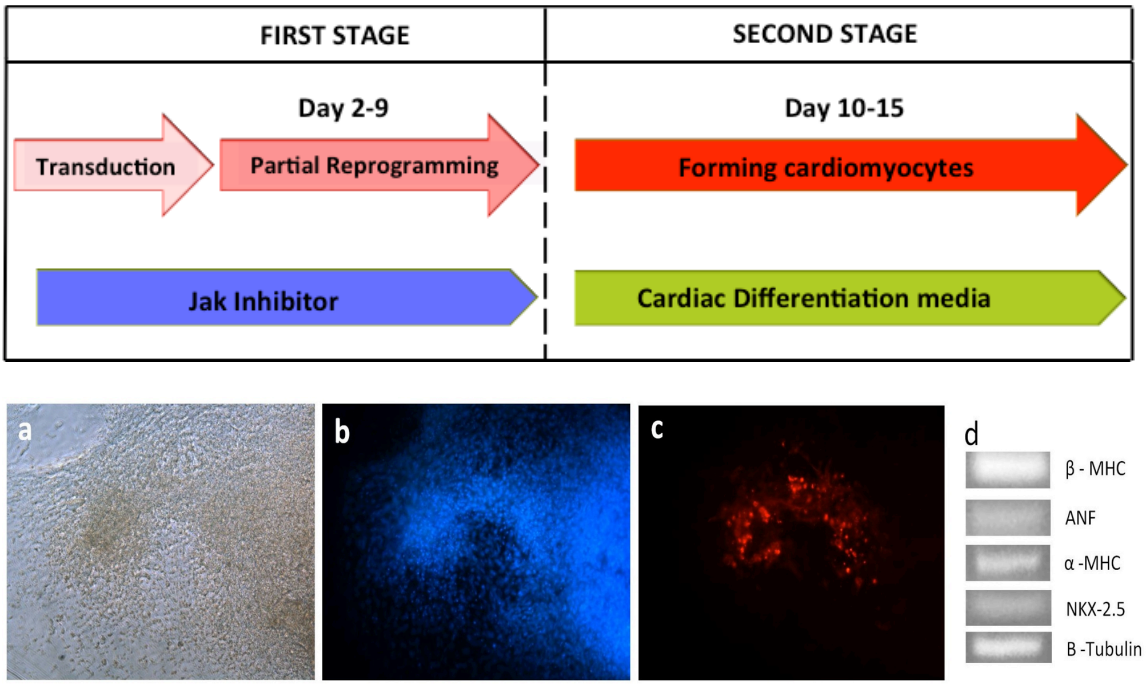

Figure 9. Schematics of transdifferentiation based on initial activation of OSKM (plastic state) follow by direct conversion into cardiomyocytes in cardiac differentiation media 15% FBS, in the presence of ascorbic acid and BMP4 a). b) Beating cells were observed on day 6, immediately after being exposed to cardiac differentiation media. c) nuclear staining with TOTO-3, d) the presence of MF-20 (α-MHC) was found by immunofluorescence. e) cardiac gene markers β-MHC, α-MHC, ANF, NKX-2.5 and β-Tubulin were also found expressed in transdifferentiated cells. Scale bar 100μm.

Transdifferentiation of MEFs into cartilage aggregates

After successful transdifferentiation of MEFs into cardiomyocytes, the same procedure was used to transdifferentiate MEFs into cartilage aggregates. This process was divided in two stages: 1) partial reprogramming, and 2) exposure to cartilage differentiation media (Figure 10). Much like the cardiomyocyte transdifferentiation experiment described above, MEFs were first transduced with OKSM retroviral particles, to be partially reprogrammed for 9 days in the absence of LIF and the presence of a JAK inhibitor (JI1). Unlike the Efe protocol however, on day 10 cells were passaged to form high cell density micromass cultures for ongoing chondrocyte differentiation, as per our previous chondrocyte differentiation protocol (Yamashita et al., 2010). Following the formation of micromasses each containing 100,000 cells, media that supported chondrocyte differentiation was added to the cells. This media had chondrocyte-defined factors (BMP2 and TGF-β), to complete transdifferentiation into chondrocytes and final formation of cartilage aggregates (Figure 10) (Yamashita et al., 2010).

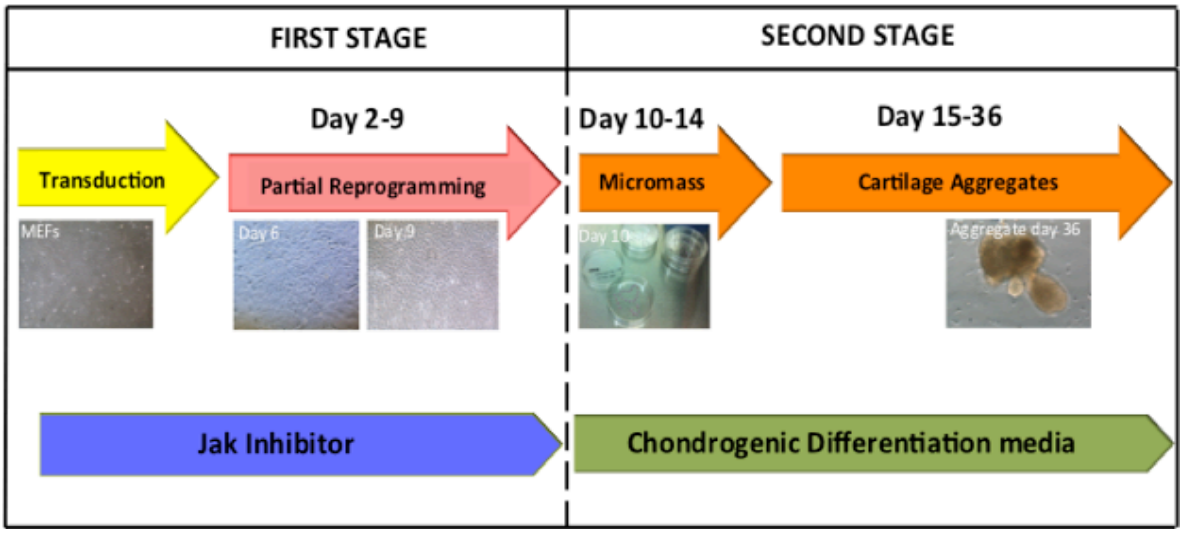

Figure 10. Schematics of transdifferentiation approach as outlined in this study. First after transduction, from day 2-9 is the partial reprogramming (First stage) where the OSKM factors bring cells in permissive plastic form and at the same time, the lack of LIF and presence of Jak inhibitor, arrest the pluripotency formation of these cells. Later, in the second stage (day 10-36) the cells are exposed to chondrocyte differentiation media in the presence of growth factors (TGF-β and BMP2) that will give the cues for the cells to turn into chondrocytes for cartilage formation.

MEFs are effectively transduced with viral particles

The partial reprogramming occurring during the first stage is driven by the effect of the exogenous factors. Viral particles carry the OSKM factors that once inside of the MEFs, are transcribed to be double stranded to be able to be inserted and integrate into MEF's to later integrate in their genome. Thus, in order to assure that the viral particles were transducing the OSKM factors to the MEFs, viral particles containing a Ds-Red gene in the same backbone vector as the OSKM factors, was introduced into the cells. Thus, upon expression and translation of this gene, MEFs would fluoresce red and would provide evidence that the transduction with the OSKM factors is working (Figure 11a). After transduction, MEFs were exposed to a laser that exited the Ds-Red protein under a fluorescent microscope at different days. The numbers of positive cells (in red) were quantified by flow cytometry. At day 1 after transduction, no Red signal was found in the cells. At day 3, cells were shown to be fluorescent, indicating that there was a successful transduction. At day 5, the number of cells that fluoresced red increased, and peaked at day 7. Finally, at day 9 the number of positive red cells stayed steady, decreasing just by a small amount (Figure 11b and c). This information indicated that MEFs are successfully transduced with the OSKM factors. It also provided an estimation of the time frame when the OSKM factors are active and able to start the reprogramming of the cell.

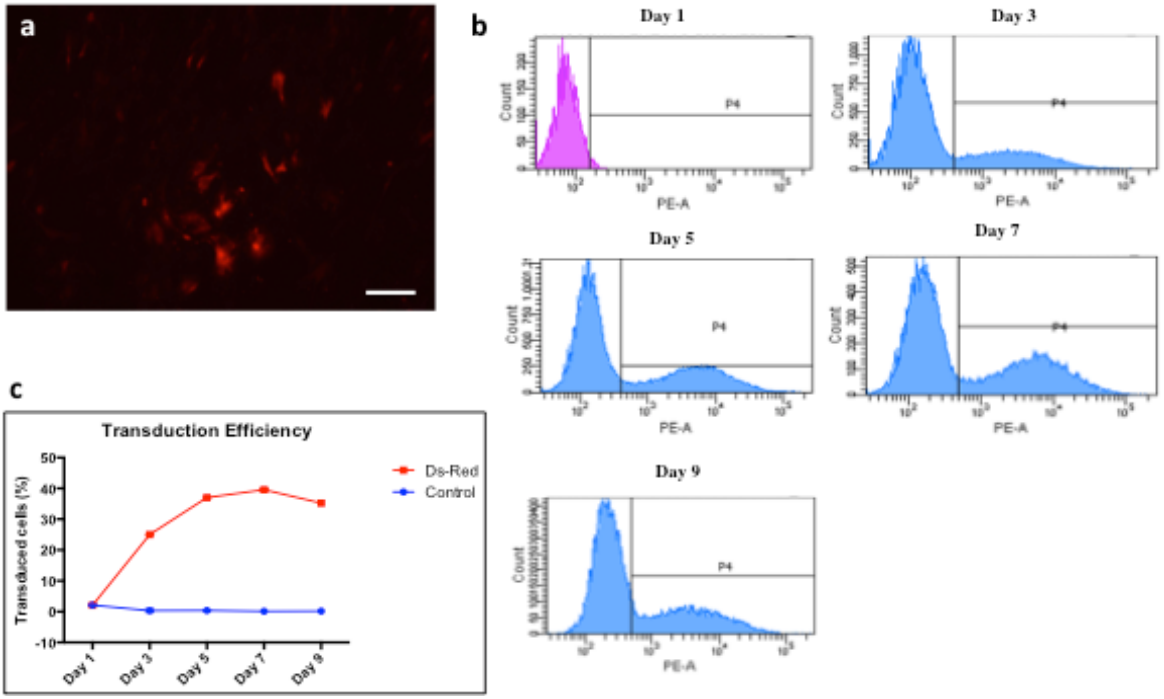

Figure 11. Transduction efficiency of MEF using lentiviral vectors. To evaluate the efficiency of transduction, we utilized the fluorescence reporter gene Ds-Red and transduced MEF as outlined in material and methods. Overall, 40% of the cells were transduced. Transgene expression reached a maximum on Day 7 and maintained the signal until day 9. Scale bar 50µm.

MEFs were successfully transdifferentiated into chondrogenic aggregates

Cells that underwent the whole process of chondrocyte transdifferentiation were collected at day 36 (Yamashita et al., 2010) and characterized by assessing at chondrocyte gene markers Sox9, Col2, Col10 and Mmp13 (Figure 12a-c). Finally, for a more definitive chondrogenic assessment, the morphology of the aggregates, histology of cartilage-like tissue and glycosaminoglycan (GAG) deposition was analyzed (Figure 12a-e).

Chondrocyte marker gene expression fold difference of Sox9, Col2, Col10 and Mmp13 relative to mouse chondrocytes was assessed. Significant upregulation in Sox9 ((P= 0.0131, *P<0.05), Col2 (P=0.0008, ***P<0.05), Col10 (P=0.0106, *P<0.05) and Mmp13 (P=<0.0001, *P<0.05), was observed on –LIF+JI aggregates when compared to MEFs.

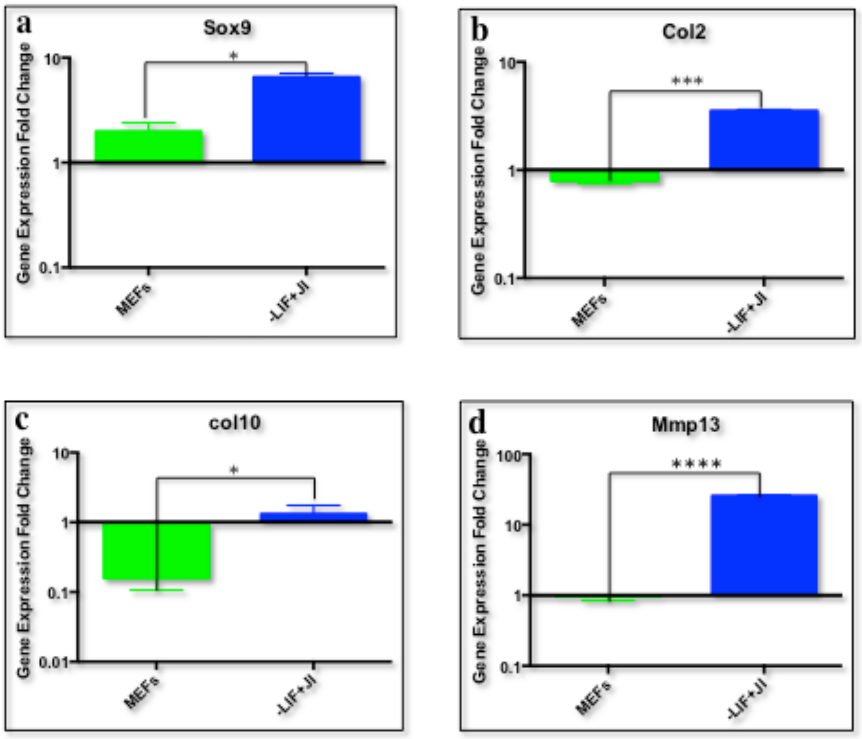

Figure 12. Chondrocyte gene expression is different on transdifferentiated cells compared to MEFs. OSKM-transduced MEFs were transdifferentiated (-LIF+JI) and then analyzed for chondrogenic gene expression (Sox9, Col2, Col10 and Mmp13) with comparison to MEFs (starting cell population). Gene expression data normalized to chondrocytes. Figure shows that the expression of Sox9 was 5 times higher in transdifferentiated cells than in MEFs, with a significant upregulation ((P= 0.0131, *P<0.05). b) Col2 (P=0.0008, ***P<0.05), c) Col10 (P=0.0106, * P<0.05) and d) Mmp13 (P=<0.0001, * P<0.05), was observed in –LIF+JI aggregates when compared to MEFs.

Upon sectioning and staining resulting aggregates with H&E, clear hyaline and hypertrophic cartilage (Figure 13b and c). At the same time, the aggregates stained with Alcian blue and Safranin-o, demonstrated the presence of glycosaminoglycans (GAG), which confirmed cartilage origin (Figure 13d and e).

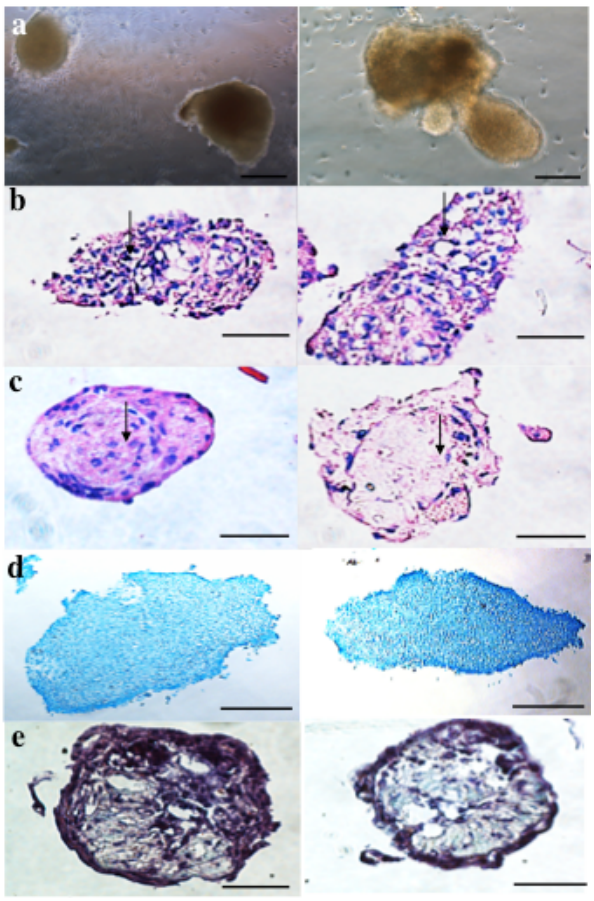

Figure 13. Cartilage structure and glycosaminoglycan (GAG) are present in transdifferentiated aggregates. OSKM-transduced MEFs were transdifferentiated (-LIF+JI) then analyzed for cartilage structure formation and the presence of GAG a) compact cartilage aggregates in culture, b) histology of hypertrophic cartilage-like tissue stained by H&E. This one has porous characteristics similar to a structure ready to mineralized and vascularized for bone formation, and c) hyaline cartilage–like tissue stained by H&E., with sparse chondrocytes. In the lacunae structure (Scale bar 25μm). d) Low levels of glycosaminoglycan (GAG) deposition stained by alcian blue, most likely because of the degradation of aggrecan for bone formation, and e) safranin-o staining showing the same results of low levels of GAGs. (Scale bar 100μm).

Finally, transplantation of Day 36 transdifferentiating aggregates into SCID mice resulted in the formation of cartilage three weeks post-transplantation (Figure 14) Interestingly, it was observed that similar to the aggregates, lacunae structures of hyaline (Figure 14a) and hypertrophic cartilage (Figure 14b and c) were formed.

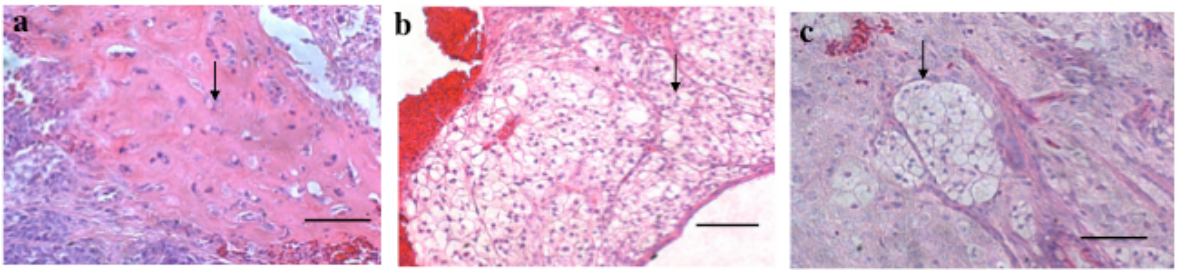

Figure 14. Cartilage structure found *in vivo* from teratoma formation in mice. OSKM-transduced MEFs were transdifferentiated (-LIF+JI). (Day 36) chondrocyte aggregates were injected subcutaneously into the inner thigh of SCID mice and by three weeks post injection formed teratomas were isolated and analyzed for cartilage structure formation. Figures show a) hyaline cartilage tissue structure with sparse chondrocytes and uniform lacunae stained with H&E and b) and c) hypertrophic cartilage formation with porous structures stained with H&E. No other type of tissue was detected by H&E. Scale bar 25μm.

Chondrocytes are formed by transdifferentiation

Starting population of MEFs does not contain mature chondrocytes

The presence of contaminating chondrocytes among the mouse embryonic fibroblast (MEFs) population was assessed by flow cytometry. Two markers were used for the analysis, one for fibroblast (H2kk) and the other one for chondrocytes (Col2). In addition to those markers, gene expression fold change relative to mouse chondrocytes for the same chondrogenic marker (Col2) was examined. The gene expression for Col2 in MEFs was compared to the transdifferentiated chondrogenic aggregates (Day 36). In the flow cytometry study, 96.6% of the MEFs population was positive for only the fibroblastic marker H2kk (Figure 15a and b). The percentage of cells expressing both markers, Col2 and H2kk was 0.8%. For, Col2 alone there were only 0.08% of positive cells. In the gene expression analysis, the chondrocyte marker Col2 was found to be downregulated in MEFs compared to transdifferentiated chondrocyte aggregates. These results ruled out the possibility that the MEF population contained a significant population of mature chondrocytes that might demonstrate a false positive in the transdifferentiation protocol.

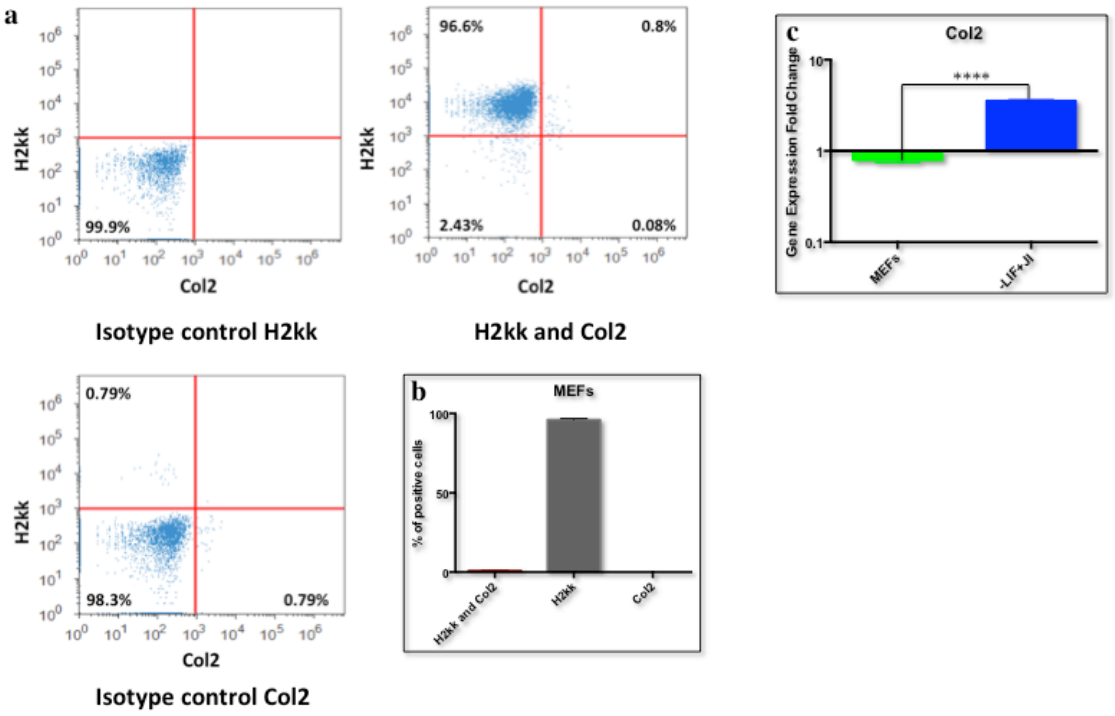

Figure 14. Negligible number of chondrocytes are found in the starting MEFs population. 2-D histogram plotted with fluorescent intensity from fibroblast (MEFs) marked with H2kk (APC) and Col2 (FITC) antibodies analyzed by flow cytometry a). The horizontal-vertical lines divide the cells in four quadrants, which represent cells that were not marked or did not have expression of those proteins (bottom left), cells with H2kk expression (top left), cells with Col2 expression (bottom right) and cells with expression of both proteins (top right). b) 96.6% of the MEF population stained positive for H2kk, whereas 0.8% positive for both H2kk and Col2 c) the fold change of Col2, was significantly (****P<0.05) upregulated in experimental sample (-LIF+JI) compared to MEFs, where it was downregulated.

There is no intermediate forming iPSCs during the process of transdifferentiation

One of the advantages of transdifferentiation is that the step of going to iPSCs is avoided. Thus, in order to demonstrate that intermediate iPSCs did not form, we analyzed the level of pluripotency marker gene expression during days six to nine in reprogramming vs. transdifferentiation (Figure 16a and b) Samples at day 2, 6 and 9 from both conditions were collected for RNA isolation and subsequent gene expression analysis. The gene expression fold change for the pluripotency markers Nanog and Rex-1 were analyzed relative to MEFs. Cells under iPSCs conditions (+LIF-JI), showed a higher expression of Nanog overall compared to the other group (transdifferentiation –LIF+JI), with significant upregulation ($P = <0.0001$ $*P<0.05$) at day 6 (Figure 16a). Similarly, Rex1 was highly expressed at every time point under the iPSC condition and significantly ($P = <0.0001$ $*P<0.05$) higher at day 6 when compared to the transdifferentiation group (-LIF+JI) (Figure 16b). These results indicated that LIF removal combined with the JI addition avoided intermediate iPSC formation.

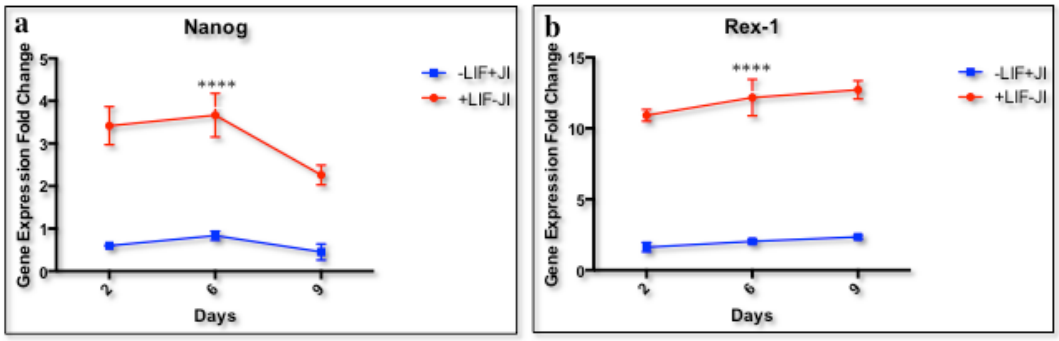

Figure 16. Pluripotency gene expression is upregulated in the presence of iPSC-inductive media. OKSM-transduced MEFs were cultured in both iPSC reprogramming media (+LIF/-JI) and transdifferentiation media (-LIF/+JI) then analyzed for quantitative gene expression of pluripotency markers Nanog and Rex-1. The figure shows that both Nanog and Rex-1, are significantly (****P<0.05) upregulated 3 and 10 fold,respectively in cells under iPSC reprogramming media.

Endogenous and Exogenous Oct4 assessment to determine the potential formation of iPSCs intermediates

In another study to assess if there was intermediate iPSC formation during OSKM transdifferentiation in the presence of JI, we correlated the levels of expression of endogenous and exogenous Oct4. MEFs used for the transdifferentiation experiment, were isolated from embryos of mice from the strain B6; 129S4-Pou5f1tm2Jae/J, where the GFP gene is expressed upon the activation of Oct4 promoter. MEFs from this strain would fluoresce green if endogenous Oct4 was to be expressed. The positive control for the endogenous expression of Oct4 was shown when these cells were reprogrammed into iPSCs (Anokye-Danso F. et al., 2011) (Figure 17a). Exogenous Oct4 expression was measured by staining cells with a monoclonal antibody that targeted Oct4. MEFs were subjected to transdifferentiation and the number of positive cells for endogenous and exogenous Oct4 was measured by flow cytometry on days 2, 6 and 9. There were no positive cells detected for the expression of endogenous Oct4 on days 2, 6 and 9. Exogenous Oct4 however, showed a signal starting at day 2. Here it appears that the signal of exogenous Oct4 peaked at day 9 (Figure 17d). Levels of retroviral signal are in accordance to the levels of Ds-Red experiment (Figure 10). Finally the histogram log shift comparison among the exogenous Oct4 and endogenous Oct4 on days 2, 6 and 9 showed changes only on the exogenous Oct4 (Figure 17e). It was observed that there was not signal from endogenous Oct4 compared to signal on exogenous Oct4 starting at day 2.

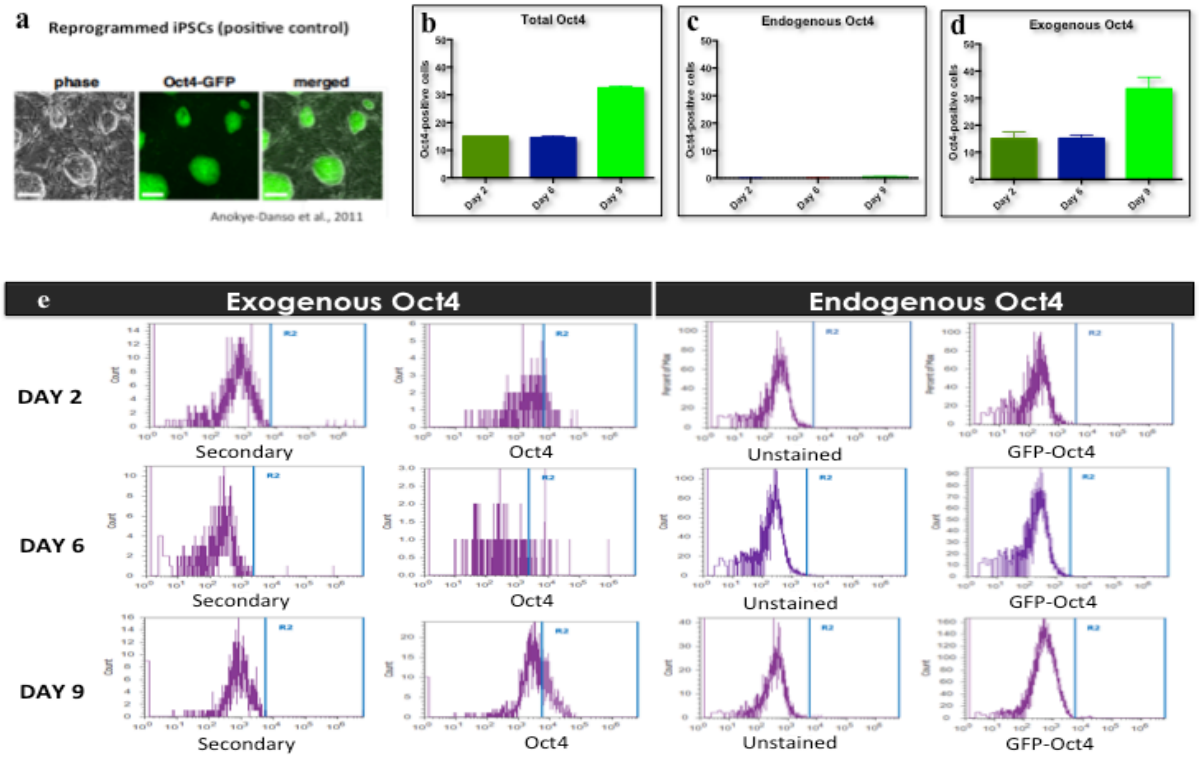

Figure 17. Exogenous, but not endogenous Oct4 is expressed during the first stage of transdifferentiation. The figure shows: a) GFP expression in MEFs upon Oct4 promoter is active in the reprogramming of iPSCs (positive control for endogenous Oct4) (Anokye-Danso et al., 2011). b) The total signal of Oct4 (exogenous and endogenous) at every time point. c) Cells were not expressing endogenous oct4 at any point in time (figure 16); d) Exogenous Oct4 signal was present since day 2 with the highest signal shown at day 9 indicating that partially reprogramming is occurring, and e) histogram demonstrating the log change in exogenous Oct4 compared to the absence of log change in endogenous Oct4. The absence of log change on endogenous Oct4 and the log change on exogenous Oct4 demonstrated that there was not intermediate iPSCs formation.

JI has no effect on chondrogenesis

In the first stage of transdifferentiation, JI was added to contribute to the partial reprogramming. However, as part of the protocol standardization, we needed first to demonstrate that JI was not contributing to chondrogenesis in non-transduced MEFs. Thus, non-transduced MEFs were cultured in a conducive media (+JI) for 9 days and the cells were analyze by looking at the gene expression of chondrogenic marker. These cells were compared to non-transduced MEFs cultured in a non-conducive media (-JI) for the period of 9 days. Non-transduced cells from the two conditions were taken at days 2, 6 and 9. Their RNA was isolated for further gene expression analysis. The transcription factor Col2, is essential for normal embryonic skeleton development and is one of the genes that Sox9 (transcription factor for chondrogenesis) activates (Sugimoto Y et al., 2013). Hence, the gene expression for Col2 was observed during days 2, 6 and 9. Here, Col2 in MEFs treated with –LIF+JI at day 2 (Figure18a) was shown to be significantly downregulated ($P= 0.012$, $*P<0.05$) compared to the other group that did not have JI. For days 6 and 9, there was not significant difference in gene expression among any of the groups (Figure 18b and 18c).

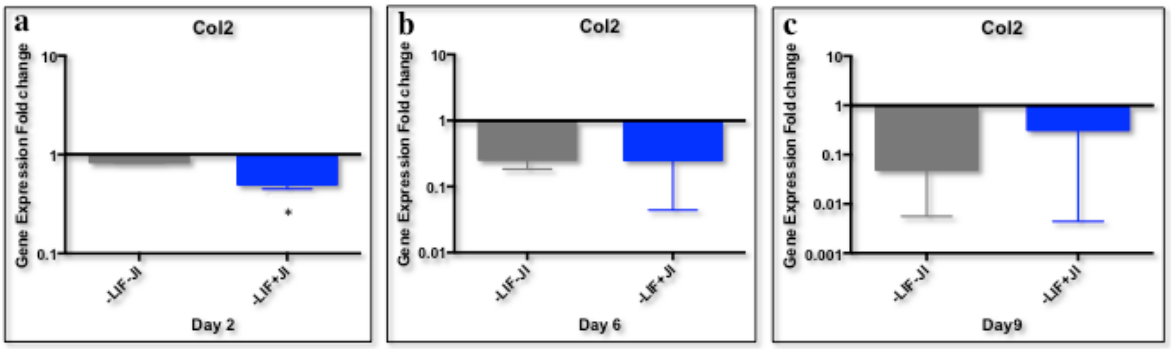

Figure 18. Chondrocyte gene expression is downregulated in the presence of JAK inhibitor on non-transduced MEFs. Non-transduced MEFs were cultured in the presence of JI and without JI, and later compared and analyzed for the presence of the chondrogenic marker Col2 at days 2, 6 and 9. The figure shows that Col2 was downregulated at days 2 (P= 0.012, *P<0.05), 6 and 9 in both conditions, demonstrating that there is effect of JI on MEFs.

MEFs do not undergo spontaneous chondrogenesis after transduction and in the presence of LIF or JI

After observing the effect of JI on MEFs, it was decided to determine whether any spontaneous chondrogenesis occurred during the first stage of transdifferentiation, before the chondrocyte media was present. Therefore, three groups of transduced MEFs: +LIF/–JI, LIF/+JI, and -LIF/-JI were grown for 9 days. Samples at day 2, 6 and 9 were collected for RNA isolation and subsequent gene expression analysis. The transcription factor Sox9 was analyzed at all the time points. These results indicated that there was no significant difference in Sox9 gene expression in any of the groups at any day (Figure19a). At the

same time, to indirectly observe if there was any activation of a cartilage protein, Col2 gene expression was also analyzed. Col2 did not show any up regulation in any group at any time (Figure 19b)

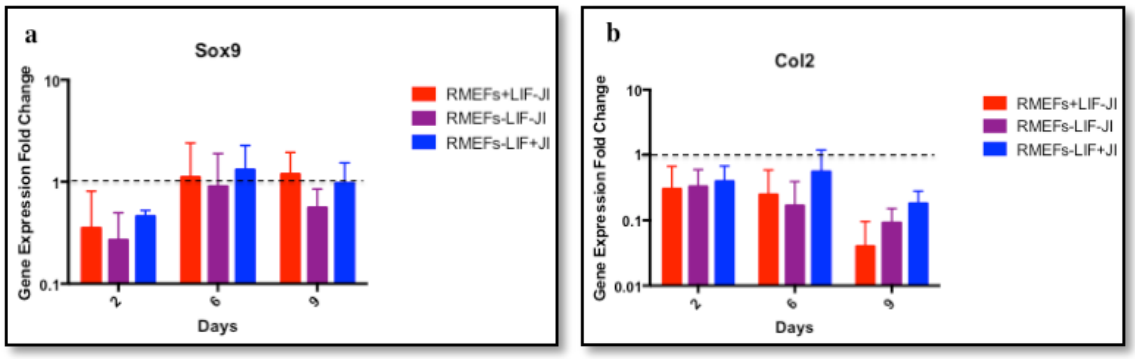

Figure 19. MEFs do not undergo spontaneous chondrogenesis during the first stage of transdifferentiation either in the presence or absence of LIF or JI. OSKM-transduced MEFs were cultured in three different condition (-LIF/+JI, -LIF/-JI and +LIF-JI) and samples at days 2, 6 and 9 were analyzed for chondrogenic gene expression (Sox9 and Col2). Figure shows that both Sox9 and Col2 genes were not significantly upregulated at any time and in any condition. There was a noticeable, but not apparent downregulation of Col2 among groups, which is consistent throughout the days assessed.

Transdifferentiation Kinetics

After analyzing the effect of JI and characterizing the chondrocyte aggregate formed by transdifferentiation**,** a gene expression profile of how the chondrocyte markers were expressed during this process was determined. Transduced MEFs were transdifferentiated under the same conditions mentioned above. Cell samples were collected at days 2, 6, 9, 12, 15 and 36, and RNA was isolated from each of the samples for later gene expression analysis. Gene expression fold change of chondrocyte markers *Sox9, Col2, Col10* and *Mmp13* relative to mouse embryonic fibroblast (MEFs) was assessed for each of the time points.

During the first stage, at days 2 and 6 the four-chondrocyte markers were down regulated as expected because no chondrogenic differentiation media was there to support the expression of these genes (Figure 20). At day 9 the gene Col10 appears to be the only one upregulated (Figure 20). At this point the rest of the markers remained low. During the second stage, cells are exposed to chondrogenic growth factors. Here genes Sox9, Col10 and Mmp13 were immediately upregulated as shown at day 12 (micromass). Interestingly, Col2 remained downregulated even in the presence of chondrogenic factors. At day15, a reduction in the expression of Sox9 and Col10 and a concomitant increase in the expression of Mmp13 was observed (Figure 20a). Finally, by day 36 the profile is maintained with a slight reduction on the expression of Sox9. The expression of the genes Col10 and Mmp13 genes remained similar to what was observed on day 15 (Figure 20a).

The effect of JI was also analyzed by comparing transdifferentiated MEFs in a conducive media (+JI) to transdifferentiated MEFs in a non-conducive media (-JI). Here cells in the absence of JI showed that Sox9 and Col2 maintained the same pattern as cells in the presence of JI (Figure 20b), whereas Col10 was downregulated. Mmp13 showed less expression under no JI conditions but at the same time was the only upregulated gene (Figure 20b). Overall, Mmp13 was the only gene upregulated in the absence of JI by day 15.

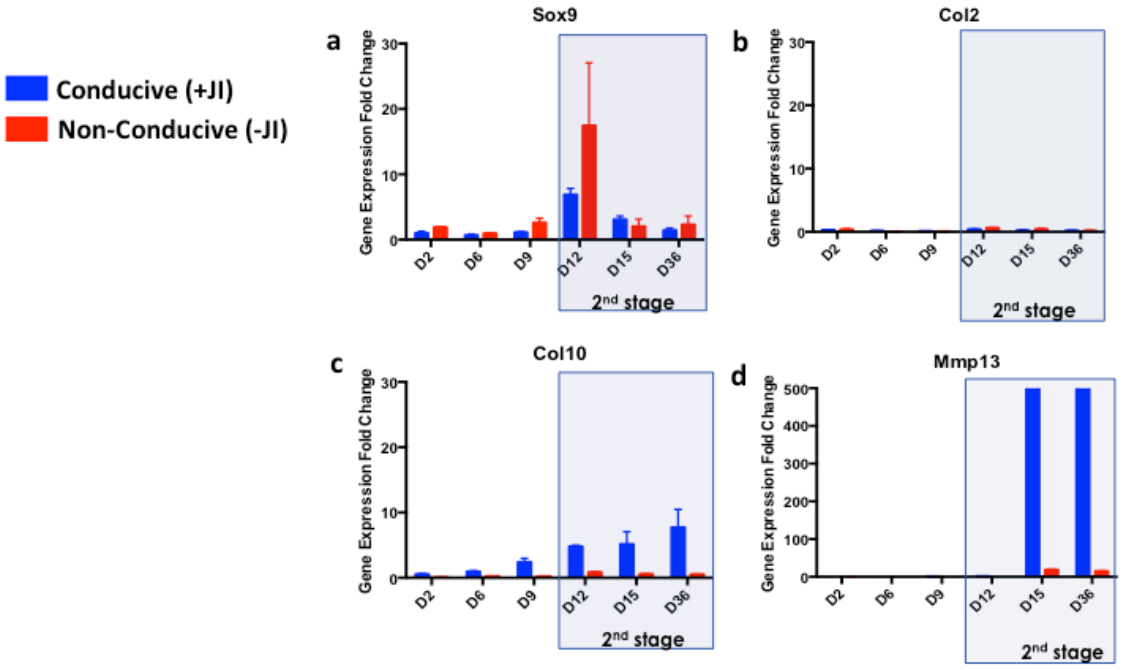

Figure 20. Effect of JI to the chondrogenic gene expression kinetics of transdifferentiated aggregates. Cells were transdifferentiated (day 2 to 36) under a conducive and non-conduce media and compared a) Sox9 expression kinetics, demonstrated its upregulation at day 12 (2^{nd} stage) with later gene expression reduction towards the end of the process. Levels of expression were similar for both conditions. b) Col2 expression kinetics was not upregulated in either condition at any time in the process. c) Col10 was higher expressed on cells under a conducive media and increased with time. Col10 was never expressed under a non-conducive media. d) Finally Mmp13 gene upregulated under a conducive media at day 15 and was maintained until day 36. Mmp13 gene was not present on a non-conducive media.

JI increases efficiency in transdifferentiation into cartilage by avoiding bone formation

Understanding how each chondrocyte marker behaves during transdifferentiation provide us with a good understanding of what stage in chondrogenesis is occurring during the process. Interestingly, it was observed that as soon as cells were exposed to a chondrogenic differentiation media, there was an upregulation of the gene Mmp13 (Figure 20a). Mmp13 is an enzyme and degrades the cartilage matrix for bone formation, thus this transdifferentiation process might lead to bone formation. For that reason, MEFs that were subjected to transdifferentiation under the same conducive conditions (-LIF+JI) were analyzed for bone markers. As a control, MEFs that were transdifferentiated in a non-conducive media (-LIF-JI) underwent the same transdifferentiation process. Cell samples were collected at Day 36. RNA was isolated from each of the samples for later gene expression analysis. Gene expression fold change of the bone markers RunX2, Sp7 and ACP5 was analyzed relative to mouse embryonic fibroblast (MEFs). Surprisingly, RunX2, Sp7 and ACP5 gene expression was significantly lower compared to the non-conducive (-JI) condition. ACP5 showed the highest expression in the non-conducive condition. These results might suggest that the presence of JI increases the efficiency of transdifferentiating MEFs into cartilage by avoiding bone formation.

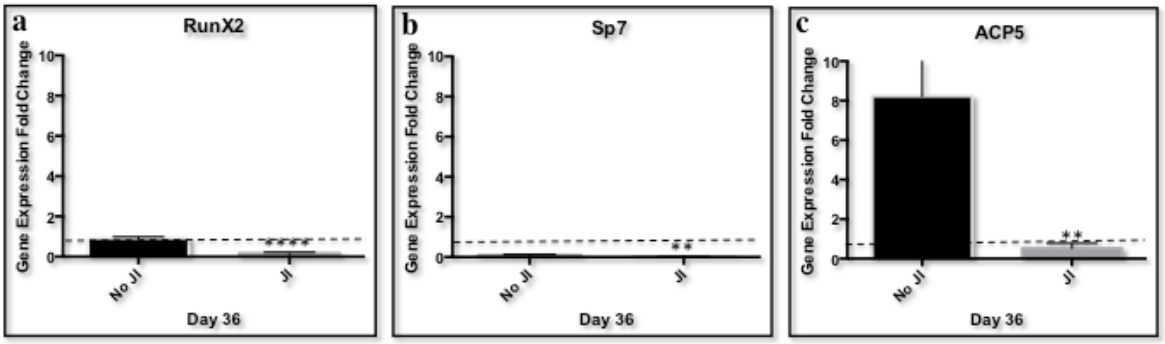

Figure 21. Bone markers gene expression is stimulated in the absence of JI during transdifferentiation. OSKM-transduced MEFs were cultured in two different conditions: a conducive (+JI) and in a non-conducive media (-JI), then analyzed for bone marker gene expression (RunX2, Sp7 and ACP5). Gene expression was normalized to MEFs. Figure shows that in both conditions, Runx2 and Sp7 are downregulated, however the levels are less in the presence of JI. The marker ACP5 is highly upregulated (7-fold) in a non-conducive media compared to the conducive conditions. This might indicate that JI makes transdifferentiation into cartilage more efficient by not letting the cell form bone.

CHAPTER 5

Discussion

Terminally differentiated cells have the ability to be reprogrammed to become iPSCs, which are close to embryonic stem cells (ESCs) in mouse. This provides the possibility to attempt personalized medicine without causing immune-rejection. Nevertheless, generating iPSC-derived cells and tissues is an inefficient and slow process.

Transdifferentiation is a reprogramming process that uses lineage specific transcription factors as a faster alternative to derive tissue-specific cells. With this process, a terminally differentiated cell reprograms into a desired differentiated cell type. In order to use transdifferentiation for clinical applications, the molecular and epigenetic changes during this reprogramming process need to be understood. Currently, transdifferentiation approaches use different transcription factor cocktails that rely on a specific cell type (Ieda et al., 2010, Hiramatsu et al., 2013). Thus, the process of reprogramming is different with every transcription factor cocktail. For this reason, a set of transcription factors that can be used in the transdifferentiation of every type of cell would be a good solution (Zhang Y. et al., 2012). The Yamanaka (OSKM) factors were proposed for transdifferentiation into any type of cell. These factors cause a series of modifications in the nuclei driving cells into a plastic open state, and upon direction allowing them to transform into the desired cell type. Because of the importance of the OSKM factors, the reprogramming process is already well understood, bringing the possibility to introduce transdifferentiation to the clinic.

The objective of these studies was to demonstrate the successful transdifferentiation of mouse embryonic fibroblasts into chondrocytes and cartilage via two stages. First, using the Yamanaka (OSKM) transcription factors to partially reprogram fibroblasts, by including a Jak Inhibitor (JI) and not adding LIF. Secondly, these cells were directed to a chondrogenic lineage by exposing them to a chondrogenic differentiation media early on in the process of reprogramming. Evidence of true transdifferentiation process is provided, together with the assessment of transdifferentiation kinetics by gene expression profiling.

Establishing Transdifferentiation

Prior to transfecting cells with OKSM factors, we investigated the ability to transfect MEFs with a retroviral vector that we packaged in house. Accordingly, cells were first transduced with a vector that contained DsRed protein. This vector had the same backbone as the vectors containing the pluripotency factors. After transduction, MEFs were observed under a fluorescent microscope at different days to determine the expression of Ds-red, and also this signal was quantified by flow cytometry. MEFs fluoresced red starting at day 3. The first day where fluorescence was detected is in agreement with other studies where transduction was undertaken with vectors that had the same backbone (Hiramatsu et al., 2013 and Takahashi K. et al., 2006). The highest signal detected by flow cytometry was at day 7, and maintaining it until day 9. This information was a good indicator that MEFs were being transduced with the viral particles that contained the OSKM factors.

Preliminary transdifferentiation study of MEFs into cardiomyocytes

Results from the preliminary study with MEF transdifferentiation into cardiomyocytes were very successful. MEFs were able to be transdifferentiated into cardiomyocytes using the OSKM factors, obtaining beating cells by day 15. These results turned out to be 10 days faster than when using the cardiac differentiation protocol on iPSCs generated in the laboratory (Shafa M. et al., 2012). Efe and colleagues (2011) generated cardiomyocytes by MEF transdifferentiation with OSKM also under partial reprogramming conditions, but applying different cardiac growth factors (BMP4). Similarly, they obtained higher efficiency in cardiomyocyte formation compared to cardiac differentiation from iPSCs (Efe et al., 2011).

The presence of the markers ANF, α-MHC, and NKX2.5 confirmed the effective transdifferentiation. Among the cardiac markers that Efe et al. (2011) analyzed, they also assessed NKX2.5 (transcription factor that regulates heart development) and found that NKX2.5 was upregulated at day 9. In contrast, in this preliminary study, it was found that NKX2.5 was first upregulated later, by day 11. The cardiac media used for this preliminary study might have delayed the upregulation of the NKX2.5 marker given that cells react differently depending the media to which they are exposed.

Small differences in media can affect cell proliferation of the same type of cell by (Adamzyk C. et al., 2013). For example, this study reported that a culture media composition used for human MSCs adversely affected the cell proliferation of ovine MSCs and because of that media components had to be changed. Specific factors, such as the fetal calf serum (FCS) content and the addition of supplements have to be adjusted for the type

of cell (Adamzyk C. et al., 2013). Nonetheless, the upregulation of NKX2.5 in our preliminary experiment demonstrated that the protocol for transdifferentiation was effective. Also based on the literature, it is suggested that the time for the full transdifferentiation might depend on the growth media to which the cells are exposed.

MEF transdifferentiation into chondrocytes

Cartilage aggregates were obtained after undertaking the same strategy of transdifferentiation outlined above, only using a chondrocyte differentiation media instead of cardiomyocyte differentiation media. The differentiation media used in this current study was previously developed by our group (Yamashita et al., 2009), and was reported to generate chondrogenic gene expression.

In this study, during the first state of transdifferentiation, JI was necessary to completely eliminate cells that could be responsive to LIF. LIF is not present in this transdifferentiation approach but some other cytokine from the same family such as neurotrophic factor (Peterson W. M. et al., 2000) and oncostatin M (Levy J. B. et al., 1996) can activate the (JAK/STAT) pathway (important in pluripotency reprogramming). In a similar transdifferentiation approach, Kim J. and colleagues (2011) found that when turning MEFs into neural progenitor cells, even in the absence of LIF cells these was still low expression of the pluripotency markers (Nanog and Rex-1) when compared to iPSCs. Therefore they decided to use a JI to inhibit this small number of LIF-responsive cells (Kim J. et al., 2011). From this evidence, to further reduce the possibility of intermediate iPSCs forming, it was decided to include a JI in the first stage of transdifferentiation.

Chondrocyte differentiation media with micromass formation was more effective in this transdifferentiation approach than with ESC-chondrocyte differentiation. For the second stage (day 10), cells were plated in 10 µl micromass cultures according to Yamashita and collaborator's protocol (2009). At this time, it was not clear whether the pre-iPSCs generated in stage 1 of this transdifferentiation protocol would be able to form micromasses. Yamashita et al., (2009) reported that when differentiating ESCs into chondrocytes using this approach, discordant cells die by apoptosis, undergoing a selection process where only cells that will turn into those of the chondrogenic lineages survive (Yamashita et al., 2009). We observed that forming micromass did not compromise the MEF-chondrocyte transdifferentiation. Also, in contrast to Yamashita's et al., (2009) results, cells successfully formed cartilage aggregates and did not separate from the micromass and die. This observation suggested that using a micromass approach in transdifferentiation for chondrocyte derivation is more efficient than deriving chondrocytes from ESC differentiation via micromass culture (Yamashita et al. 2009).

The presence of chondrogenic markers in transdifferentiated aggregates confirmed the successful chondrocyte formation. The markers Sox9, Col2, Col10 and Mmp13 were chosen for the characterization of these cartilage aggregates because they are present at different stages of chondrogenesis. Sox9 is a transcription factor that orchestrates the expression of collagen genes such as Col2 and Col10. Col2 along with others, form the cartilage specific collagens that will later serve as a scaffold. Next, proliferating chondrocytes express Col10 gene among others markers (Goldring 2012). Mmp13 participates at the end of the process by degrading the cartilage matrix for mineralization to be initiated (Murphy G., 1995 and Goldring 2012). These results were compared to the

ESC-chondrocyte differentiation work of Yamashita et al., 2010, for two reasons: 1) they differentiated ESCs using micromass; and 2) the differentiation media that they used, is the same used for this transdifferentiation work. Upregulated Sox9 and Col2 markers on aggregates collected at day 36 demonstrated that MEF were transdifferentiated into chondrocytes. Col10 and Mmp13 were also upregulated, indicating that the collagen matrix may be being degraded for bone formation. In accordance with this assessment, Yamashita et al., 2010 at day 40, observed the expression of these same markers. They also reported that this gene expression pattern indicated that these cells were prone to form hypertrophic chondrocytes for later bone formation given that Mmp13 was upregulated.

Further, a cartilage structure was observed by staining of transdifferentiated aggregates. H&E stained sections of transdifferentiated aggregates clearly showed a cartilage like structure for hyaline and hypertrophic cartilage, with no other type of tissue detected throughout the aggregates. Interestingly, transdifferentiated aggregates showed similar structure to aggregates that were characterized at different stages of ESC-differentiation into cartilage (Yamashita et al., 2010). This suggests that transdifferentiation develops similar to ESC-cartilage differentiation. Also, transdifferentiated aggregates clearly showed glycosaminoglycan (GAGs) deposition when stained with alcian blue or safranin-o. Upon transplantation of transdifferentiating aggregates into SCID mice, only hyaline and hypertrophic cartilage was observed. These structures are consistent with the structures found in the *in vitro* sectioned aggregates.

Chondrocytes arose from fibroblast transdifferentiation

Transdifferentiation is a process where one somatic cell is reprogrammed into another somatic cell. Therefore, a key step is to have a pure population at the beginning of the process. MEFs in this case are isolated from embryos; therefore these might carry contaminating chondrocytes that could give false positive results following transdifferentiation.

MEFs are likely to carry other types of cell given that they come from the embryo. However our results indicated that the starting MEF population was very uniform and did not contain a significant number of chondrocytes. The flow cytometry results showed that 96% of MEFs expressed the fibroblastic marker H2kk only. Also, 0.8% of the population appeared to be expressing both fibroblastic H2kk and chondrogenic marker Col2.

The remaining chondrogenic cells likely were not able to interfere with transdifferentiation because of their limited ability to survive. It has been previously observed that chondrocytes are difficult to expand because they rarely divide (Watt F. M., 1988). In addition to that, when chondrocytes are cultured in a monolayer at low densities, they tend to differentiate into fibroblasts (Watt F. M., 1988). In this study, there were only a few chondrocytes detected with MEFs. Hence, when cultured at low densities, chondrocyte survival would be minimal because the media used would not contain the proper growth factors to promote cell division and survival.

On the other hand, if chondrogenic residual cells survive in the MEF population, once plated for transdifferentiation, they are not likely to be reprogrammed with the OSKM factors. The RNA double stranded viral particles used for transduction need to convert the RNA to DNA and then integrate into the host DNA. Furthermore the reverse transcriptase

from the host cell (MEFs) must convert this strand into DNA, and for that, the cells need to be able to divide.

For transdifferentiation, MEFs were plated at 3.5 X 10^4 and if we consider the 0.8% of chondrocytes, there are approximately 350 chondrogenic cells present at the beginning. Since chondrocytes only double after 3 days, and considering that these might not divide (quiescent G0 state), they would not be able to reverse transcribe the RNA from the virus. Chondrocytes then, most likely die and the MEFs are the only cells that are being partially reprogrammed (Adler K. et al., 1999).

Nonetheless, MEFs as well might contain some multipotent cells (mesenchymal cells). These cells could either have been partially reprogrammed and transdifferentiated. Also, cell fusion of multipotent cells with partially reprogrammed MEFs during micromass culture could have also contributed to chondrocyte formation. Therefore, for better transdifferentiation assessment another source of fibroblast should be used and compared. For example, fibroblast specific GFP marked transgenic mice could be used to isolate fibroblasts from MEF populations via flow cytometry. Alternatively, a different source of fibroblasts could be used, for example from mouse-tail tips or skin.

Transdifferentiated MEFs into chondrocytes do not go through an iPSCs intermediate state

Pluripotency markers Nanog and Rex-1 are not upregulated during Transdifferentiation

The four OSKM transcription factors have been used to reprogram cells to pluripotency. However, using them to partially reprogram cells (no LIF and with JI) for transdifferentiation raises the question of whether these factors still have the capacity to form intermediate iPSCs. When comparing transdifferentiated cells under the above conditions to MEFs undergoing pluripotency (iPSCs), a higher expression of Nanog and Rex-1 genes was observed on MEFs undergoing pluripotency. The same trend in gene expression was found in a similar study where MEFs were transdifferentiated into neural progenitors with the OSKM factors (Kim J., et al., 2011). In that study, they compared the expression of Nanog and Rex-1 by differentiating iPSCs into neural progenitor cells to transdifferentiating MEFs into neural progenitor cells. In differentiating iPSCs, the expression levels of Nanog and Rex-1 were significantly higher and were expressed early in the process compared to transdifferentiating cells. In transdifferentiated cells, expression of Nanog and Rex-1 did not appear at any time (Kim J., et al., 2011).

There is no endogenous Oct4 signal during the partial reprogramming stage

Oct4 has been determined to be a good marker for intermediate iPSCs (Mikkelsen T. S. et al., 2008). This is also one of the factors that is used for the partial reprogramming in transdifferentiation. Although this is an indirect assessment, we wanted to characterize

the population in order to fully determine the presence of intermediate iPSC. Thus we decided to observe the signal of exogenous and endogenous Oct4 during transdifferentiation by flow cytometry.

Flow cytometry results from days 2, 6 and 9 (first stage of transdifferentiation) showed a signal from exogenous Oct4 and no signal from endogenous Oct4, thus supporting the gene expression results presented above. These findings suggest that no intermediate iPSCs are formed, since endogenous Oct4 was never present during this stage of transdifferentiation. In addition to that conclusion, it was assumed that partial reprogramming was happening while bringing the cells to an accessible chromatin state because an exogenous Oct4 signal was present.

Lack of tumor formation following chondrogenic transdifferentiation

Compared to iPSC chondrocyte differentiation, chondrocyte transdifferentiation can substantially reduce the risk of tumor formation. Normally, iPSCs (and ESCs) have the potential to form teratomas when transplanted this a major limitation slowing the clinical application of pluripotent stem cells (Amariglio N., et al. 2009 and Jiang Z. et al., 2014). This can occur when residual cells within a differentiating population are refractory to differentiation, remain pluripotent and are not removed from the population. Because JI prevents the formation of iPSCs, this could explain why we did not observe evidence of teratoma formation, which would have been easily observed by the presence of cells from lineages other than chondrocytes. Of course, since only two mice were transplanted with transdifferentiated chondrocytes, we cannot be emphatic about this conclusion. Future

studies to follow up this observation would have to include many more mice, which were also studied for considerably longer periods of time.

In addition to teratoma formation, iPSCs present a risk of giving rise to other tumor types. Using the mouse model, Yamanaka demonstrated that chimeras generated from certain lines of iPSCs developed cancer in adulthood (Okita K. et al., 2007). He later demonstrated that this potential for tumor formation could be significantly curbed by removing c-Myc from the reprogramming cocktail (Okita K. et al., 2007).

Recently, Hiramatsu and collaborators (2013) developed a chondrocyte transdifferentiation strategy by transducing dermal mouse fibroblasts with 3 transcription factors (Klf4, c-Myc and Sox9). Their direct conversion process was successful in finding a window of opportunity for hyaline cartilage formation, however some disadvantages have been suggested. Following evidence where c-Myc was reported to be responsible for iPSCs tumorigenesis (Okita K. et al., 2007), it is believed that the presence of c-Myc might be the reason for tumor formation in their studies. Even though is a very promising study which obtained hyaline cartilage was obtained, this transcription factor cocktail is not very likely to reach the clinic because of the presence of c-Myc.

Others investigators have argued that other reprogramming factors can play roles in tumorigenesis making it necessary to eliminate the integration of all reprogramming genes in both reprogramming and transdifferentiation. In a recent study, our lab has demonstrated that certain hiPSCs give rise to abnormal growths resembling adenocarcinomas when differentiated into chondrocytes using the micromass protocol presented in this thesis (Yamashita *et al.,* 2013). Although we did not observe similar tumor formation in our current transdifferentiation protocol, the studies were not

exhaustive. Moreover, our approach was not clonal, meaning that different starting MEFs procured different OKSM retroviral integration events making it difficult to detect tumorigenic events.

Nonetheless non-viral alternatives for reprogramming and transdifferentiation are better approaches to make this process available for clinical applications. Regularly, researchers look for non-viral alternatives aiming to avoid intermediate iPSCs, and rather obtain ultimate iPSCs. Non-viral options for the delivery of OSKM factors can be readily adapted to the transdifferentiation approach. In transdifferentiation, cells only need the stimulus to start the chromatin-remodeling cascade. Some of these non-viral approaches include using plasmids with the factors and delivering them with nanoparticles (Soldner F. et al., 2009), mRNAs (Yisraeli J. K. and Melton D. A. 2009), on minicircle DNA episomal vectors (Jia F. et al., 2010).

Transdifferentiation of MEFs into chondrocytes is a faster process compared to iPSCs derivation

Transdifferentiation of MEFs, formed mature chondrocytes by Day 15 (Figure 20), which is half the time required for reprogramming MEFs into iPSCs and then differentiating them into chondrocytes. We defined mature chondrocyte formation according to the characterization of the differentiation stages of the conversion of ESC into chondrogenic lineages described by Yamashita et al. (2010). In their work, hypertrophic chondrocytes exhibited the expression of the Col10 and Mmp13 markers.

Results in this transdifferentiation approach displayed the expression of Col10 and Mmp13 genes by day 15, which likely indicates hypertrophic chondrocyte formation. It has

been reported that reprogramming experiments with iPSCs formation, requires around 10-15 days for their first appearance in culture (Ramos-Mejia et al., 2012), which in comparison to transdifferentiation, is the same amount of days for transdifferentiated MEFs to become hypertrophic chondrocytes. In another study, turning osteoarthritic chondrocytes into iPSCs and then differentiating them into healthy chondrocytes took on average 34 days (Yiyong Wei, et al., 2012). This is double the time required for obtaining chondrocytes with the transdifferentiation approach. Overall, our results suggest that transdifferentiation of MEFs into the chondrogenic lineage using OSKM factors is faster than conventional methods of using iPSCs.

On the other hand, partial reprogramming with OSKM factors is a slower process when compared to other chondrogenic transdifferentiation approaches. In Hiramatsu and collaborators (2013) reported the direct conversion of MEFs into chondrocytes using Klf4, c-Myc and Sox9 transcription factors, and they found that their system led to form chondrocytes by day 7. In contrast to our protocol where we only found mature chondrocytes by day 15, suggests that their approach is faster, albeit problematic because of potential tumorigenicity. Our system might not be tumorigenic because it only does a partial reprogramming. The effect of c-Myc might not be fully present.

Characterization of transdifferentiation Kinetics

Transdifferentiation of MEFs into chondrocytes using the OSKM factors is a promising protocol that can easily be driven to form articular cartilage for regeneration (Hiramatsu et al., 2013). By having a good understanding of the gene expression profile of the chondrocyte markers in the procedure, it might be possible to find a window during the

process where cells can be manipulated in order to shift their fate to form articular cartilage.

Transdifferentiation does not have a window in time for hyaline cartilage formation

During the process of transdifferentiation, we observed an early upregulation of Col10 on day 6, as well as a later upregulation of Mmp13 on Day 12. This observation suggests that our transdifferentiation method may be more inclined towards the formation of hypertrophic chondrocytes for later development into bone (Zuscik M. J. et al., 2008). Although endochondral ossification does not occur *in vitro*, it could occur *in vivo* upon transplantation. Yamashita and colleagues observed this when ESCs were differentiated to chondrocytes in micromass culture (Yamashita et al., 2010). Hence, longer periods of incubation following transplantation would have also allowed us to better study whether cartilage transdifferentiation could lead to endochondral ossification following transplantation. However, this approach has the potential to find this window for hyaline cartilage formation with the advantage that it could be tumor free. A good example is that this window has been found before in another chondrocyte transdifferentiation approach.

Hiramatsu and colleagues (2013) were able to find this window in their protocol for hyaline cartilage formation by retaining expression of Sox9 as it has been reported that it delays hypertrophy (Akiyama H. et al., 2004). In their work, they used viral particle transduction together with doxycyclin inducibility of the transgenes. Hence, when Sox9 expression was maintained, cells remained in a hyaline cartilage-like state. In contrast, when Sox9 expression was not maintained, cells could move into hypertrophy.

Transdifferentiation into cartilage is more efficient under the presence of JI

Using similar transdifferentiation approaches, JI was reported to enhance transdifferentiation efficiency in cardiomyocytes and neural progenitor cells (Efe et al., 2011 and Kim J., et al., 2011). However, it has never been reported for chondrogenic transdifferentiation and thus, the effects are unknown. In this study, the gene expression profile of Sox9, Col2, Col10 and Mmp13 on cells that were transdifferentiated in a conducive media (+JI) were compared to transdifferentiated cells in a non-conducive media (-JI). Interestingly, when comparing the gene expression profile of transdifferentiated cells in conducive to a non-conducive media, Sox9 was present with similar pattern in both conditions. On the other side, Col10 showed to be upregulated in a conducive media whereas not present in a non-conducive media. Similarly, Mmp13 expression was higher in a conducive media compared to the non-conducive media (-JI). Overall, under a conducive media, Sox9, Col10 and Mmp13 were upregulated whereas; in a non-conducive media there was only a small amount of expression of Mmp13 gene. Mmp13 has been found to be involved in the degradation specifically of collagen type II, as it has been found to be overexpressed in OA cartilage tissue (Mitchell P. G. et al., 1996). In this scenario it might be that transdifferentiation without JI leads to poorer cartilage structure. This in consequence highlights how transdifferentiation efficiency increases upon the usage of a JI.

The gene expression profile of chondrocyte transdifferentiation suggests that it takes a similar path as does ESC chondrocyte differentiation, one that later leads to bone formation. Thus, it was decided to investigate expression of some bone markers on

transdifferentiated aggregates. The marker RunX2, which determines osteoblast lineage, Sp7 that directs the fate of cells to chondrocytes by blocking their differentiation into chondrocytes and ACP5, which is an acid phosphatase 5, tartate resistant protein were assessed. The results were compared to transdifferentiated cells cultured in the absence of a JI or non-conducive condition. ACP5 resulted highly expressed in the absence of JI compared to transdifferentiated cells in the presence of JI. The Sp7 and RunX2 genes were expressed slightly higher in the non-conducive (-JI) condition when compared to the condition with JI. These results suggest that the presence of JI is making transdifferentiation into chondrocytes more efficient by keeping the cells from going to a bone fate. JI benefits chondrogenic transdifferentiation in that it may keep the cells from continuing to bone formation. Thus, there is the potential to tailor this transdifferentiation approach into a hyaline cartilage fate perhaps by adjusting the JI exposure time. Other approaches to obtain hyaline cartilage can also be applied. For example, as previously mentioned, Sox9 while overexpressed in chondrocytes of mouse embryos, results in delayed hypertrophic chondrocyte differentiation (Akiyama H, et. al., 2004). Thus, the manipulation of Sox9 gene expression might be a good pathway to direct transdifferentiation into a more hyaline cartilage fate.

In this project, it was proposed that JI was directly inhibiting the downstream activation of the LIF-Jak/Stat3 pathway (Figure 7). Without a JI, Jak would activate Stat3 to be translocated in the nucleus and activate the transcription factor Klf4 as many others. In this case Klf4 would later participate in the activation of the pluripotency circuitry (Sox2, Oc4, Nanog) (Figure 7). Among the transgenes (OSKM factors) there is Klf4 and there was previously showed that, transgene expression is present since day 2 with a higher

level on day 9. Thus, it is possible that exogenous Klf4 is activating the pluripotency circuitry. On that note, there might be two possibilities 1) that exogenous Klf4 is not acting on LIF-Jak/Stat3 pathway and that JI is indeed blocking it and avoiding the activation of the pluripotency circuitry. 2) Klf4 is contributing to the activation of the pluripotency circuitry, but then JI is acting in a different fashion, and maybe not through this pathway. However, it is evident that JI is having a positive effect on the chondrogenic transdifferentiation, but the specific mode of action is unknown.

All together, the results discussed above demonstrate that transdifferentiation with OSKM factors with a partial reprogramming stage and direction with chondrogenic growth factors, can result in formation of a chondrocyte-cartilage tissue without going through an iPSC intermediate state, and is faster than using the iPSC route. In addition to that the findings suggest that transdifferentiation may follow a path towards bone formation, however JI seems to make the transdifferentiation more efficient into cartilage since it appears to be compromising the path to bone. Finally maybe the mode of action of JI in benefit of transdifferentiation is through another pathway (different that LIF-Jak/Stat3).

CHAPTER 6

Limitations, Conclusion and Future Directions

Limitations

Transdifferentiation of MEFs into chondrocytes demonstrated to be a more effective method than using conventional iPSCs approaches and more efficient based on no apparent tumor formation. Nevertheless, there were some limitations to complement the chondrocyte transdifferentiation approach with OSKM factors.

One aspect of this transdifferentiation protocol that could have been useful to obtain is, the number of cells that turned into chondrocytes at the end of the process. With that, efficiency under a different aspect could've been determined making this protocol stronger and more prone to be applied in humans.

We attempted to determine the number of positive transdifferentiated chondrocytes, however our approach was not successful. The biggest hurdle was that we obtained a tight cartilage aggregate, and because of that we could not manage to dissociate the cells from it. Hence we were not able to characterize the population by flow cytometry. A good alternative for this was to have used a genetically modified mouse that had a reporter gene after a chondrogenic marker as a tool to easily observe the exact time when cells started to acquire chondrogenic phenotype and estimate the number of cells turning into chondrocytes to determine efficiency.

Conclusion and Future direction

The main conclusions presented in this thesis are: 1) Utilizing OSKM pluripotency transcription factors to partially reprogram MEFs successfully gave rise to chondrocytes after exposing them to differentiation media; 2) Transdifferentiation did not rely on the formation of iPSCs during the transdifferentiation process; 3) transdifferentiation proved to be faster than forming iPSCs followed by differentiation, as well as being more efficient since no tumor formation was observed; and 4) JI may increase the efficiency of transdifferentiation by preventing bone formation. Overall this protocol of transdifferentiation demonstrated hypertrophic cartilage formation.

Transdifferentiation using the OSKM factors has some room for improvement. One is to replicate the system with an integration free approach to assure the safety of this method. Studies on the exposure times to JI in the process can also be assessed as this might help in finding a window for optimal hyaline cartilage formation.

Given the success of OSKM transdifferentiation in the mouse, OSKM transdifferentiation in human cells is likely to be effective as well, however studies on compromising reprogramming have to be carried. The transition of the method to human cells would not be a difficult one. Our laboratory focuses in the derivation of human iPSCs by non-viral routes, either with episomal plasmids or with small molecules. Therefore, the partial reprogramming part with human cells would be easy to adapt. For the second part of the transdifferentiation, a well studied chondrocyte differentiation media will be needed. In our laboratory, Yamanaka et al., (2013) were able to differentiate human iPSCs lines into cartilage. Hence, a differentiation system for human cells is available to direct human cells

into chondrogenesis. The importance of this transdifferentiation approach relies on its regenerative potential in joints. Thus, future human studies are necessary to prove its advantages and clinical applicability.

Finally, transdifferentiation has the potential to be taken to a bioreactor environment for the development of a bioprocess. Our laboratory innovator in using stirred suspension bioreactor for ESC expansion, and iPSCs derivation and expansion. Thus, this technology has the potential to evolve new diagnostic/therapeutic approaches in the era of personalized medicine with transdifferentiation.

In general, our transdifferentiation approach shows that partially reprogramming MEFs is sufficient to partial reprogram these cells to induce lineage specific cells in this case chondrocytes. This process has proven to have great potential to generate cartilage that could be used for tissue regeneration in a faster and more efficient manner than using iPSC alternatives.

APPENDICES A

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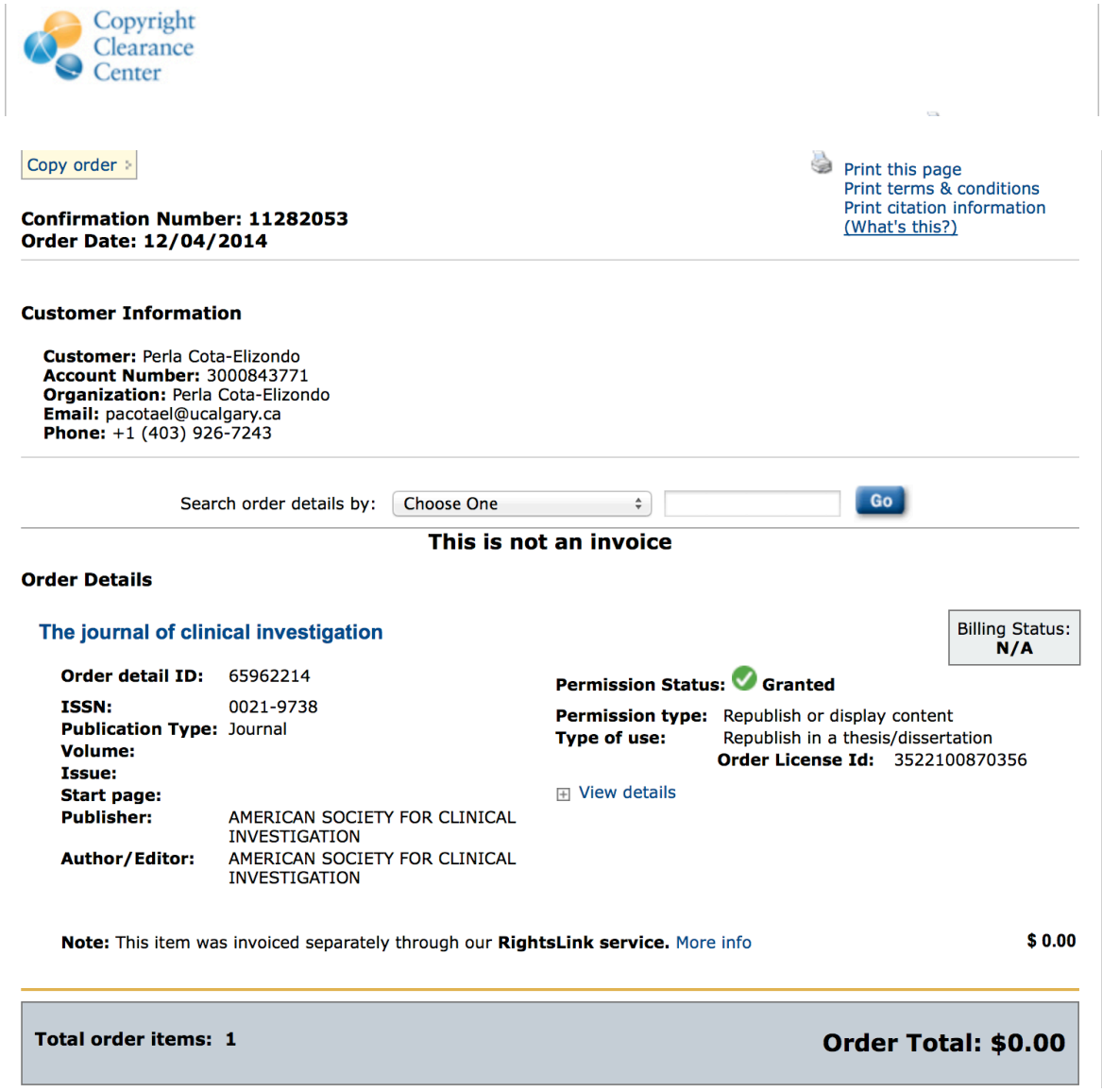

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