

**FUSION OF BOVINE FIBROBLASTS TO MOUSE EMBRYONIC STEM CELLS:  
A MODEL TO STUDY NUCLEAR REPROGRAMMING**

Maria Cristina Villafranca Locher

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Willard H. Eyestone, co-chair  
Roderick V. Jensen, co-chair  
Colin E. Bishop  
William R. Huckle  
Hehuang “David” Xie

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Blacksburg, Virginia

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## ACADEMIC ABSTRACT

### FUSION OF BOVINE FIBROBLASTS TO MOUSE EMBRYONIC STEM CELLS: A MODEL TO STUDY NUCLEAR REPROGRAMMING

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The cells from the inner cell mass (ICM) of an early embryo have the potential to differentiate into all the different cell types present in an adult organism. Cells from the ICM can be isolated and cultured *in vitro*, becoming embryonic stem cells (ESCs). ESCs have several properties that make them unique: they are unspecialized, can self-renew indefinitely in culture, and given the appropriate cues can differentiate into cells from all three germ layers (ecto-, meso-, and endoderm), including the germline, both *in vivo* and *in vitro*. Induced pluripotent stem cells (iPSCs) can be generated from adult, terminally differentiated somatic cells by transient exogenous expression of four transcription factors (Oct4, Sox2, Klf4, and cMyc; OSKM) present normally in ESCs. It has been shown that iPSCs are equivalent to ESCs in terms of morphology, gene expression, epigenetic signatures, *in vitro* proliferation capacity, and *in vitro* and *in vivo* differentiation potential. However, unlike ESCs, iPSCs can be obtained from a specific individual without the need for embryos. This makes them a promising source of pluripotent cells for regenerative medicine, tissue engineering, drug discovery, and disease modelling; additionally, in livestock species such as the bovine, they also have applications in genetic selection, production of transgenic animals for agricultural and biomedical purposes, and species conservancy. Nevertheless, ESC and iPSC lines that meet all pluripotency criteria have, to date, only been successfully produced in mice, rats, humans, and non-human primates.

In the first part of this dissertation, we attempted reprogramming of three types of bovine somatic cells: fetal fibroblasts (bFFs), adult fibroblasts (bAFs), and bone marrow-derived mesenchymal stem cells (bMSCs), using six different culture conditions adapted from recent work in mice and humans. Using basic mouse reprogramming conditions, we did not succeed in inducing formation of ESC-like colonies in bovine somatic cells. The combination of 2i/LIF plus ALK5 inhibitor II and ascorbic acid, induced formation of colony-like structures with flat morphology, that occasionally produced trophoblast-like structures. These trophoblast-like vesicles did not appear when an inhibitor of Rho-associated, coiled-coil containing protein kinase 1 (ROCK) was included in the medium. We screened for expression of exogenous OSKM vector with RT-PCR and found upregulation of OSKM vector 24h after Dox was added to the medium; however, expression was sharply decreased on day 2 after Dox induction, and was not detectable after day 3. In a separate experiment, we induced reprogramming of bFF and bAFs using medium supplemented with 50% of medium conditioned by co-culture with the bovine trophoblast CT1 line. These cells expressed both *OCT4* and the OSKM vector 24h after Dox induction. However, similar to our previous observations, both markers decreased expression until no signal was detected after day 3. In summary, we were unable to produce fully reprogrammed bovine iPSCs using mouse and human protocols, and the exact cause of our lack of success is unclear. It is possible that a different method of transgene expression could play a role in reprogramming. However, these ideas would be driven by a rather empirical reasoning, extrapolating findings from other species, and not contributing in our understanding of the particular differences of pluripotency in ungulates. Our inability to produce bovine iPSCs, combined with the only partial reprogramming observed by others, justifies the need for in depth study of bovine pluripotency mechanisms, before meaningful attempts to reprogram bovine somatic cells to pluripotency are made.

Therefore, we focused on getting a better understanding of bovine nuclear reprogramming. This would allow us to rationally target the specific requirements of potential bovine pluripotent cells.

Cell fusion is a process that involves fusion of the membrane of two or more cells to form a multinucleated cell. Fusion of a somatic cell to an ESC is known to induce expression of pluripotency markers in the somatic nucleus. In the second part of this dissertation, we hypothesized that fusion of bFFs to mouse ESCs (mESCs) would induce expression of pluripotency markers in the bFF nucleus. We first optimized a cell fusion protocol based on the use of polyethylene glycol (PEG), and obtained up to 11.02% of multinucleated cells in bFFs. Next, we established a method to specifically select for multinucleated cells originated from the fusion of mESCs with bFFs (heterokaryons), using indirect immunofluorescence. With this in place, flow cytometry was used to select 200 heterokaryons which were further analyzed using RNA-seq. We found changes in bovine gene expression patterns between bFFs and heterokaryons obtained 24h after fusion. Focusing on the bovine transcriptome, heterokaryons presented upregulation of early pluripotency markers *OCT4* and *KLF4*, as well as hypoxia response genes, contrasted with downregulation of cell cycle inhibitors such as *SST*. The cytokine *IL6*, known to increase survival of early embryos *in vitro*, was upregulated in heterokaryons, although its role and mechanism of action is still unclear. This indicates that the heterokaryon cell fusion model recapitulates several of the events of early reprogramming, and can therefore be used for further study of pluripotency in the bovine. The cell fusion model presented here can be used as a tool to characterize early changes in bovine somatic nuclear reprogramming, and to study the effect of different reprogramming conditions on the bovine transcriptome.

## GENERAL AUDIENCE ABSTRACT

### **FUSION OF BOVINE FIBROBLASTS TO MOUSE EMBRYONIC STEM CELLS: A MODEL TO STUDY NUCLEAR REPROGRAMMING**

Maria Cristina Villafranca Locher

The cells of an early embryo have the potential to give rise to any cell type found in the adult body. When these cells are transferred to a culture dish and kept under the right conditions, they become Embryonic Stem Cells (ESCs), and they retain the same developmental potential as the original embryonic cells they were derived from. In 2006, researchers in Japan found that it is possible to “reprogram” the cells of an adult individual (for example, fibroblast skin cells taken from a biopsy) to an embryonic state, by forcing the cells to express extra copies of genes that are normally active in embryos. These reprogrammed cells are called induced Pluripotent Stem Cells (iPSCs), and similarly to ESCs, they also have the potential to produce any cell type found in an adult organism. Lines of iPSCs from livestock species have possible applications in agriculture, species conservancy, biomedical industry, and veterinary and human health. Unfortunately, for reasons that are to date not fully understood, the technology to produce iPSCs has, so far, only worked in mice, rats, humans, and non-human primates.

We first attempted to produce bovine iPSCs by adapting methods and conditions used to derive iPSCs in mice and humans. We observed partial reprogramming of bovine cells, but were ultimately not able to produce true bovine iPSCs. This suggests that the bovine requires alternative/additional factors to induce reprogramming in adult cells. However, not knowing exactly what conditions or reagents will induce the reprogramming process in the cow, we decided to take a different approach. We focused on trying to understand how nuclear reprogramming works in the bovine. This would allow us to rationally target the specific requirements of potential bovine pluripotent cells.

It is known that the fusion (“merging”) of an adult cell with a stem cell, causes the adult cell to change its gene expression pattern to resemble a stem cell. We therefore fused mouse ESCs with bovine fibroblasts, and observed changes in bovine gene expression pattern as early as 24 hours after fusion. The gene expression changes observed resemble those found during early reprogramming of human and mouse iPSCs, and are accompanied by silencing of fibroblast specific genes. This suggests that our cell fusion model recreates the changes that happen during reprogramming, and can therefore be used as a tool to better understand pluripotency in the cow. The cell fusion method described in this dissertation can in theory be adapted to other species; by fusing somatic cells from other species to mouse ESCs, this model can be used to find species specific relevant pluripotency genes.



## DEDICATION AND ACKNOWLEDGEMENTS

*To everyone who in one way or another was part of this journey that hasn't ended yet.*

*--Azifri*



## OVERVIEW OF DISSERTATION

Following is a short description of each section contained in this dissertation:

### **Chapter 1. Pluripotency and how to induce it: Where does the cow stand in 2018?**

Literature review providing background information on the concepts and ideas that are relevant to understand the work presented in this dissertation. Also illustrates the current issues that we attempted to solve with this project, and the technologies available to do so.

### **Chapter 2. Reprogramming mouse embryonic fibroblasts to pluripotency using a polycistronic, doxycycline-inducible lentiviral vector**

A proof of principle chapter, in which we describe production of mouse induced pluripotent stem cells (iPSCs) in our lab following other researcher's protocols, so this technique can later be adapted to reprogram bovine somatic cells to pluripotency.

### **Chapter 3. Early loss of exogenous gene expression during bovine somatic cell reprogramming with a doxycycline-inducible polycistronic vector**

Describes the different attempts made to obtain bovine iPSC lines, using methods adapted from the literature. In the end, this approach failed to produce stably reprogrammed iPSCs, which serves as a rationale to focus on understanding bovine nuclear reprogramming instead of extrapolating methods in an empirical fashion.

### **Chapter 4. Fusion of murine and bovine fibroblast monolayers using polyethylene glycol (PEG)**

This chapter summarizes the optimization of a cell fusion protocol based on the use of polyethylene glycol (PEG), using mouse fibroblast cells first and then adapting it to bovine fibroblasts.

### **Chapter 5. The nuclei of bovine somatic cells fused to mouse embryonic stem cells express pluripotency markers similar to early reprogramming events**

Includes two main sections: (1) our efforts to specifically label and isolate fused cells composed of a mouse embryonic stem cell and a bovine fibroblast using several methods, and (2) RNA-seq analysis of mouse-bovine heterokaryons, in which we observed upregulation of some bovine-specific pluripotency markers as well as downregulation of fibroblast genes.

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## CHAPTER 1. PLURIPOTENCY AND HOW TO INDUCE IT: WHERE DOES THE COW STAND IN 2018?

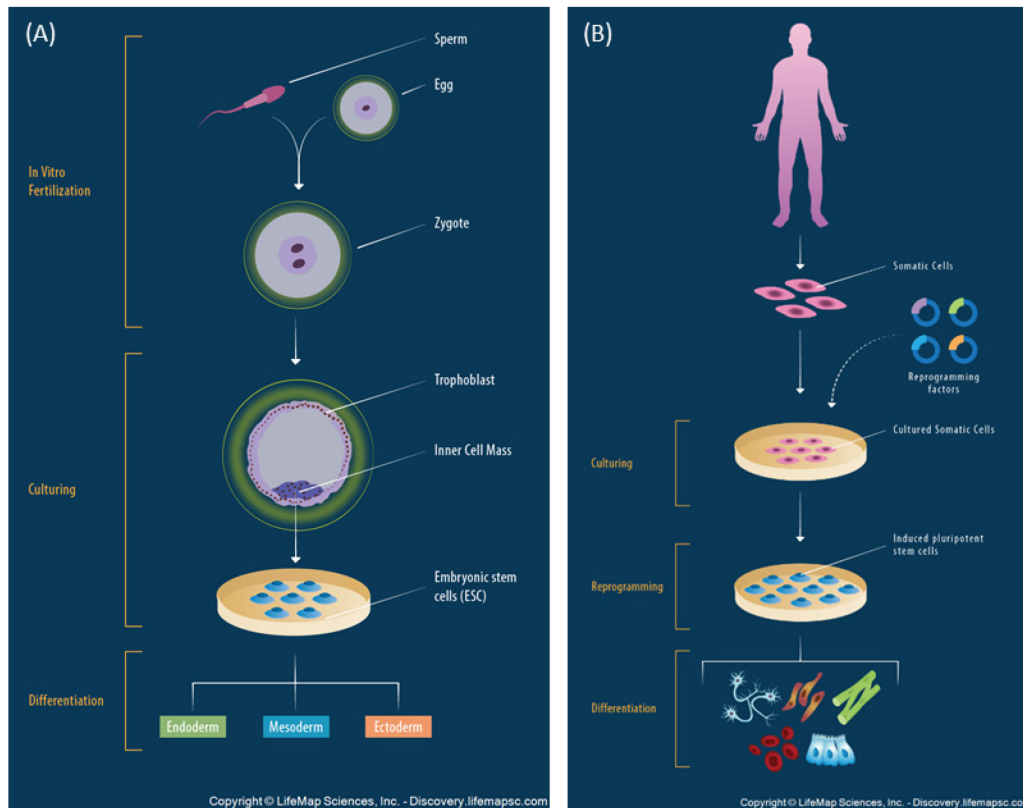
### 1.1 ABSTRACT

Pluripotency is a transient cellular state that occurs briefly during early embryo development. Pluripotent cells therefore have the capacity to give rise to all cell types present in an adult organism. If removed from the embryo and cultured *in vitro* under the right conditions, these cells become embryonic stem cells (ESCs), and retain their original capacity to generate cells from any lineage. Pluripotent cells can also be induced in adult, differentiated cells, by forcing the cell to express ESC genes; this causes “reprogramming” of the adult cell into induced pluripotent stem cells (iPSCs). Both ESCs and iPSCs are functionally equivalent; they can be cultured in the lab indefinitely, and given the appropriate cues can differentiate into any cell type *in vitro* and *in vivo*. These technologies, particularly iPSCs, have had an enormous impact in biomedical research for the species for which they have been derived. However, to date, ESCs and iPSCs only exist for a limited number of mouse strains, rats, humans, and non-human primates. In cattle and in many other species, no true ESCs or iPSCs have been produced, restricting the potential of this species for biomedical and agricultural purposes. Mammalian early embryo development is relatively conserved across species, and it has been assumed that methods used for reprogramming mouse and human somatic cells to a pluripotent state can be extrapolated to other species. However, despite many efforts, isolation of ESCs and iPSCs in the bovine has remained unsuccessful. This can be partially attributed to our limited understanding of the molecular mechanisms driving pluripotency and cell reprogramming in this species. If available, cattle iPSC lines could be used in agriculture, species conservancy, biomedical industry, as well as veterinary and human medicine. Here, we go over the concept of pluripotency in the bovine and review previous attempts to capture this transient state in a culture dish.

### 1.2 INTRODUCTION

Pluripotency can be defined as the capacity of a cell to differentiate into any cell type present in an adult organism. Pluripotent cells exist for a short time during early embryo development, and if these cells are isolated and maintained *in vitro* using the right conditions, they become **embryonic stem cells (ESCs)** ([Figure 1.1A](#)). ESCs have several properties that make them unique: (1) they can self-renew indefinitely in culture, (2) they are unspecialized, and (3) they can differentiate into specialized cells from all three germ layers (ecto-, meso-, and endoderm), including the germline. To date, ESC lines have been successfully derived from the embryonic inner cell mass of mice, humans, and non-human primates. However, in large domestic animals, particularly the cow, attempts to establish ESC lines from bovine embryos have not been entirely successful. We know from **somatic cell nuclear transfer experiments (SCNT; cloning)** that the nucleus of a somatic, terminally differentiated cell, can be reprogrammed to a totipotent state when implanted into an enucleated oocyte<sup>1-3</sup>, and can ultimately lead to the development of an adult organism. Although this demonstrated the plasticity of the cell’s nucleus, it was generally perceived that nuclear reprogramming was a complicated process, involving hundreds of genes and pathways. However, Takahashi and Yamanaka proved in 2006 that mouse adult fibroblasts can be reprogrammed to pluripotency by forced expression of four transcription factors expressed normally in embryonic stem cells<sup>4</sup>. These **induced pluripotent stem cells (iPSCs)** are morphologically and functionally equivalent to ESCs: they can be maintained in culture indefinitely, and can produce chimeric offspring when injected into early embryos, with contribution to tissues from all lineages ([Figure 1.1B](#)). The success of iPSC

technology first in mouse, and later in humans<sup>5</sup>, was greeted with enthusiasm by researchers studying other species, with the hope that this technology could bypass the lack of ESCs. However, to date, generation of iPSCs in species such as cattle has not been possible. In this review, we describe the current state of the art of bovine pluripotent cell lines (ESCs and iPSCs), with reference to mouse and human pluripotent cells, since these two species are usually taken as reference to develop technologies in cattle. We also describe the many uses that iPSC technology would have in veterinary and human medicine, agriculture, animal conservation, and biomedical industry. The need for pluripotent cell lines in the cow was the motivation behind the research described in the following chapters of this dissertation.



**Figure 1.1 Sources of pluripotent cells. (A)** Pluripotent cells exist for a short time in the inner cell mass (ICM) of mammalian early embryos. If these cells are properly isolated and cultured in the lab, they can be maintained in vitro as embryonic stem cells (ESCs). ESCs are able to self-renew in culture, and given the appropriate signals can differentiate into cells from all three germ layers. ESCs can also be implanted into an embryo, where they can resume development. **(B)** Induced pluripotent stem cells (iPSCs) can be generated by forced expression of transcription factors expressed in ESCs, using adult, terminally differentiated cells. iPSCs are equivalent to ESC in their morphology, pluripotency, and differentiation potential. Illustrations reproduced courtesy of LifeMap Sciences, Inc. (discovery.lifemapsc.com).

### 1.3 PLURIPOTENCY DURING EMBRYO DEVELOPMENT

Cells can be classified according to their **developmental potency**, this is, the different cell types they can potentially differentiate into. During early mammalian embryo development, the zygote and subsequent blastomeres are considered **totipotent**: they can produce cells from all three embryonic germ layers (ecto-,



meso-, and endoderm), the germline, and the placenta. After several rounds of cleavage divisions, the embryo forms a blastocyst: a spherical structure with a fluid filled cavity (blastocoel) and two distinct cellular structures: the inner cell mass (ICM) and the trophectoderm (TE). Cells from the TE will later form the placenta, whereas the ICM cells will give rise to cells from all three germ layers and the germ lineage. This more restricted developmental capacity of the ICM is defined as **pluripotency**. The ICM subsequently differentiates into two cell types: epiblast (cells closest to the TE) and hypoblast (in contact with the blastocoel). At this stage, the epiblast contains the cells that retain the capacity to produce the embryo proper, whereas the hypoblast is a transient structure which contributes to the development of extraembryonic structures and influences the axial patterning of the embryo. The epiblast is also considered to be pluripotent. From here on, the epiblast will continue its development and differentiation into cell types with a more restricted developmental potential<sup>6</sup>.

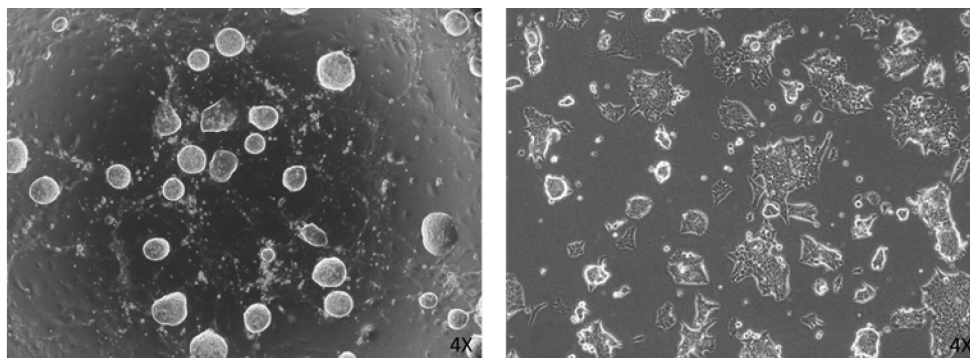
As a generalization, mammalian early preimplantation development is relatively conserved between species. However, timing of morphogenic events, as well as patterns of gene expression, are species specific. Mice and humans are both species where early embryo development has been studied extensively, and descriptions obtained from their study are often extrapolated to other species. However, embryo development in the bovine has some unique features that set it apart from other species. In mice and humans, the embryo implants into the uterus shortly after formation of epiblast and hypoblast; the bovine and many other domestic ungulates have a longer pre-attachment period (often referred to as delayed implantation), with extensive proliferation of extraembryonic tissue before attachment. Species differences are also reflected at the gene expression level. Although key pluripotency markers and regulatory networks are shared between mammalian species, there are important differences in timing and presence or absence of specific factors. For example, in mice, *OCT4*, *SOX2*, and *NANOG* (the “core” pluripotency regulators) are co-expressed in morulae and the ICM of blastocysts, whereas in human and cattle, only *OCT4* and *SOX2* are present in morulae, and co-expression of all three factors is only found in late (expanded) blastocysts. Moreover, *OCT4* in the bovine is not restricted to the ICM as in mice and humans, but is found in both ICM and TE.

## 1.4 EMBRYONIC STEM CELLS FROM EMBRYOS

From the previous section we know that, during mammalian embryo development, pluripotent cells are found for a short time in the ICM of the blastocyst. In 1981 two independent research groups described the isolation and *in vitro* culture of pluripotent cell lines from mouse ICM cells<sup>7,8</sup>. These *in vitro* adapted ICM cells were named ESCs. When kept in culture under the appropriate conditions, ESCs are capable of indefinite *in vitro* proliferation while maintaining a normal karyotype. Given the appropriate signals, they can differentiate into cells from all three germ layers *in vitro* (by formation of embryoid bodies) and *in vivo* (formation of teratoma when injected into immunodeficient mice). The most stringent criterion of pluripotency is the capacity to form a chimeric individual with germline incorporation following injection of ESCs into a morula or blastocyst. Formation of chimeras with germline transmission has only been proven for murine ICM derived ESCs, which are considered “true” (naïve) pluripotent ESCs. When mouse pluripotent cells are isolated from the epiblast of late, post implantation blastocysts, their capacity to form chimeras is reduced. Although still pluripotent, this distinct type of epiblast-derived (“primed”) ESC presents characteristics that set them apart from naïve ESCs ([Figure 1.2](#)).

The isolation of mouse ESCs (mESCs)<sup>7,8</sup> revolutionized mouse genetics. ESCs can be maintained in culture without risk of senescence, and genetic modifications can be introduced in the cells, which can later be selected, expanded, and transferred to an egg or blastocyst to produce clones or chimeras. Alternatively,

mouse ESCs (mESCs) can also be induced to differentiate *in vitro*, serving as a laboratory model to study genetic modification in different tissues. Since mESCs have the capacity to produce germline chimeras, this has permitted the generation of hundreds of genetically modified mice, making the mouse the primary mammalian model in biomedical research<sup>9</sup>.



	Naïve	Primed
Colony morphology	Dome-shaped	Flat
Disaggregation to single cell	Yes	No
Media supplements	LIF, 2i	FGF2, Activin A
Metabolism	OxPhos, glycolysis	Glycolysis
Chimera formation	Germline competent	Limited
X chromosome inactivation	No	Yes
Nanog expression	high	Low
Adhesion molecules	E-cadherin	N-cadherin

**Figure 1.2 Mouse naïve and primed ESCs.** Top left image: naïve, dome-shaped colonies of mouse ESCs (mouse strain C57BL/6) cultured in media supplemented with 2i/LIF, growing on mitotically inactivated mouse fibroblast feeder cells; top right image: mESC line D3 (mouse strain 129S2/SvPas) cultured in media supplemented with LIF on gelatin coated culture plates, showing a primed colony morphology with irregular borders. The table below describes some pluripotency-associated characteristics of mouse naïve and primed ESCs.

However, ESC lines from cattle and other species have proven difficult to isolate and maintain, and in many cases do not exhibit all pluripotency criteria, deeming these lines unsuitable for biomedical and pharmaceutical purposes. The isolation of bovine ESC (bESCs)-like cells was first reported in 1992 by Saito *et al.*, who described isolation of ESC-like cells from the ICM of *in vivo* produced blastocysts, cultured on mitotically inactivated mouse embryonic fibroblasts<sup>10</sup>. In this study, researchers described the propagation of slow growing cell clusters (compared to a parallel culture of mESCs) with homogeneous morphology and defined borders. These bESC-like cells presented a normal (euploid) number of chromosomes, and could be subcultured and maintained for at least four passages. Developmental potential was assessed by SCNT into enucleated *in vitro* matured oocytes. Some of the reconstituted

embryos reached the 8-16 cell stage *in vitro*, at which point the study was ended<sup>10</sup>. Since that initial report, researchers have attempted to isolate and culture ESCs from cattle using early cleavage embryos<sup>11,12</sup>, blastocysts from different stages<sup>13-17</sup>, ICM, and epiblast cells<sup>18</sup> ([Table 1](#)).

The choice of culture media used to derive bESC-like cells has been mostly adapted from work in mESCs. Freitas *et al.* (2011) described propagation and maintenance of bovine ICM cells derived from day-7 blastocysts for 6 passages in mESC culture conditions supplemented with leukemia inhibitory factor (LIF), on a layer of mitotically inactivated mouse embryonic fibroblasts (mEFs), before spontaneous differentiation occurred<sup>13</sup>. In this study, cells expressed pluripotency markers *OCT4* and *STAT3*, but their inability to sustain long term culture suggests that standard mESC culture conditions do not maintain bovine ICM in an undifferentiated state. The use of additives that have been found to promote stemness in mouse and human ESCs has greatly improved the quality of the obtained bESC-like cell lines. Verma *et al.* (2013) produced bESC-like cells in media supplemented with LIF and two small molecules that inhibit differentiation signals: glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 and mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) inhibitor PD184352; so called “2i”), and using laminin (a component of the basal lamina) coated plates instead of feeder cells. These cells expressed pluripotency markers *OCT4* and *NANOG* but were not able to produce teratomas<sup>19</sup>. It has been described that inhibition of Rho kinase (ROCK) activity enhances survival and colony formation of human ESCs (hESCs) after cryopreservation<sup>20</sup>. Furusawa *et al.* (2013) described isolation of bESCs directly from inner cell masses that were cultured on MEFs in the presence of 2i/LIF and ROCK inhibitor. These cells expressed pluripotency markers *OCT4*, *SOX2*, and *KLF4*, and formed embryoid bodies *in vitro*. However, the embryoid bodies did not express mesodermal markers<sup>21</sup>. Recently, mouse and human ESCs have been isolated in “3i” inhibitor system: PD, CHIR, and FGF receptor inhibitor SU5402. Kim *et al.* 2016 also used 3i to produce bESC-like cells that expressed both primed and naïve markers. These cells persisted for at least 50 passages, had a normal karyotype, formed embryoid bodies (EBs) *in vitro*, and teratomas in nude mice<sup>22</sup>. Thiazovivin (Tzv) is an even more potent ROCK inhibitor, known to improve derivation of mouse and human ESCs and iPSCs. Park *et al.* 2015 demonstrated a positive effect of Tzv in isolation, subculture and attachment of bESCs *in vitro*, with co-expression of pluripotency markers<sup>16</sup>. The *in vivo* differentiation potential of these cell lines has yet to be addressed.

With some exceptions<sup>19</sup>, putative bESCs are feeder-dependent<sup>10,12-18,21-26</sup>. Interestingly, work by Jin *et al.* (2012) showed that bESCs perform better when plated on mouse feeders instead of bovine fibroblast feeders<sup>14</sup>, which is attributed to the secretion of LIF by the mouse embryonic fibroblasts. More recently, Cong *et al.* (2014) cultured and propagated ESC-like cells derived from day-6 – day-11 whole blastocysts plated onto different types and combinations of feeder layers and found that mEFs and bEFs (at a ratio of 1:1) as well as STO cells, were superior to mEF alone in terms of colony morphology, cell adhesion, and number of passages (up to 10 passages) with demonstrated expression of pluripotency markers *OCT4*, *SOX2* and *NANOG*<sup>15</sup>.

**Table 1.1** Summary of publications describing isolation of bovine ESC-like cell lines.

Source of cells	Culture media additives*	Feeders/coating	Pluripotency indicators*	Length of culture	<i>In vitro</i> differentiation	<i>In vivo</i> differentiation	Reference
ICM (day 7)	LIF	MEF	Morphology, normal karyotype	4 passages	EBs	Clones to 16 cell stage	Saito <i>et al.</i> 1992
ICM (days 8-9)		STO	Morphology, AP+	not amenable to passage	EBs		Talbot <i>et al.</i> 1995
morula and ICM		MEF	Morphology	50 passages	EBs	Clones and chimeras not to term	Stice <i>et al.</i> 1996
ICM (day 7)		MEF	Morphology, AP-	>1 year		Germline chimeras to term	Cibelli <i>et al.</i> 1998
8-cell		MEF	Morphology, SSEA-1, SSEA-3, SSEA-4	>3 years	EBs	Unable to form teratomas	Mitalipova <i>et al.</i> 2001
ICM	LIF, EGF	STO	Morphology, AP+, Oct4, SSEA1, STAT3	15-20 passages	EBs	Clones to term	Saito <i>et al.</i> 2003
ICM	LIF, FGF2	MEF	Morphology, AP+, normal karyotype, Oct4, SSEA4	>1 year	EBs		Wang <i>et al.</i> 2005
Blastocyst (day 7)	LIF	MEF	Morphology, AP+, Oct4, Stat3		EBs		Freitas <i>et al.</i> 2011
Hatched blastocysts	LIF, bFGF	MEF	Morphology, AP+, Oct4, SSEA1, SSEA4	13 passages	EBs		Jin <i>et al.</i> 2012
ICM (days 7-8)	LIF, 2i, ROCK inhibitor	MEF	Morphology, AP+, Oct4, Sox2, Klf4, LIFR, SSEA-1	>15 passages	EBs (no mesoderm)	Chimeras not to term	Furusawa <i>et al.</i> 2013
ICM (days 7-8)	LIF, 2i	Laminin	Morphology, normal karyotype, SOX2, NANOG	8 passages	EBs	Unable to form teratomas	Verma <i>et al.</i> 2013
Blastocyst (days 8-11)	LIF, FGF	MEF, BFF, STO	Morphology, AP+, Oct4, Sox2, Nanog, SSEA1	10 passages	EBs		Cong <i>et al.</i> 2014
Blastocyst and ICM (day7)	3i, Thiazovivin	STO	Morphology, AP+, OCT4, SOX2, NANOG, e-cadherin, TRA-1-60	25 passages	EBs		Park <i>et al.</i> 2015
Blastocyst	3i	STO	Morphology, AP+, OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA-1-81	>50 passages	EBs	Teratoma	Kim <i>et al.</i> 2016
Blastocyst and ICM	CTFR, FGF2, IWR1	MEF	Morphology, AP+, Oct4, Sox2	>70 passages		Teratoma and cloned blastocysts	Bogliotti <i>et al.</i> 2018

\*List is not comprehensive. Refer to original publication for full details.

Abbreviations: **MEF**: mouse embryonic fibroblasts; **STO**: immortalized fibroblast cell line; **BFF**: bovine embryonic fibroblasts; **AP**: alkaline phosphatase; **EBs**: embryoid bodies; **CTFR**: custom growth factor-free media, **IWR1**: canonical Wnt signaling pathway inhibitor; **2i** (CHIR99021, PD184352), **3i** (CHIR99021, PD18435, SU5402), **ROCK inhibitor** (Y-27632).

Retention of a normal karyotype after long term *in vitro* culture is another concern. Mitalipova *et al.* (2001) generated a line of feeder-dependent bESCs that proliferated in culture for at least 150 passages (three years) at the time of publication<sup>12</sup>. Although these cells expressed pluripotency markers SSEA-1, SSEA-3, SSEA-4, and the c-Kit receptor, they exhibited an abnormal number of chromosomes (aneuploidy). Recent studies by Bogliotti *et al.* (2018) reported the generation of bESCs using a custom serum-free media supplemented with fibroblast growth factor 2 (FGF2) and an inhibitor of the canonical Wnt-signaling pathway (IWR1)<sup>17</sup>. With these conditions, the obtained bESC-like cells have retained a normal karyotype for over 70 passages, which is the longest time point reported so far.

Ultimately, the final “proof” of pluripotency is the generation of a reconstituted SCNT or chimeric offspring. Despite the difficulties for long term culture, many bESC lines have exhibited developmental potential both *in vitro* (embryoid body formation) and *in vivo* (teratoma and/or chimera formation). Stice and coworkers (1996) isolated both blastomeres and ICM, and using low passage putative bESCs tested their developmental potential by SCNT and chimera formation. Although pregnancies were confirmed, fetuses were eventually aborted possibly due to placental formation failure. The authors were able to confirm the bESC origin of the SCNT fetuses, and observed ~50% of bESC contribution in chimeric offspring<sup>11</sup>. Placental abnormalities and pregnancy loss is still, to date, an issue observed in SCNT derived animals<sup>27</sup>. Two years later, Cibelli *et al.* (1998) were able to obtain phenotypically normal live calves produced by injection of bESCs-like cells in day-3 *in vitro* produced embryos. Animals were chimeric for at least one tissue, including the germline<sup>25</sup>. More recently, Furusawa *et al.* (2013) described generation of chimeric blastocysts and fetuses that showed chimerism in the majority but not all of the analyzed tissues<sup>21</sup>. These observations indicate that bESC-like cells have limited *in vivo* differentiation potential, which is a concern if this technology is to be used to produce germline chimeras. Still, the lack of efficient germline transmission is not required to exploit other uses of bESCs, since they can be used to study *in vitro* development, differentiation, and disease modelling, as well as to generate transgenic cells<sup>28</sup>. Unfortunately, another aspect that hinders the usefulness of current bESC-like cells is their capacity to be dissociated to a single cell suspension. Mouse ESCs can be separated to single cells, which facilitates genetic manipulation of the cells and clonal selection of transgenic lines. Putative bESCs are very sensitive to enzymatic passaging<sup>12,21,23</sup>, and have to be manually disaggregated by pipetting or cutting of the colonies.

Finally, another aspect worth considering is the lack of commercially available bovine-specific reagents. For example, although short lived, most bESC-like cells have been generated using LIF<sup>10,13,15</sup>. However, the lack of commercially available bovine LIF (bLIF) means researchers have to use heterologous sources of LIF such as human (hLIF) or murine (mLIF). It has been reported that heterologous LIF sources such as human and murine have a detrimental effect on bovine blastocyst formation<sup>29</sup>. This adds an extra layer of complexity to an outstanding problem.

From the work described here, we can conclude that even though the technical procedures to isolate specific cell types from an embryo has improved considerably over the past decades, the knowledge of the particular species-specific mechanisms that promote and maintain pluripotency are still largely unknown, and often this knowledge is extrapolated from mouse and human research. In summary, most of the bESC lines described so far have not proven to be “true” ES cells, and even the lines that have been cultured for extensive periods of time and seem to have most if not all characteristics of pluripotency, have not yet been used as a biotechnological tool in a similar fashion to mouse and human ESCs, nor are these lines commercially available like their mouse and human counterparts<sup>30,31</sup>.

## 1.5 INDUCED PLURIPOTENT STEM CELLS FROM ADULT CELLS

Induced pluripotent stem cells (iPSCs) are pluripotent cell lines generated from adult cells (generally fibroblasts) by ectopic, forced expression of transcription factors found in mESCs. These cell lines are functionally equivalent to ESCs and, similarly to ESCs, have only been successfully established in mice<sup>4</sup>, humans<sup>5,32</sup> and non-human primates. Attempts to induce reprogramming of somatic cells from many species followed shortly after the initial reports of iPSC generation in mice and humans. In the bovine, researchers used similar or slightly adapted procedures to reprogram adult or fetal fibroblasts. In all cases, the same combination of transcription factors (*OCT4*, *SOX2*, *KLF4*, and *cMYC*: OSKM)<sup>33–36</sup> was used, sometimes including *NANOG*<sup>37</sup> and *LIN28*<sup>38,39</sup> (Table 2). The exact combination of transcription factors, growth factors and inhibitors required to reprogram bovine cells is still unclear, since none of the cell lines described so far meet the criteria of true iPSCs. Some researchers have found that OSKM is insufficient to generate bovine iPSCs (biPSCs), and have used *NANOG* and *LIN28* in addition to OSKM, hypothesizing that the bovine needs these additional factors and possibly others for successful reprogramming<sup>37–39</sup>. In contrast, others have reported the production of biPSCs using OSKM only, but with addition of small molecules in the culture media<sup>33,35</sup>. In addition to LIF, other agents such as bFGF, and 2i, forskolin (an activator of protein kinase A (PKA)) have been reported to have a beneficial effect on reprogramming<sup>35</sup>.

In the mouse, residual expression of exogenous transcription factors makes the cells prone to genomic instability<sup>40</sup> and prevents them from acquiring a stable transcriptional program akin to mESCs<sup>41</sup>. All the biPSC lines reported so far are unable to completely silence exogenous gene expression. Moreover, Kawaguchi *et al.* (2015) used doxycycline (Dox)-inducible piggyBac vectors containing OSKM. In a Dox-inducible gene expression system, transgenes are only active when Dox is present in the culture media (Figure 1.3). In this report, biPSC lines were cultured for over 65 passages in media supplemented with 2i/LIF plus forskolin. However, when Dox was not supplied in the culture media, iPSCs changed their morphology and differentiated, no longer expressing endogenous pluripotency genes such as *OCT4*, thus showing a clear dependence on a constant supply of exogenous pluripotency factors<sup>35</sup>. This finding reinforces the idea of incomplete endogenous gene activation in biPSCs.

Despite the differences in reprogramming methods used to produce biPSCs, the body of work accumulated so far has consistently shown that: (1) biPSCs are unable to completely silence exogenous gene expressions, (2) do not have stably activated endogenous pluripotency networks and are therefore (3) dependent on constant exogenous supply of pluripotency factors to maintain a pluripotent state. This last point makes it impossible to derive footprint-free iPSCs that lack integration of viral vector sequences. Similar to ESCs, iPSCs have to be capable of unlimited proliferation *in vitro*, as well as the ability to generate all adult cell types, including functional gametes. These characteristics have not been demonstrated in the bovine by using mouse-adapted protocols. In depth understanding of nuclear reprogramming in cattle is therefore fundamental to adapt iPSC technology to this species.

**Table 1.2** Summary of publications describing generation of bovine iPSC lines.

Cell type reprogrammed	Reprogramming factors	cdDNA sequence	Vector	Colony formation	Feeders/coating	Culture media additives*	Vector silencing	Pluripotency indicators*	Length of culture	<i>in vitro</i> differentiation	<i>in vivo</i> differentiation	Reference
BAF	OSKM + Nanog	human	Retrovirus	12 days	MEF	LIF, bFGF	No	Morphology, AP+, normal karyotype, Oct4, Sox2, Nanog, SSEA-1, SSEA-4	>10 passages	EBs	Teratoma	Sumer et al. 2011
BFF (d55 fetus)	OSKM + Nanog + Lin28	Bovine	Retrovirus	21 days	MEF	bFGF	No	morphology, AP+, normal karyotype, Oct4, Sox2	>16 passages	EBs	Teratoma, cloned blastocysts	Han et al. 2011
BEF (d40 embryos)	OSKM	Bovine	Plasmid	10 days	Laminin	LIF, 2i	No	Morphology, normal karyotype, AP+, SOX2, Oct4, Nanog, Sall4	quiescent cells	EBs	Teratoma	Huang et al. 2011
BFF (2.5 - 4 month fetus)	OSKM	Human (O), porcine (SKM)	Lentivirus	4 days	MEF	LIF, bFGF	No	Morphology, AP+ (variable intensity), normal karyotype, Oct4, Klf4, Nanog, SSEA-1	>10 passages	EBs	Teratoma	Cao et al. 2012
BFF (2-3 month old fetus)	OSKM + Nanog + Lin28	murine	PiggyBac transposon	14 days	MEF	LIF, bFGF	No	Morphology, normal karyotype, AP+, OCT4, SSEA-1, and SSEA-3, endogenous OSKM, Nanog, Rex1	>40 passages	EBs	Teratoma	Talluri et al. 2015
bovine amnion-derived cells	OSKM	murine	PiggyBac transposon	8 days	SNL	2i, bovine LIF, forskolin	No	Morphology, AP+, normal karyotype, OCT3/4, SOX2, NANOG	>65 passages	EBs	Chimeric embryos (germline)	Kawaguchi et al. 2015
BFF (d40 fetus)	OSKM	bovine	PiggyBac transposon	8 days	STO	LIF, bFGF	not assessed	Morphology, AP+ (variable), normal karyotype, OCT4, NANOG, SOX2, E-CADHERIN, SSEA1 and SSEA4	50 passages	EBs	Teratoma	Zhao et al. 2017

\*List is not comprehensive. Refer to original publication for full details.

Abbreviations: **BAF**: bovine adult fibroblast; **BFF**: bovine fetal fibroblasts; **OSKM**: OCT4, SOX2, KLF4, cMYC; **MEF**: mouse embryonic fibroblast; **STO**: immortalized fibroblast cell line; **AP**: alkaline phosphatase; **EBs**: embryoid bodies; **2i** (CHIR99021, PD184352).

## 1.6 THE NEED FOR BOVINE PLURIPOTENT CELLS

Early attempts to obtain pluripotent cell lines from cattle was driven by the ultimate purpose of using them as a tool to produce transgenic animals and to study embryonic differentiation<sup>42</sup>. The lack of bESC lines that meet all the criteria of pluripotent cells led researchers to use alternative techniques to introduce genetic modifications in the cells. Animal cloning technology in the late 1990s was to a certain extent used as an alternative to produce genetically modified embryos; somatic cells were genetically modified and transplanted into enucleated oocytes, thus bypassing the need for pluripotent cells. However, technical difficulties and low efficiencies, as well as the limited lifespan of donor somatic cells, were major drawbacks of this approach. Later, the development of iPSC technology led to the thought that livestock iPSCs could replace embryo-derived ESCs, but similarly to ES cells, no biPSC cell method has so far given a reliable result. Recent advances in “designer” gene editing technologies (zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)), which unlike previous technologies can efficiently target one or more specific loci can, to a certain extent, circumvent the need for pluripotent cells. For example, CRISPR/Cas9 has been used to successfully introduce genetic changes in the early embryo. However, this does not address the absence of pluripotent cells to maintain and differentiate *in vitro*, with all the potential this technology has for agriculture, conservancy, and animal and human health. Therefore, iPSC technologies are still a need if large animal models such as the cow are to be used to their full potential. Here, we briefly describe the niches that we believe would benefit the most from bovine pluripotent cell lines.

### 1.6.1 GENETICALLY MODIFIED CATTLE FOR AGRICULTURE AND PRECLINICAL STUDIES

Although the subject of debate, biPSCs can be used as a starting point to create transgenic animals to improve agricultural production and/or create disease resistant animals. Because of their body size and lifespan, transgenic animals could also be used as animal models for preclinical studies. Humanized mice are still widely used as models in the biomedical field, despite the growing body of evidence suggesting that commonly used rodent models are inadequate for human comparative studies.

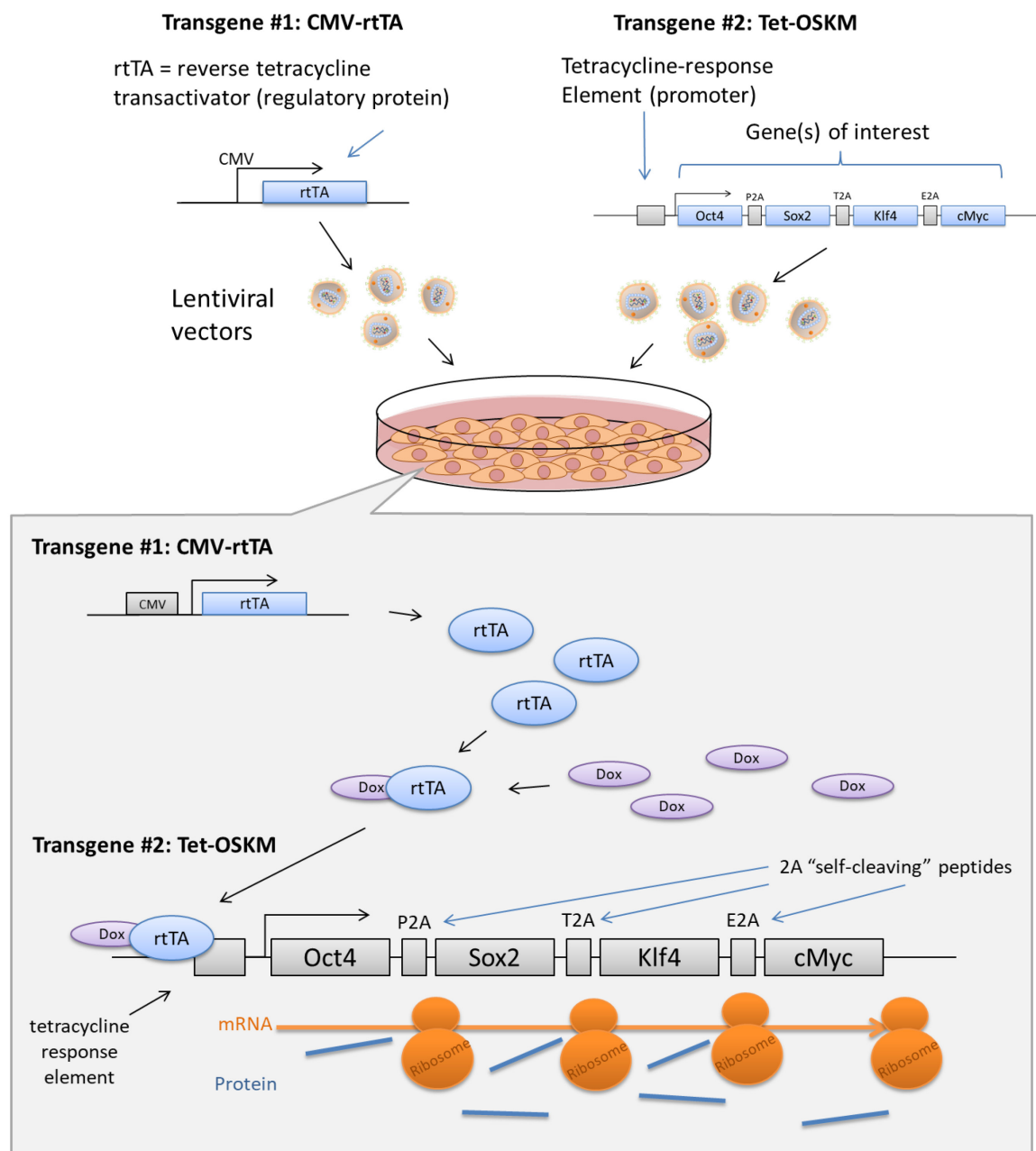
### 1.6.2 THE COW AS A BIOREACTOR FOR PRODUCTION OF THERAPEUTIC COMPOUNDS AND TISSUES

Due to their size and therefore large blood volume, transgenic large animals can be used as bioreactors for production of recombinant proteins. Although still in preliminary phases, several authors have already reported the generation of transgenic cattle producing human therapeutic polyclonal antibodies<sup>43,44</sup>, human lactoalbumin, human lactoferrin, and human bile salt stimulated lipase. The same gene editing technology can also be used to remove/replace potentially immunogenic or pathogenic proteins such as the prion protein<sup>45</sup>.

### 1.6.3 NATURALLY-OCCURRING ANIMAL MODELS

As stipulated in the laws of many countries, animal studies must always be performed before any clinical trial is realized in humans<sup>46</sup>. The vast majority of animals currently used in biomedical research are rodents, particularly mice. Mice are relatively inexpensive to house and maintain, reach maturity quickly, and breed rapidly. There are also hundreds of strains carrying a wide range of natural and directed mutations available, and several research facilities and companies offer services to produce transgenic mice *on demand*. However, results obtained in rodent models do not always translate to humans, and therapies shown to be successful in mice have often failed in humans<sup>47</sup>.





**Figure 1.3 Representation of the Dox-inducible Tet-On gene expression system.** Two lentiviral vectors harboring the rtTA driven by a constitutive (a.k.a. "always on") CMV reporter, and OSKM under the Tetracycline response promoter, are transduced into the cells. When Dox is added to the culture media, Dox and rtTA activate the tet response element and permit transcription of the OSKM into one mRNA. Sequences in the mRNA are separated by 2A self-cleaving peptides, which cause the ribosome to interrupt translation, producing four proteins out of one mRNA. The "self-cleaving" 2A peptides prevent the ribosome from covalently linking a new amino acid without interrupting translation. This is often referred to as "ribosomal skipping". This system permits the production of four proteins out of one mRNA. The method described in this figure is later used in [chapters 2](#) and [3](#).

Some species and breeds are naturally affected by diseases and conditions that are also found in humans. In cattle, bovine cutaneous neurofibromatosis presents resemblance with human neurofibromatosis type 1<sup>48</sup>, and recent studies have shown that bovinds have co-evolved and developed resistance to lentiviral proteins akin to primate lentiviral molecules<sup>49</sup>. The study of a disease that occurs naturally, rather than being lab-created, is often more predictive of how that condition will behave and ultimately respond to treatment. The information gained can benefit both human and animal health. In addition, if a naturally occurring disease can be studied *in vitro*, it would reduce cost and decrease the number of animals used. For domestic species, the study of naturally occurring diseases is not only humane but can improve the life of animals and inform human clinical trials<sup>50</sup>.

#### 1.6.4 DEVELOPMENTAL BIOLOGY

Mouse and human pluripotent cell lines have been essential to study development and differentiation, as well as the effect of chemical compounds during development. *In vitro* cattle embryo development is an active area of research that could benefit from bovine ESCs and iPSCs. Study of development *in vitro* can significantly reduce cost, and having cells of the same species as intended to study would increase the relevance of the results more than extrapolating results from one species to another.

#### 1.6.5 IN VITRO PRODUCTION OF GAMETES

As mentioned previously, pluripotent cells have the potential to produce cells from any lineage, including the germline. Mouse iPSCs have already been used to produce oocytes<sup>51</sup>, sperm, or both. The availability of this technology in cattle could have a significant impact in agriculture. For example, a breeding scheme could be developed using several rounds of *in vitro* gamete production, fertilization, and derivation of pluripotent lines that can in turn be used to generate new gametes. Genomic selection, instead of phenotypic selection, could be therefore used to produce genetically superior cattle within a shorter generational interval.

In addition, oocytes are routinely required in laboratories that study cattle embryo development and *in vitro* fertilization (IVF) technologies. For this purpose, oocytes are usually collected in abattoirs. Major drawbacks of this source of oocytes is the seasonality (bovine fertility decreases over hot months, and research labs often suspend IVF studies during the summer due to low yields), long transportation times, and often no knowledge of the phenotype or genetic background of the animals. *In vitro* produced oocytes would represent a source of research material with less genetic and environmental variability, aspects that are essential in early studies.

#### 4.6.6 CONSERVATION OF ENDANGERED SPECIES AND BREEDS

*In vitro* gamete production, in conjunction with assisted reproduction technologies, could also aid in the conservation of endangered species and breeds. Reduced population sizes result in genetic homogeneity due to inbreeding, which leads to detrimental effects such as poor reproductive performance<sup>52,53</sup>, increased neonatal mortality<sup>54</sup>, and increased susceptibility of diseases. Gametes could be produced from iPSCs derived from animals otherwise unable to reproduce, thus increasing the genetic pool. This approach has already been introduced to endangered species such as the white rhinoceros (*Ceratotherium simum cottoni*)<sup>55</sup>, drill (*Mandrillus leucophaeus*)<sup>55</sup>, and snow leopard (*Panthera uncia*)<sup>56</sup>. Researchers have produced iPSCs from these species, with the hope that gametes could later be produced. Additionally, pluripotent cell lines from endangered species can be used as an *in vitro* model to test and develop therapeutic applications for captive animals.

Rare and endangered cattle breeds could also benefit from these technologies. The Domestic Animal Diversity Information System of the Food and Agriculture Organization of the United Nations (FAO) indicates that there are more than 3200 reported cattle breeds<sup>57</sup>, of which around 30% are endangered. Breeds adapted to their local environments are a genetic reservoir of traits of ecological, agricultural, and economical relevance. Native cattle strains possess adaptive traits that give them a competitive edge over imported breeds, such as heat and drought tolerance, resistance to ticks and tick borne diseases, and resistance to trypanosomiasis. Having access to animal genetic diversity permits farmers to select traits or develop new breeds in response to changing environmental and economic conditions.

### 1.6.7 CARNICULTURE

Pluripotent cell lines with myogenic potential could be used as a starting point for developing carniculture, this is, *in vitro* cultured meat production<sup>58</sup>. The obtainment of cultured meat from satellite cells and adipose tissue-derived stem cells has already been demonstrated<sup>59</sup>, setting the base for this technology. However, the cell types used have limited proliferation capacity; the availability of true self-renewing bESCs or iPSCs that could be expanded extensively before differentiating them into skeletal muscle and fat tissue would give this technology its full potential<sup>9</sup>.

## 1.7 WHAT WE HAVE LEARNED SO FAR AND WHERE TO GO FROM THERE

Despite decades of effort, true bESCs or iPSCs that meet all pluripotency criteria and are therefore useful for biomedical applications have, so far, not been produced. This lack of *in vitro* bovine pluripotent cell lines means the full potential this species can offer has yet to be unlocked. SCNT experiments have shown that the nucleus of a somatic cell can be fully reprogrammed to an embryonic pluripotent state, and from there it can develop into an adult organism. From this we can infer that the lack of success in isolating bESC or generating iPSCs originates from the lack of knowledge we have in understanding the species-specific differences in pluripotency, as well as the pathways involved in pluripotency, specific growth factors, and culture conditions in this species. It is therefore fundamental to understand the particularities of bovine pluripotency and nuclear reprogramming, before meaningful attempts to generate pluripotent cells are made. Moreover, the low efficiency of reprogramming observed in mice and humans, indicates that current reprogramming protocols in those species can still be improved. As new understanding of the pluripotent state is made in mice and humans, it is possible that those findings can also be extrapolated to the bovine and other species, improving the derivation of pluripotent cells. However, considering the already known species differences, this approach is rather empirical, and does not directly contribute in our understanding of this species.

Researchers have shown for decades that the fusion of cells from different lineages and differentiation states results in changes of gene expression in the nuclei of the fused cells<sup>60</sup>. Interestingly, fusing somatic cells with pluripotent cells such as embryonic stem cells (ESCs)<sup>61-63</sup>, embryonal germ cells (EGCs)<sup>64</sup>, or embryonal carcinoma cells (ECCs) induces reprogramming of the somatic nucleus, suggesting that the reprogramming activity of the pluripotent cell is dominant over the gene expression pattern of the somatic cell. Changes in gene expression in cells fused to pluripotent cells happen in the absence of cell division, which makes cell fusion a powerful tool to study early modifications in gene expression. This approach is currently very relevant in understanding reprogramming<sup>65</sup> and differentiation<sup>66</sup>, both processes that constitute one of the fundamentals of regenerative medicine that are, to date, inefficient and poorly understood<sup>67</sup>.

At the beginning of this project, a series of publications reporting factors and conditions that increased efficiency of reprogramming in mice and humans, as well as reports of generations of biPSCs, were published. This, together with reports of successful derivation of biPSCs, motivated us to attempt reprogramming of bovine somatic cells using these described methods, but we were unsuccessful in producing biPSCs. Therefore, we diverted our research focus from trying to generate biPSCs with current methods, to understand early changes in bovine nuclear reprogramming. For this, we used cell fusion to mouse pluripotent cells (mESCs) to study the effect of the stem cell over the somatic nucleus. Due to differences in sequences between mice and cows, differential gene expression patterns can be detected. We anticipate that these differentially expressed genes will provide a better insight into cattle cell reprogramming, and hopefully be used to improve current ESC and iPSC protocols. In addition, this cell fusion model can, in theory, be adapted to study nuclear reprogramming in other species for which current iPSC technologies have failed, or to have better understanding of the process in species for which iPSCs exist but their efficiencies are low.

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## 1.9 ATTRIBUTIONS

This chapter was written by the author.

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## CHAPTER 2. REPROGRAMMING MOUSE EMBRYONIC FIBROBLASTS TO PLURIPOTENCY USING A POLYCYSTRONIC, DOXYCYCLINE-INDUCIBLE LENTIVIRAL VECTOR

### 2.1. ABSTRACT

Induced pluripotent stem cells (iPSCs) can be generated from fibroblast cells by transient exogenous expression of four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *cMyc*; OSKM) present normally in embryonic stem cells (ESCs). It has been shown that iPSCs are morphologically and functionally equivalent to ESCs, can proliferate in culture indefinitely, and differentiate into cell types from the three germ layers *in vivo* and *in vitro*. One method to control the expression of exogenous pluripotency genes in somatic cells during reprogramming is the use of Doxycycline (Dox)-based externally regulatable systems. Particularly, the Dox-inducible Tet-On system permits controlling the expression of transgenes because the activation of the promoter is dependent on the presence of Dox in the culture media. When reprogramming “difficult” species such as the bovine, knowing the exact timing of exogenous gene expression can be critical to understanding the reprogramming process. This makes the Dox-inducible Tet-On system an ideal option for monitoring the effect of gene overexpression over a set period of time. Our lab has not used these technologies in the past; therefore, as a proof of principle, we reprogrammed mouse embryonic fibroblasts harboring an Oct4-GFP reporter to pluripotency using a Dox-inducible polycistronic vector expressing OSKM. Pluripotent cells were alkaline phosphatase positive and we confirmed expression of pluripotency markers by RT-PCR and immunohistochemistry. Our mouse iPSCs were able to differentiate *in vitro* and express markers from all three germ layers. This work sets the foundation for our reprogramming attempts in the bovine species.

### 2.2. INTRODUCTION

Mouse<sup>1</sup> and human<sup>2</sup> induced pluripotent stem cells (iPSCs) can be generated by ectopic expression of four transcription factors present normally in embryonic stem cells (ESCs), namely: Pou5f1 (Oct4), Sox2, Klf4, and cMyc (hereafter referred to as OSKM). These cells are considered functionally equivalent to ESCs in terms of morphology, self-renewal and pluripotency. Although differences at the epigenetic and transcriptional level have been described<sup>3-5</sup>, it has recently been reported that these differences are due to variations in the genetic background of the cells<sup>6</sup>. In either case, these differences do not seem to affect pluripotency and differentiation potential of iPSCs, characteristics that make iPSCs a fundamental tool in regenerative medicine and the biomedical field in general<sup>7,8</sup>.

During reprogramming, following initial ectopic expression of OSKM the cells enter a transient early phase in which exogenous factors are gradually silenced whereas endogenous pluripotency genes become activated. Fully reprogrammed iPSCs are able to not only activate but also maintain endogenous pluripotency regulatory networks and are morphologically indistinguishable from ESCs<sup>9</sup>. It has been reported that iPSCs that fail to silence ectopic reprogramming factors are prone to genomic instability including chromosomal abnormalities<sup>10</sup>. One way to control transgene expression is to use a tetracycline-regulatable promoter<sup>11</sup>. Here, we used a four-factor (4F) doxycycline (Dox)-inducible polycistronic vector encoding cDNA sequences for murine OSKM separated by three different 2A peptides ([Figure 1.3](#))<sup>11</sup>. This Dox-inducible Tet-On system allows control over the time that the transgenes are expressed because the promoter is active only when Dox is present in the culture media. To function, the Tet-On system requires



the presence of two individual DNA constructs to activate transcription: the transcription regulatory unit (rtTA) driven by a constitutive cytomegalovirus (CMV) promoter, and the responsive element (promoter) linked to the OSKM coding region. In the presence of Dox, the rtTA binds to the responsive element which will activate transcription<sup>12</sup>. The OSKM sequences in the coding region are separated by “self-cleaving” 2A peptides, which are short (~18-22 aminoacids long) oligopeptides that prevent the ribosome from covalently linking a new amino acid, without interrupting translation. This results in a continuous translation process that yields to equal levels protein expression for the genes encoded in the same mRNA<sup>13</sup>.

Our lab has not used these techniques in the past. Therefore, to demonstrate their feasibility we tested the aforementioned reprogramming system in the mouse. We were able to obtain ESC-like cells and confirmed expression of pluripotency markers Oct4, Sox2, Klf4, cMyc, and Nanog by RT-PCR, and OCT4, SOX2, NANOG, TRA-1-60, and TRA-1-81 by immunohistochemistry. Colonies of iPSCs were also alkaline phosphatase positive. Pluripotent cells were able to differentiate *in vitro* and we observed expression of markers from all three germ layers: endoderm (Gata4), ectoderm (Gfap and Gata6), and mesoderm (Col1a1 and CD34). This work provides the foundation for transcription factor-based reprogramming procedures in the bovine species, which is described in Chapter 3.

## 2.3 MATERIAL AND METHODS

### 2.3.1 CELL CULTURE

Mouse embryonic fibroblasts (mEFs) harboring an Oct4-GFP reporter gene downstream of the endogenous Oct4 locus (Oct4-GFP mEFs) were purchased from PrimCells (mEFs (Oct4-GFP), Catalog # PCEMM03). mEFs were purchased from Millipore (PMEF-CLF-P1). The cell line 293T was purchased from ATCC (CRL-3216). Oct4-GFP mEFs, mEFs, and 293T cells were expanded in Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; HyClone) and 50 µg/ml gentamicin (Lonza) at 37°C in 5% CO<sub>2</sub> incubator. Medium was replaced every three days. Subculture of mEFs and 293T cells was done with 1X 0.25% Trypsin/EDTA (Corning) before they reached 90% confluence.

The mouse ESC line D3 (mESC-D3) was obtained from ATCC (CRL-1934) and first expanded on STO feeders (also from ATCC; CRL-1503) pretreated with 10 µg/ml Mitomycin C (Sigma), in medium consisting of DMEM supplemented with 15% ESC-qualified FBS (Gibco), 1X Non-Essential Amino Acids supplement, 1X Glutamax, 1,000 U/ml of ESGRO, 0.55 mM beta-mercaptoethanol, and 50 µg/ml Gentamycin. Media of ESC-D3s was changed every other day; passaging was performed with 1X 0.05% trypsin/EDTA (Corning) when colonies appeared dome-shaped but before they were able to reach each other. Cultures were trypsinized and plated (1:4 split) onto 0.1% gelatin-coated tissue culture plates in their appropriate culture media.

Induced pluripotent stem cells (iPSCs) were cultured on gamma irradiated mEFs (IRR-mEFs, GlobalStem) in medium consisting of KnockOut Dulbecco's minimal essential medium (KO-DMEM; Gibco) supplemented with 20% KnockOut Serum Replacement (KO-SR; Gibco), 1X Non-Essential Amino Acids supplement (HyClone), 1X Glutamax (Gibco), 1,000 U/ml of ESGRO (Millipore), 0.55 mM beta-mercaptoethanol, and 50 µg/ml Gentamycin at 37°C in 5% CO<sub>2</sub>. For passaging of initial iPSC colonies, colonies were manually selected using a glass capillary tube under a magnifying glass, transferred individually to one well of a 96 well plate containing 50 µl of 1X 0.05% trypsin/EDTA for 5 min, and pipetted gently to disaggregate cells to a monocellular suspension. An equal volume of iPSC media was used to inactivate trypsin and cells were pelleted by centrifugation, resuspended in iPSC media, and plated over

IRR-mEFs. Following this initial selection step, frequency of media changes and method of subculture was performed as described for mESC-D3.

### 2.3.2 LENTIVIRAL VECTOR PREPARATION

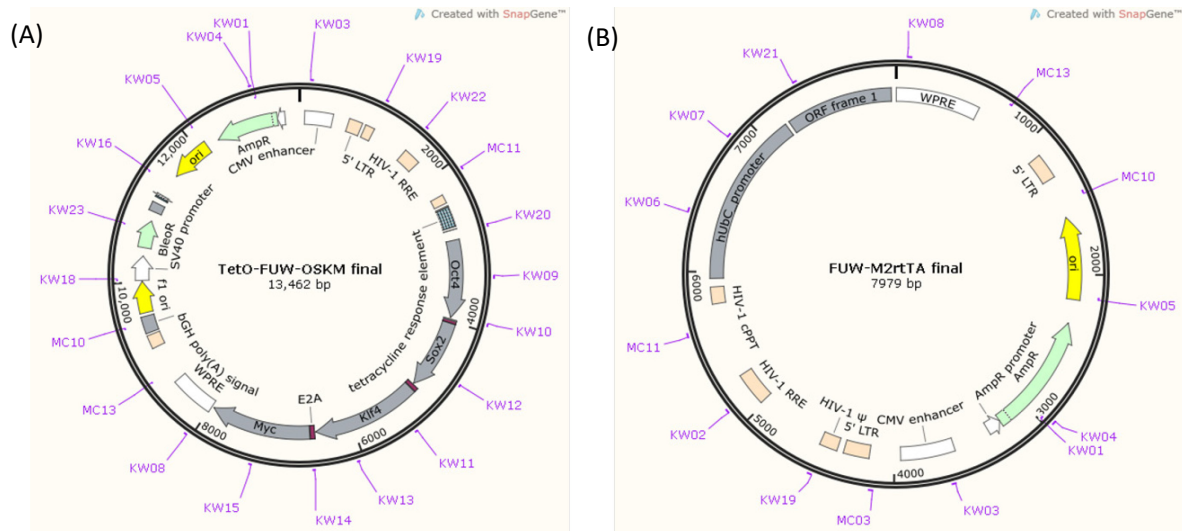
The reprogramming plasmids TetO-FUW-OSKM<sup>11</sup> and FUW-M2rtTA<sup>14</sup> have been previously published and were purchased from Addgene.com (Addgene plasmids #20321 and #20342, respectively). Upon arrival, bacterial stabs were cultured in Luria-Bertany (LB) agar plates supplemented with 100 mg/ml Ampicillin and incubated overnight at 37°C. Individual colonies were manually selected with a toothpick and grown in LB broth with 100 mg/ml Ampicillin overnight at 37°C. Plasmid DNA was extracted using the Zippy Plasmid Miniprep Kit (Zymo Research) following the manufacturer's instructions. Concentration and quality of DNA was measured using a Nanodrop 1000 spectrophotometer. DNA sequence of three clones was corroborated via Sanger sequencing (performed at the Virginia Bioinformatics Institute at Virginia Tech) using custom designed primers ([Table 2.1](#), [Table 2.2](#), and [Figure 2.1A and B](#)). Chromatograms were aligned in Lasergene SeqMan Pro. Large quantities of plasmid from one selected clone were obtained using PerfectPrep EndoFree Plasmid Maxi Kit (5Prime) according to the manufacturer's instructions.

**Table 2.1** Primers used for Sanger sequencing of TetO-FUW-OSKM.

<b>KW01</b>	<b>GCTATGTGGCGCGGTATTAT</b>	<b>KW15</b>	<b>TCGTCCGATTCCACGGCCTT</b>
<b>KW03</b>	CCCATATATGGAGTTCCGCG	<b>KW16</b>	ACCGAGCGCAGCGAGTCAGT
<b>KW04</b>	GAGTTGCTCTTGCCCGGCGT	<b>KW18</b>	CCACACCCTAACTGACACAC
<b>KW05</b>	TTCTGCGCGTAATCTGCTGC	<b>KW19</b>	AAGGAGAGAGATGGGTGCGA
<b>KW08</b>	CGCTATGTGGATACGCTGCT	<b>KW20</b>	GAACCGTCAGATCGCCTGGA
<b>KW09</b>	CTCACCTGGGCGTTCTCT	<b>KW22</b>	GTTCTTGGGTTCTTGGGAG
<b>KW10</b>	TGTTCCCGTCACTGCTCTGG	<b>KW23</b>	ACACGACCTCCGACCACTCG
<b>KW11</b>	AGACCGAGGAGTTCAACGAC	<b>MC10</b>	CACCCCCAGAATAGAATGACACC
<b>KW12</b>	CCTGCAGTACAACCTCATGA	<b>MC11</b>	TTCACCATTATCGTTTCAGACC
<b>KW13</b>	ACTGTCACCCTGGCCTGCCT	<b>MC13</b>	TAAGATCTACAGCTGCCTTG
<b>KW14</b>	TGGAGATGTTGAGAGCAACC		

**Table 2.2** Primers used for Sanger sequencing of FUW-M2rtTA.

<b>KW01</b>	<b>GCTATGTGGCGCGGTATTAT</b>	<b>KW08</b>	<b>CGCTATGTGGATACGCTGCT</b>
<b>KW02</b>	GTCTGGTATAGTGCAGCAGC	<b>KW19</b>	AAGGAGAGAGATGGGTGCGA
<b>KW03</b>	CCCATATATGGAGTTCCGCG	<b>KW21</b>	CTGCTCGATGCCCTGCCAAT
<b>KW04</b>	GAGTTGCTCTTGCCCGGCGT	<b>MC03</b>	CGTGTACGGTGGGAGGTCTA
<b>KW05</b>	TTCTGCGCGTAATCTGCTGC	<b>MC10</b>	CACCCCCAGAATAGAATGACACC
<b>KW06</b>	CTTGGTGAGTAGCGGGCTGC	<b>MC11</b>	TTCACCATTATCGTTTCAGACC
<b>KW07</b>	AGGTGTGCGGTAGGCTTTTC	<b>MC13</b>	TAAGATCTACAGCTGCCTTG



**Figure 2.1** Full schematic view of the (A) TetO-FUW-OSKM and (B) FUW-M2rtTA plasmids, showing Sanger sequencing primer annealing sites

To produce replication-incompetent Lentiviral vectors, we cultured 293T cells and passaged 3-4 times to ensure proper cell growth. Cells presented ~70% confluence at the time of transfection. Lentiviral plasmids TetO-FUW-OSKM or FUW-M2rtTA, together with four packaging plasmids encoding the lentiviral genes Gag-Pol (Addgene plasmid #12260), Tat (Addgene plasmid #14654), Rev (Addgene plasmid #12253) and VSV-G (Addgene plasmid #12259) were transfected with Lipofectamine 2000 (Invitrogen) or Xfect transfection reagent (Clontech) in 293T cells. Viral supernatants from 293T cell cultures producing each of the two vectors were harvested at 24, 48, and 72 h, pooled, centrifuged for 5 min at 400 g to remove floating cells, syringe-filtered through a 0.45  $\mu$ m filter (Millipore) and concentrated by ultracentrifugation at 4°C for 4 h at 16,500 g. Viral pellets were resuspended in Hanks balanced salt solution (HBSS; HyClone) overnight and stored at -80°C for up to 6 months. To calculate the titer of the lentiviral preparation, we transduced 293T cells and mEFs with several ten-fold serial dilutions of lentiviral vector suspension. Expression of the vector was assessed by indirect immunofluorescence of Oct4 ([Table 2.4](#); see [section 2.3.6](#) for immunostaining protocol). Cells expressing Oct4 were automatically counted using ImageJ. Viral titer was calculated using the formula: viral titer (IU/ml) = [infected cell number in a well] x [EGFP+%/100] / [amount of virus used (ml)].

### 2.3.3 TRANSDUCTION AND DOX INDUCTION

Cells to be reprogrammed were seeded the day before transduction in appropriate culture medium. After 24 hours, medium was replaced with media containing Polybrene (Millipore) at a final concentration of 8  $\mu$ g/ml and Lentiviral suspension. The dosage of lentiviral vector added to the cells was controlled by calculating the multiplicity of infection (MOI), using the formula:  $MOI = \text{viral titer (IU/ml)} \times [\text{amount of virus used for infection (ml)}] / [\text{target cell number}]$ . For all reprogramming experiments described in this chapter we used an MOI of 1. After 8-16 h the medium was replaced and cells were allowed to recover for 24 hours in mEF medium, before mouse iPSC medium supplemented with 1  $\mu$ g/ml Dox was added. Reprogramming in Oct4-GFP mEFs was replicated twice, and one colony was randomly selected for further analysis<sup>1</sup>.

<sup>1</sup> More colonies were initially selected, but the cells died during storage at -80°C when the freezer accidentally thawed over a weekend, before we had time to analyze them.

### 2.3.4 DIFFERENTIATION

Embryoid bodies (EBs) were formed by culturing disaggregated iPSC colonies in hanging drops<sup>15</sup>. Briefly, monocellular suspension of iPSCs was pipetted onto the lid of a Petri dish (Falcon) in a medium composed of half mEF medium and half iPSC medium without ESGRO. Cells aggregated together within 24-48 hours. The hanging drops were then washed off and seeded onto a Petri dish in fibroblast media for 7 days before RNA was extracted.

### 2.3.5 GENE DETECTION BY RT-PCR

For RT-PCR analysis, cells were first harvested with trypsin at 37°C for 5 min and pelleted by centrifugation. The pellet was washed once with DPBS and RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research). Concentration and purity RNA was assessed using a NanoDrop 1000 spectrophotometer. Synthesis of cDNA using total RNA was performed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Primers for mouse endogenous pluripotency genes (OSKM and Nanog), 4F reprogramming vector, ectoderm (GFAP and Pax6), mesoderm (Col2A1 and CD34) and endoderm (Amylase and Gata4) are shown in [Table 2.3](#). When possible, primers were designed to span across an exon-exon junction. End point PCR was carried with Taq PCR Master Mix (Qiagen) on a thermal cycler (Bio-Rad) according to the manufacturer's protocols. Following the reaction, samples were resolved on a 1% agarose gel with 1X Tris/Borate/EDTA (TBE) buffer.

**Table 2.3** Primers used for RT-PCR in this study.

Gene	Primers (5' -> 3')	Amplicon length (bp)
<b>Oct4</b>	F: CTAGGTGAGCCGTCTTTCCA R: CTCCACCTCACACGGTTCTC	763 bp
<b>Sox2</b>	F: ACCAGCGCATGGACAGCTAC R: CTCCCCTTCTCCAGTTCGCA	523 bp
<b>Klf4</b>	F: TGAATCCAAAGAAGGATCTCGGGC R: CACTGATGACCGAAGGGCTG	882 bp
<b>cMyc</b>	F: AGCTTACAGTCCCAAAGCCC R: TCGTCCGATTCCACGGCCTT	883 bp
<b>Nanog</b>	F: AAATCCCTTCCCTCGCCATC R: AAGGCTTCCAGATGCGTTCA	613 bp
<b>4F</b>	F: GTAGACTGCACATGGCCCAG R: GGGACCGCCTCTTGCTTAAT	997 bp
<b>Amylase2b</b>	F: GGGAGGACTGCTATTGTCCAC R: CACCACCCAGATCAATAACCTTG	520 bp
<b>Gata4</b>	F: AGACACCCCAATCTCGATATGTT R: GCGATGTCTGAGTGACAGGA	655 bp
<b>GFAP</b>	F: TCGACAACCTGGGTACCATGC R: TCTCCTCTCCAGCGATTCA	506 bp
<b>Pax6</b>	F: AGGGGGAGAGAACACCAACT R: GCATAGGCAGGTTGTTTGCC	485 bp
<b>Col1A1</b>	F: GGGTATGACGAGAAGGCTGG R: CTGGGGCCTTGATCACCTT	855 bp
<b>CD34</b>	F: TCATCTTCTGCTCCGAGTGC R: CAGCCTTCTCTGTAGGGC	483 bp

### 2.3.6 IMMUNOHISTOCHEMISTRY

Sterilized round 12 mm coverslips (Fisher) were placed into the wells of a 24-well plate (Falcon). Culture of iPSCs was performed as described in section 2.3.1. For staining, culture media was removed and cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and permeabilized with 0.2% Triton-X-100. Cells were blocked with 0.5X Odyssey blocking buffer (Licor biosciences. Diluted 1:1 in PBS) for 1 h at room temperature and then incubated with primary antibodies diluted in 0.5X blocking buffer for 1 h at room temperature, or overnight at 4°C. Following incubation, cells were washed three times with PBS and incubated with appropriate fluorescently labeled secondary antibodies diluted in 0.5X blocking buffer for 1 h at room temperature. Finally, samples were washed twice with PBS and the coverslips were carefully removed from the 24-well plates using forceps, and placed upside down on a glass slide with a drop of DAPI mounting media (Invitrogen). Specimens were analyzed on an Olympus fluorescence microscope and images were acquired with an Infinity 3 camera. The list of primary and secondary antibodies and their working dilutions are shown in [Table 2.4](#).

**Table 2.4** Primary and secondary antibodies used in this study.

Target	Antibody	Dilution	Manufacturer and catalog number
Oct4	Primary: Goat anti-human Oct4 polyclonal IgG	1:100	SantaCruz #sc-8629
	Secondary: Donkey anti-goat IgG	1:400	ThermoFisher #A11055
Sox2	Primary: Goat anti-human Sox2 polyclonal IgG	1:100	SantaCruz #sc-17320
	Secondary: Donkey anti-goat IgG	1:400	ThermoFisher #A11055
Nanog	Primary: Rabbit anti-mouse Nanog polyclonal IgG	1:200	SantaCruz #sc-33760
	Secondary: Donkey anti-rabbit IgG	1:400	ThermoFisher #A21206
Tra-1-60	Primary: Mouse anti-human Tra-1-60 monoclonal IgM	1:100	Millipore #MAB4360
	Secondary: Goat anti-Mouse IgM-TR	1:80	SantaCruz #sc-2983
Tra-1-81	Primary: Mouse anti-human Tra-1-81 monoclonal IgM	1:100	Millipore #MAB4381
	Secondary: Goat anti-Mouse IgM-TR	1:80	SantaCruz #sc-2983

### 2.3.7 ALKALINE PHOSPHATASE STAINING

Staining for alkaline phosphatase was performed with the Alkaline Phosphatase Detection Kit (Sigma-Aldrich) according to the manufacturer's instructions. Nuclei were stained with DAPI mounting media (Invitrogen).

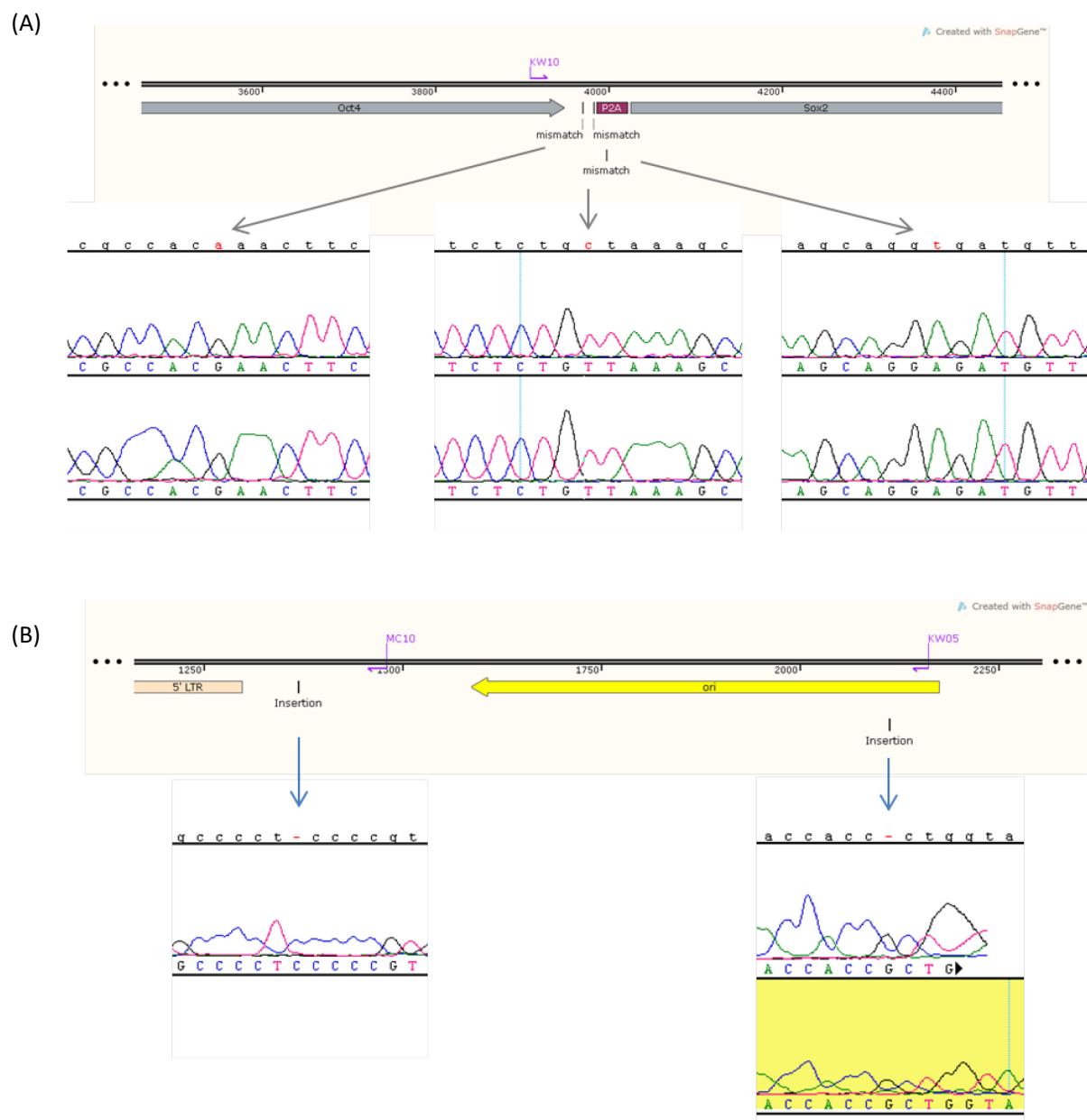
## 2.4 RESULTS

### 2.4.2 LENTIVIRAL PLASMID VECTORS PRESENT DISCREPANCIES WITH THE ADDGENE SEQUENCES

Full Sanger sequencing of both plasmid vectors revealed three base pair substitutions in TetO-FUW-OSKM ([Figure 2.2A](#)) and two insertions in FUW-M2rtTA ([Figure 2.2B](#)) when compared with the sequences provided in Addgene. As seen later, these discrepancies with the reference sequences did not interfere with the functionality of the plasmids or Lentiviral vectors.

### 2.4.1 LENTIVIRAL VECTOR SYSTEM PRODUCES HIGH YIELD OF LENTIVIRAL PARTICLES

Lentiviral particles were generated by transfection of TetO-FUW-OSKM, FUW-M2rtTA and four helper plasmids in 293T cells in the presence of Lipofectamine 2000 or Xfect. Cells appeared viable throughout the process and no excessive cell death was observed. Titration of TetO-FUW-OSKM and FUW-M2rtTA was carried once in 293T cells as a positive control, and later on every new batch of lentiviral vectors was titrated in mEFs before using. [Table 2.5](#) shows the titer of five batches of TetO-FUW-OSKM and FUW-M2rtTA in mEFs.



**Figure 2.2. Single base discrepancies found after Sanger sequencing of TetO-FUW-OSKM and FUW-M2rtTA plasmids. (A)** Location of three mismatched bases in TetO-FUW-OSKM; two are located in the linker region between the Oct4 sequence and the P2A region, whereas a third mismatch is located in the P2A sequence itself. **(B)** Location of the two extra bases found by Sanger sequencing of FUW-M2rtTA plasmid; both are located outside the viral portion of the plasmid, one in a linker region and the second one in the plasmid origin of replication.

**Table 2.5** Lentiviral vector titer (IU/ml) obtained from 5 independently produced batches of TetO-FUW-OSKM and FUW-M2rtTA vectors in MEFs. Batches 1 to 4 were produced using Lipofectamine 2000, whereas batch 5 was made with Xfect.

Volume	1	2	3	4	5
0.01 $\mu$ l		$5 \times 10^6$	$2 \times 10^7$		$2 \times 10^{11}$
0.1 $\mu$ l		$4 \times 10^6$	$6 \times 10^6$	$1 \times 10^6$	$2 \times 10^{10}$
1 $\mu$ l	$4 \times 10^5$	$3 \times 10^6$	$3 \times 10^6$	$3 \times 10^6$	$2 \times 10^9$

#### 2.4.2 DOX INDUCIBLE VECTOR CAN REPROGRAM MOUSE GFP FIBROBLASTS TO PLURIPOTENCY

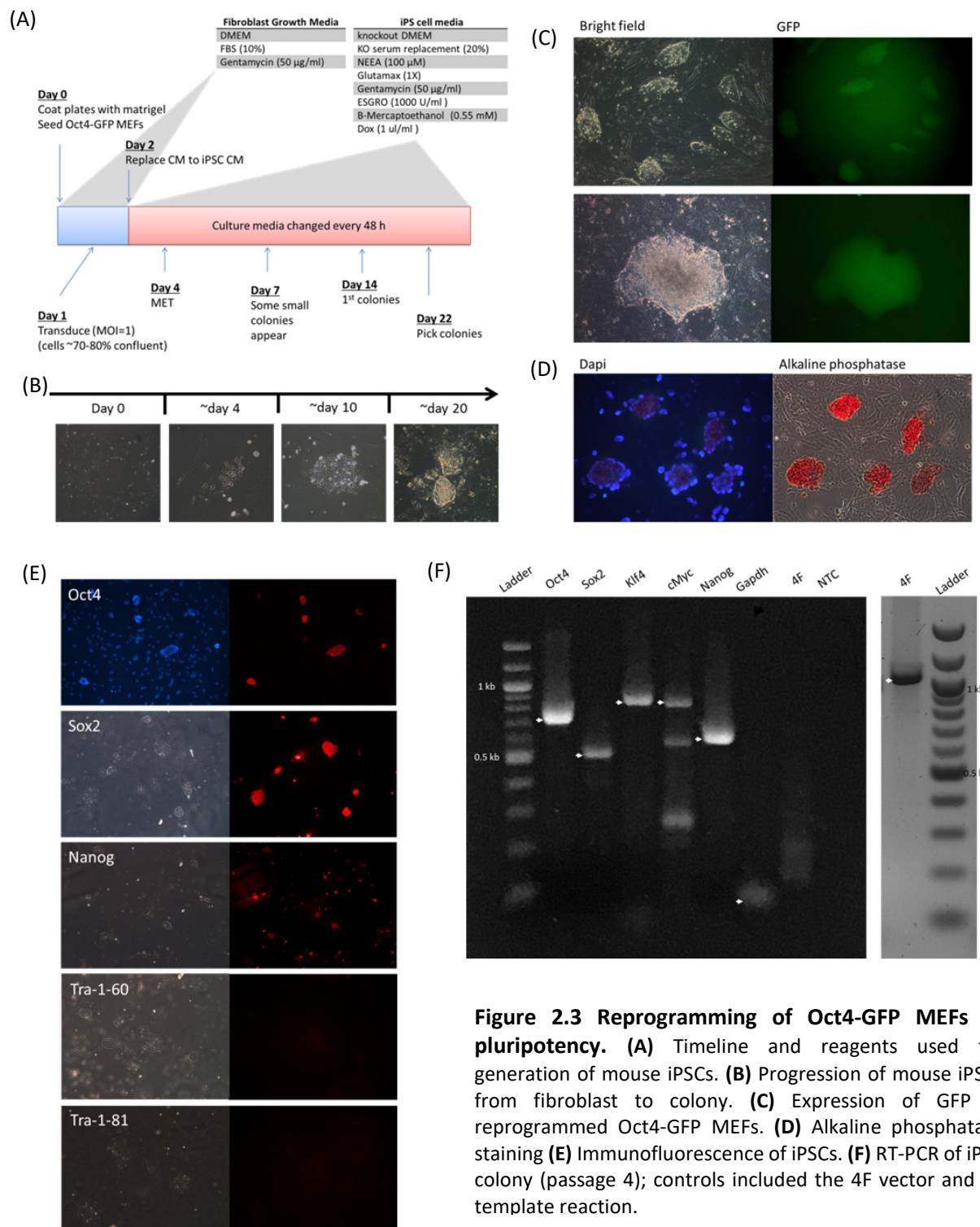
We reprogrammed mouse Oct4-GFP fibroblasts to pluripotency during 14 days using a standard mouse reprogramming protocol (Figure 2.3A). We observed several clusters of small polygonal shaped cells ~4 days after Dox induction. Several of these cells later formed ESC-like colonies. After 14 days Dox was removed from the media and cells were allowed to grow in iPSC media until tightly packed colonies were observed (Figure 2.3B). We observed Oct-GFP expression around day 14 (Figure 2.3C). These GFP expressing colonies were also positive to Alkaline phosphatase staining (Figure 2.3D), as well as to Oct4, Sox2 and Nanog antibodies (Figure 2.3E); no signal was observed from the surrounding fibroblasts. Oct4-GFP mEFs derived iPSCs did not express Tra-1-60 or Tra-1-81 (Figure 2.3E).

Around day 22 after Dox induction, colonies were selected manually and plated over IRR-mEFs. Cells were passaged and cultured in iPSC media without Dox, where they continued to exhibit a stem cell like morphology as well as an immunocytochemistry pattern similar to the initial colonies. In addition, RT-PCR was performed to detect expression of endogenous key pluripotency markers Oct4, Sox2, Klf4, cMyc, and Nanog. The RT-PCR, primers were first tested using cDNA obtained from mESC-D3s, and were then used for analysis of passage 4 iPSC colonies. Passage 4 iPSCs showed expression of endogenous OSKM, but no expression of the exogenous 4F vector was detected (Figure 2.3F).

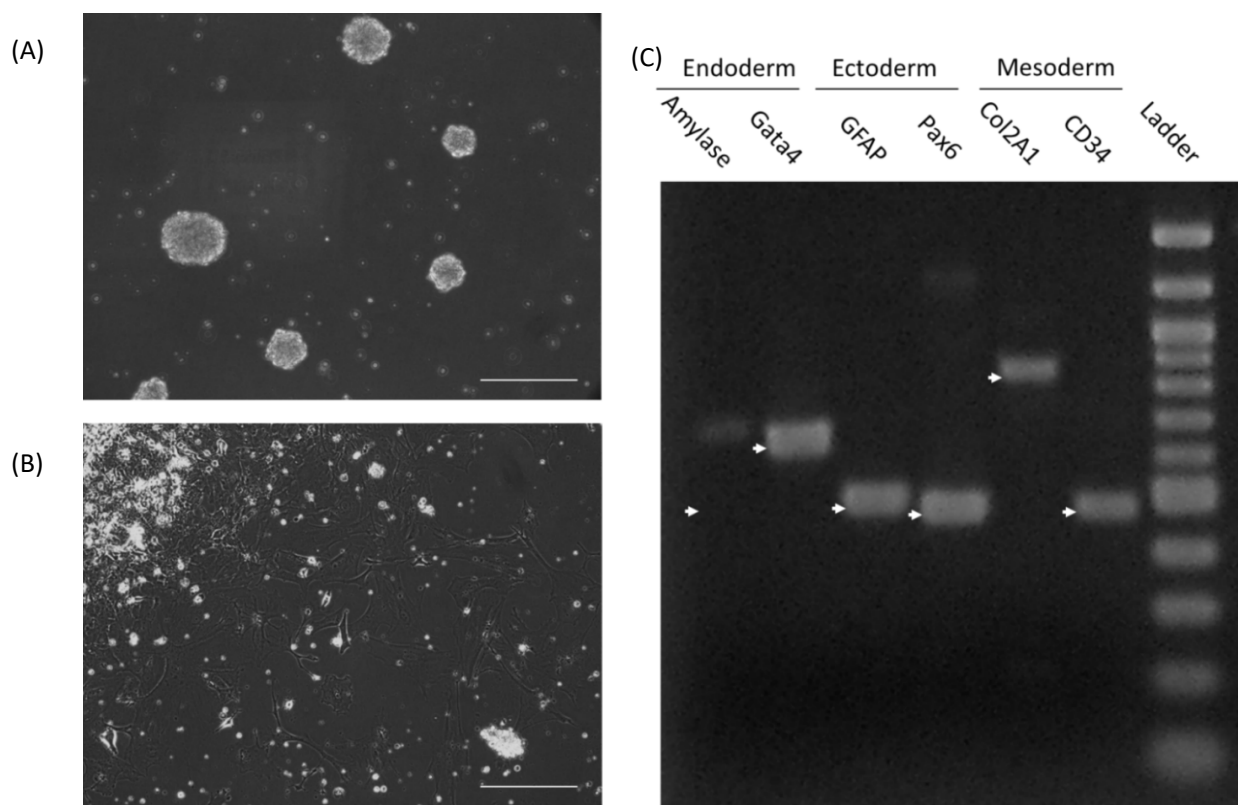
#### 2.4.3 IPSC-DERIVED EMBRYOID BODIES EXPRESS MARKERS FROM ALL THREE GERM LAYERS

We generated embryoid bodies (EBs) using the hanging drop method. Following culture in hanging drops, EBs were plated onto Petri dishes using fibroblast medium (Figure 2.4A); outgrowths from EBs were harvested after seven days (Figure 2.4B). We screened for differentiation markers from all three germ layers (Figure 2.4C).









**Figure 2.4. Embryoid body (EB) differentiation of mouse iPSCs. (A)** iPSCs were cultured in hanging drops in fibroblast media during 2 days; EBs obtained with this method have a spherical, tightly packed morphology. **(B)** EB outgrowths after 7 days of culture on Petri dishes, using fibroblast media. **(C)** RT-PCR of differentiation markers for ecto, meso and endoderm. Arrowheads indicate expected position of bands.

## 2.5 DISCUSSION

The reprogramming plasmids TetO-FUW-OSKM and FUW-M2rtTA contained three point mutations and two insertions, respectively, when compared with the sequence provided by Addgene. However, most discrepancies were in linker areas of the plasmids which are not likely to affect the functionality of the construct. As we observed later, the remaining mutations did not interfere with the functionality of the plasmids. Our Lentiviral vector system yielded viral titers in the range of  $10^5 - 10^{11}$  IU/ml, similarly to what has been described by others<sup>16,17</sup>. The use of Xfect produced a higher titer when compared to the batches generated with Lipofectamine 2000; however, additional replicates would have to be made to validate this claim.

Our experience reprogramming Oct4-GFP mEFs to pluripotency is similar to what has been widely described in the literature<sup>11,18</sup>. Early changes in mouse somatic cell reprogramming involve reverting to an epithelial phenotype, known as the mesenchymal to epithelial transition (MET)<sup>19</sup>. This was clearly observed in our reprogramming GFP-mEFs as early as day 4. After MET, the acquisition of an ESC-like morphology, as well as the silencing of exogenous factors and stable expression of the cell's own set of pluripotency genes, are considered hallmarks of true iPSCs. Our iPSCs were not dependent on exogenous gene expression, as seen by the maintenance of ESC morphology after Dox was removed from the media, as well as by the lack of transgene expression in established colonies. We also observed constant

expression of GFP in our iPSC colonies, confirming the activation of the endogenous Oct4 gene in these cells.

In conclusion, we successfully reprogrammed Oct4-GFP mEFs to pluripotency using a Dox inducible polycistronic vector. This sets the basis for reprogramming attempts in the bovine.

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## 2.7 ATTRIBUTIONS

This chapter was a collaborative effort between the author and fellow grad student Kaylyn R. Williams. Both authors contributed equally to this work.

## 2.8 ACKNOWLEDGEMENTS

Cell line 293T and lentiviral helper plasmids PMD2.G and PSPAX2 were a gift from Dr. Colin Bishop. This work was funded by the Virginia-Maryland Regional College of Veterinary Medicine.

## CHAPTER 3. EARLY LOSS OF EXOGENOUS GENE EXPRESSION DURING BOVINE SOMATIC CELL REPROGRAMMING WITH A DOXYCYCLINE-INDUCIBLE POLYCISTRONIC VECTOR

### 3.1 ABSTRACT

Induced pluripotent stem cells (iPSCs) are stem cell-like cells artificially derived from adult, differentiated cells, by forced expression of genes normally expressed in embryonic stem cells (ESCs). So far, iPSC lines that meet all pluripotency criteria have only been successfully produced in mice, humans, and non-human primates. In cattle, putative iPSCs lines suffer from three main drawbacks that limit their usefulness: (1) the use of integrative vectors that can cause genomic instability, (2) continuous expression of transgenes, and (3) dependence on this expression to maintain the pluripotent state. This indicates that even with a constant supply of exogenous pluripotency genes, these cells fail to stably activate their own endogenous pluripotency networks. Lines of iPSCs from livestock species have potential applications in agriculture, species conservancy, biomedical industry, and veterinary and human health. In this chapter, we used a Doxycycline (Dox)-inducible gene expression system driving expression of *OCT4*, *SOX2*, *KLF4*, and *cMYC* (OSKM) to attempt reprogramming of three types of bovine somatic cells: embryonic and adult fibroblasts, and bone marrow-derived mesenchymal stem cells, using six different culture conditions. Using the basic mouse reprogramming conditions, we did not succeed in inducing formation of ESC-like colonies in bovine somatic cells. The combination of 2i/LIF plus ALK5 inhibitor II and ascorbic acid induced formation of colony-like structures with flat morphology, that occasionally produced trophoblast-like structures. These trophoblast-like vesicles did not appear when an inhibitor of Rho-associated, coiled-coil containing protein kinase 1 (ROCK) was included in the medium. We screened for expression of exogenous OSKM vector with RT-PCR and found upregulation of OSKM vector 24h after Dox was added to the medium; but expression was sharply decreased on day 2 after Dox induction, and was not detectable after day 3. In a separate experiment, we induced reprogramming of bFF and bAFs using media supplemented with 50% of media conditioned by co-culture with the bovine trophoblast CT1 line. These cells expressed both *OCT4* and the OSKM vector 24h after Dox induction. However, similar to our previous observations, both markers decreased expression until no signal was detected after day 3. Our inability to produce biPSCs, combined with the only partial reprogramming observed by others, justifies the need for in depth study of bovine pluripotency mechanisms before meaningful attempts to reprogram bovine somatic cells to pluripotency are made.

### 3.2 INTRODUCTION

Induced pluripotent stem cells (iPSCs) are a type of pluripotent cell that can be produced by forced expression of stem cell genes in somatic cells. In mice, rats, humans and non-human primates, this forced expression of pluripotency genes causes a stable change in gene expression pattern from somatic to pluripotent. Unfortunately, this technology has failed to produce *bona fide* iPSC in other mammalian species, including the bovine. The availability of bovine iPSCs (biPSCs) would be a very relevant tool for the biomedical industry, agriculture, and species conservancy. Attempts to produce biPSCs have been based on methods optimized to produce mouse and human iPSCs. To produce biPSCs, researchers have used the same genes used to reprogram mouse and human iPSCs: *OCT4*, *SOX2*, *KLF4*, *cMYC*, and sometimes *LIN28* and *NANOG*, using the cDNA sequences of mouse<sup>1,2</sup>, human<sup>3</sup>, bovine<sup>4-6</sup>, or a combination of human and porcine<sup>7</sup> sequences. The resulting iPSC-like cells expressed endogenous *OCT4*,

*SOX2*, and in some cases *NANOG*. Differentiation potential was tested by formation of embryoid bodies *in vitro* and teratoma in immunodeficient mice. However, in many cases cells were not able to proliferate in culture<sup>5</sup>, or proliferation ceased after a certain number of passages. More importantly, these lines failed to silence exogenous transgenes, indicating incomplete reprogramming.

Besides the choice of transcription factors, another aspect that has been extrapolated from mouse and human research is the cell type to reprogram. A great deal has been discussed on which cell lineage is the ideal to reprogram, and epigenetic memory of the parental cell has been proposed as the cause of variable reprogramming efficiency, as well as differences in iPSC potential to differentiate into certain lineages<sup>8</sup>. Since the first report on mouse iPSCs, fibroblasts have been the cell type of choice, mostly due to the easiness to acquire them. Most mouse experiments have been performed using embryonic fibroblasts, the thought being that embryonic cells are more amenable to reprogramming, and chances of mutations are lower. Adult fibroblasts have also been used, and are the cell type of choice when reprogramming human somatic cells, the reasoning being that if iPSC technology is to be used for regenerative medicine, the easiest source to obtain patient cells is a skin biopsy. Several other cell types have been proposed as a more convenient alternative: adult peripheral blood cells, exfoliated renal epithelial cells, and keratinocytes from hair follicles<sup>9</sup>. Although more difficult to obtain, human amniotic epithelial cells have been proposed as a cell type more amenable to reprogramming than embryonic or adult fibroblasts<sup>10</sup>.

Another aspect that influences the generation of iPSCs is the environment in which the cells are maintained during reprogramming. Traditionally, iPSCs are grown on a monolayer of mitotically-inactivated mouse embryonic fibroblasts that act as feeders for the iPSCs. However, studies in human iPSCs (hiPSCs) have shown that pluripotency and developmental potential of hiPSCs is not affected after long term culture on either mouse embryonic fibroblast feeders, human dermal fibroblasts, or Matrigel coating<sup>11</sup>. Matrigel is a cell-free mixture of proteins that acts as a basement membrane, and is commonly used in ESC and iPSC cultures when a feeder-free environment is desired.

In this chapter, we describe our attempts to reprogram bovine somatic cells using six different methods adapted from the literature using the Dox-inducible polycistronic vector driving expression of OCT4, *SOX2*, *KLF4*, and *cMYC* (OSKM) used in [chapter 2](#), this time using Matrigel coated plates instead of mouse feeder cells. With this system, we first reprogramming mouse newborn fibroblasts (mNFs) and bovine fetal fibroblasts (bFFs), and observed formation of colonies in mNFs in ~2 weeks, whereas bFFs did not produce any visible morphological change and senesced. Second, we reprogrammed mNFs and three types of bovine somatic cells: bFFs, bovine adult fibroblasts (bAFs), and/or bone marrow-derived mesenchymal stem cells (bMSCs), using the Dox-inducible system in feeder-free conditions, and four different combinations of media and additives used in current mouse, human, and ungulate reprogramming protocols: leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), inhibitors of MEK/ERK and GSK pathways ("2i"), ALK5 inhibitor II (ALK5i), Rho-associated, coiled-coil containing protein kinase 1 inhibitor (ROCKi), and ascorbic acid (VitC). The two media containing either LIF plus bFGF, or 2i/LIF plus bFGF, did not induce any relevant changes in cell morphology. Reprogramming using 2i/LIF, ALK5i, and VitC, induced formation of flat, proliferative clusters of cells, which in some cases produced trophoblast-like structures. When a ROCKi was included in this last media cocktail, no trophoblast-like vesicles appeared. Using RT-PCR, we observed expression of the reprogramming construct only during the first three days after Dox induction, whereas in control reprogrammed mNFs the expression persisted for at least one week. Finally, we reprogrammed bFFs and bAFs in culture media plus 50% media conditioned by co-culture in the bovine trophoblast cell line CT1. We observed clusters of flat cells resembling primed iPSCs. We analyze the whole cell monolayer with qRT-PCR and observed co-upregulation of OCT4 and the OSKM vector; however, expression of both markers declined and was not visible after day 3.

In summary, using reprogramming methods adapted from the literature for mouse and human iPSCs, we were only able to induce morphological changes that resemble partially reprogrammed cells in the cow. Moreover, expression of the reprogramming vector was downregulated on day 3 post Dox induction. This indicates that a better characterization of pluripotency in the bovine is necessary in order to provide reliable conditions that permit reprogramming of bovine somatic cells.

### 3.3 MATERIAL AND METHODS

#### 3.3.1 CELL CULTURE

The packaging cell line 293T was purchased from ATCC (CRL-3216) and cultured in fibroblast medium composed of: high glucose Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; HyClone) and 50 µg/ml Gentamicin (Lonza) at 37°C in 5% CO<sub>2</sub> incubator. Cells were passaged with 1X 0.25% Trypsin/EDTA (Corning) before reaching 80% confluence.

Mouse newborn fibroblasts (mNFs) were isolated from the skin of a euthanized newborn (day 1) CD1 mouse, following the guidelines of the IACUC. Briefly, head and limbs were removed, skin was separated and washed briefly in 70% ethanol and DPBS (HyClone), cut into small pieces (~1-3 mm<sup>2</sup>) with a scalpel and digested in 1X 0.25% Trypsin-EDTA at 37°C for 15 min. Following incubation, trypsin was removed by allowing explants to settle on the bottom of the tube, removing the supernatant and resuspending in fibroblast medium. Explants were placed in 6-well plates, covered with a sterile coverglass to prevent them from floating, and cultured in fibroblast medium plus 2.5 µg/ml Amphotericin B (Fisher BioReagents) at 37°C in 5% CO<sub>2</sub>. Medium was replaced every 2-3 days. Cell outgrowths were harvested after ~7 and ~14 days.

Primary bovine fetal fibroblasts (bFFs) were derived from a male fetus of unknown genetic background obtained at an abattoir at gestation day 60. Primary bovine adult fibroblasts (bAFs) were obtained from the ear of a 10-month old male calf. Cell isolation from tissue explants was similar to what was described for mNFs. Both bFFs and bAFs were cultured in fibroblast medium, in a 5% CO<sub>2</sub> in air incubator at 38.5°C.

Bovine bone marrow mesenchymal stem cells (bMSCs) were obtained from the femurs of three abattoir fetuses. Fetuses were transported in heated containers to the lab, where the femurs were dissected and cut transversally in the center of the diaphysis with a saw. We used a 10 ml syringe and an 18G needle to flush the medullar cavity with warm collection medium (High glucose DMEM supplemented with 1,000 U/ml heparin, and 1X Penicillin-Streptomycin (Gibco)). Suspension was collected in a 50 ml tube and washed three times by centrifugation, resuspending the pellet in expansion medium (High Glucose DMEM supplemented with 10% FBS, 1X Penicillin-Streptomycin, and 2.5 µg/ml Amphotericin B). After the final wash step, cells in expansion medium were cultured in 10-cm tissue culture plates in a 5% CO<sub>2</sub> in air incubator at 38.5°C, until cells reached 80% confluence.

For reprogramming, mNFs, bFFs, bAFs, and bMSCs were plated in matrigel-coated plates (BD biosciences). An overview of the composition of each reprogramming media can be found in [Table 3.1](#). Detailed composition of culture media was as follows. Mouse medium (base): KnockOut Dulbecco's minimal essential medium (KO-DMEM; Gibco) supplemented with 20% KnockOut Serum Replacement (KO-SR; Gibco), 1X Non-Essential Amino Acids (HyClone), 1X Glutamax (Gibco), 1,000 U/ml of ESGRO (Millipore), 0.55 mM beta-mercaptoethanol, and 50 µg/ml Gentamycin at 37°C in 5% CO<sub>2</sub>. biPSC medium #1 (base): High glucose DMEM supplemented with 15% ESC-quality FBS, 1X Glutamax, 1X NEAAs, and 50 µg/ml

**Table 3.1.** Composition of different culture media used to attempt reprogramming of bovine cells.

	Mouse medium	biPSC medium #1	biPSC medium #2	biPSC medium #3	biPSC medium #4	Conditioned medium**
Base*	KO DMEM	DMEM	DMEM	DMEM	DMEM	DMEM
	KOSR (20%)	FBS (15%)	FBS (15%)	FBS (15%)	FBS (15%)	FBS (15%)
	Glutamax	Glutamax	Glutamax	Glutamax	Glutamax	Glutamax
	NEAAs	NEAAs	NEAAs	NEAAs	NEAAs	NEAAs
	Gentamycin	Gentamycin	Gentamycin	Gentamycin	Gentamycin	Gentamycin
		Na Pyruvate	Na Pyruvate	Na Pyruvate	Na Pyruvate	Na Pyruvate
Additives*	Dox	Dox	Dox	Dox	Dox	Dox
	LIF	LIF	LIF	LIF	LIF	LIF
	βME	βME	βME	βME	βME	βME
		bFGF	bFGF			
			2i	2i	2i	2i
				ALK5i	ALK5i	ALK5i
				VitC	VitC	VitC
				ROCKi		

\*Base media was stored for up to 7 days; additives were added fresh before use and replaced ever 48h.

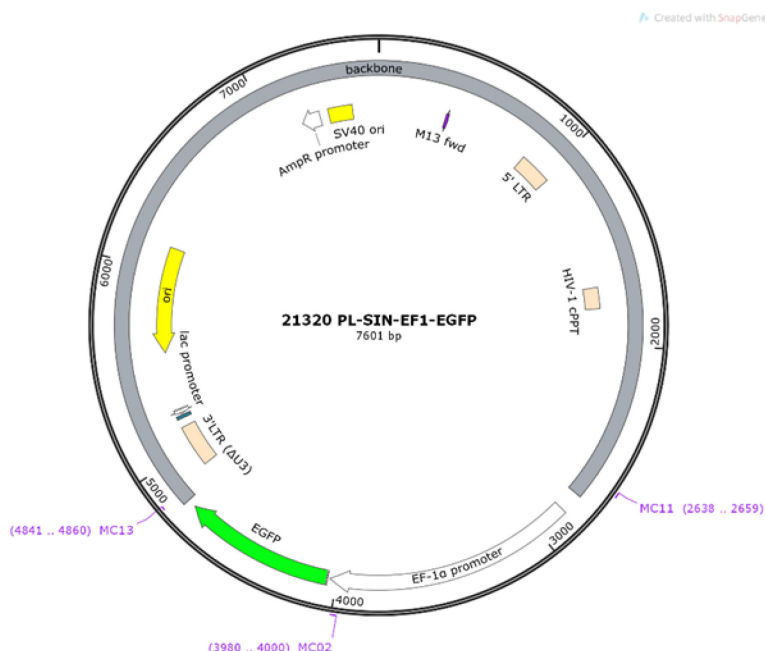
\*\*For conditioned media, base media was mixed 1:1 with CT1 supernatant. Additives were added to the resulting conditioned media.

gentamicin. biPSC medium #2, #3, #4, and conditioned medium (base): medium #1 (base) plus 1 mM sodium pyruvate (Gibco). To prepare a working solution of conditioned media, base conditioned medium was mixed 1:1 with media co-cultured for 2-days with the bovine trophoblast cell line CT1. Additives were added to the resulting conditioned media. Additives and concentrations used were: Doxycycline (Dox; 1.5 µg/ml), leukemia inhibitory factor (LIF; 1500 U/ml), B-mercaptoethanol (BME; 100 µM), basic fibroblast growth factor (bFGF;), InSolution™ MEK ½ inhibitor III, PD0325901 (PD; 1 µM), CHIR99021 (3 µM), ALK5 inhibitor II (ALK5i; 1 µM), L-Ascorbic acid (VitC; 500 µM), and ROCK inhibitor (ROCKi; 5, 10, and 15 µM).

### 3.3.2 LENTIVIRAL VECTOR PRODUCTION AND TITRATION

Reprogramming plasmids TetO-FUW-OSKM and FUW-M2rtTA were already described in [chapter 2](#).

To have an approximation of the transduction efficiency of mNFs and bFFs, we calculated the viral titer using a lentiviral reporter vector consisting of a constitutive (“always on”) EF1α promoter driving expression of the enhanced version of green fluorescent protein (EGFP). Plasmid PL-SIN-EF1α-EGFP has been previously published<sup>12</sup> and was purchased from Addgene.com (Addgene plasmid #21320). Upon arrival, bacterial stab was processed as described previously and plasmid sequence was corroborated using Sanger sequencing (Virginia Bioinformatics Institute at Virginia Tech) and custom primers MC02: TCAAGCCTCAGACAGTGGTTC, MC11: TTCACCATTATCGTTTCAGACC, MC13 TAAGATCTACAGCTGCCTTG ([Figure 3.1](#)). Chromatograms were aligned in Lasergene SeqMan Pro. Large quantities of plasmid from one clone that did not present any mismatches with the sequence deposited in Addgene were obtained using PerfectPrep EndoFree Plasmid Maxi Kit (5Prime), following the manufacturer’s instructions. To calculate the titer of the lentiviral preparation, we transduced 293T cells, mNFs, and bFFs with three ten-fold serial dilutions of lentiviral vector suspension. Culture media was replaced after 12h, and cells were incubated



**Figure 3.1** Full schematic view of the plasmid PL-SIN-EF1 $\alpha$ -EGFP. Sanger sequencing primer annealing sites are indicated.

for 72h, at which time the cells were detached and fixed in suspension with 4% paraformaldehyde. Expression of the vector was assessed by flow cytometry. Viral titer was calculated using the formula: viral titer (IU/ml) = [number of cells in well at time of transduction] x [EGFP+/100] / [amount of virus used (ml)].

### 3.3.3 TRANSDUCTION AND DOX INDUCTION

Cells to be reprogrammed were passaged at least twice to ensure proper growth, and passaged the day before transduction in fibroblast medium. After 24 hours, medium was replaced with fibroblast medium containing Polybrene (Millipore) at a final concentration of 8  $\mu$ g/ml and lentiviral suspension. The dosage of lentiviral vector added to the cells was controlled by calculating the multiplicity of infection (MOI), using the formula: MOI = viral titer (IU/ml) x [amount of virus used for infection (ml)] / [target cell number]. Unless indicated, for all reprogramming experiments described in this chapter we used an MOI of 1. After 8-16 h the medium was replaced and cells were allowed to recover for 24 hours in fibroblast medium, before reprogramming medium supplemented with 1.5  $\mu$ g/ml Dox was added.

### 3.3.5 RNA ISOLATION, REVERSE TRANSCRIPTION, AND END POINT POLYMERASE CHAIN REACTION (PCR)

Cells were harvested using Trypsin (Corning) at 37°C for 5 min and pelleted by centrifugation. Cell pellets were lysed and RNA was isolated using the Quick-RNA MiniPrep Kit (Zymo Research) following the manufacturer's instructions. We performed end point PCRs in 20  $\mu$ l reaction mixtures containing 10  $\mu$ l of Taq PCR master mix (Qiagen), template (4 ng), and custom made primers at a final concentration of 10  $\mu$ M each one. Primer sets for mouse Oct4 (mOct4), mouse Nanog (mNanog), and the reprogramming vector (4F) were already described in [chapter 2](#). Primer sets for bOct4 and bNanog are listed in [Table 3.2](#). End point RT-PCR was run on a thermal cycler (My Cycler, Bio-Rad) using the following parameters: stage 1: 1 cycle (3 min, 94°C), stage 2: 30 cycles (denaturation: 45 sec, 94°C; annealing: 45 sec, 57°C; extension:



**Table 3.2** Primers for end point RT-PCR and qRT-PCR.

Gene	Primers (5' -> 3')	Amplicon length (bp)
<b>End point PCR</b>		
bOct4	F: GTTGATCCTCGGACCTGGATG R: CACTGCTTGATCGTTTGCCCT	784 bp
bNanog	F: CACAAGCCCCAGAGTCAAAC R: AGCCTGGGTATTCTGCCATT	323 bp
<b>Real time PCR (QPCR)</b>		
bGapdh	F: TGGTGAAGGTCGGAGTGAAC R: TGTAGACCATGTAGTGAAGGTCAA	129 bp
bOct4	F: AAATTAGCCACATCGCCCAG R: TCACTGCTTGATCGTTTGCC	97 bp
4F	F: TGTCCCGTCACTGCTCTGG R: CCCGGGGTTTTCTCAACAT	109 bp

1 min, 72°C), stage 3: 1 cycle (10 min, 72°C), stage 4: hold at 4°C. Following PCR, the reaction mixture plus 1X purple loading dye (New England BioLabs) was run on a 1% agarose gel (Bio-Rad) at 6 volts/sec for 5 min and 2 volt/sec until loading dye reaches 2/3 of the gel. Gels were stained with ethidium bromide (IBI Scientific) for 15 min and de-stained in distilled H<sub>2</sub>O for 10 min. Gels were visualized on a benchtop UV transilluminator (UVP) and photographed.

### 3.3.6 REAL TIME PCR (QPCR)

Primers specific for bovine OCT4 (bOCT4) and bovine GAPDH (bGAPDH) were designed using Primer Blast<sup>2</sup> and following the directions for qPCR primers indicated in the Primer Express handbook. We validated the efficiency of the primers by performing 5-fold serial dilutions using cDNA from bovine blastocysts. Samples were analyzed in 20 µl reaction mixtures using Power SYBR Green Master Mix (Applied Biosystems) and run on a StepOnePlus™ Real-Time PCR System using the following parameters: stage 1, 1 cycle (95°C, 10 min); stage 2, 40 cycles (95°C, 15 sec; 60°C 1 min); stage 3, melt curve (95°C, 15 sec; 60°C, 1 min; 95°C, 15 sec). Samples were normalized to GAPDH as a housekeeping gene.

### 3.3.7 ALKALINE PHOSPHATASE STAINING

Staining for alkaline phosphatase in the culture plate was performed with the Alkaline Phosphatase Detection Kit (Sigma-Aldrich) according to the manufacturer's instructions.

## 3.4 RESULTS

### 3.4.1 LENTIVIRAL VECTOR TITER IS SIMILAR IN BOTH MNFS AND BFFS

Primary cell lines are considered difficult to transduce. Lentiviral vectors have high transduction efficiency even in cells types not easily transduced by methods such as lipofection or nucleofection. We transduced 293T cells (considered gold standard for their easiness to transduce), mEFs and bFFs, with a lentiviral vector harboring a construct expressing EGFP under the control of a constitutive EF1α promoter (PL-SIN-

<sup>2</sup> <https://www.ncbi.nlm.nih.gov/tools/primer-blast>

EF1 $\alpha$ -EGFP). It is generally considered that cells that express between 5-25% of reporter have single copies. Therefore, we calculated the titer based the lowest dilution of virus that will still give a positive signal within that range. As expected, the highest titer was found in 293T cells. Titer was lower but similar in both mNFs and bFFs, showing that primary cells are less amenable to transduction ([Table 3.3](#)).

**Table 3.3** Lentiviral vector titer (IU/ml) obtained from two independently produced batches of PL-SIN-EF1a-EGFP lentiviral vector. Each batch was tested in 293T cells, mEFs, and bFFs.

	293T	mEFs	bFFs
Batch #1	$9.2 \times 10^5$	$2.9 \times 10^5$	$2.5 \times 10^5$
Batch #2	$1.1 \times 10^8$	$1.8 \times 10^7$	$1.1 \times 10^7$

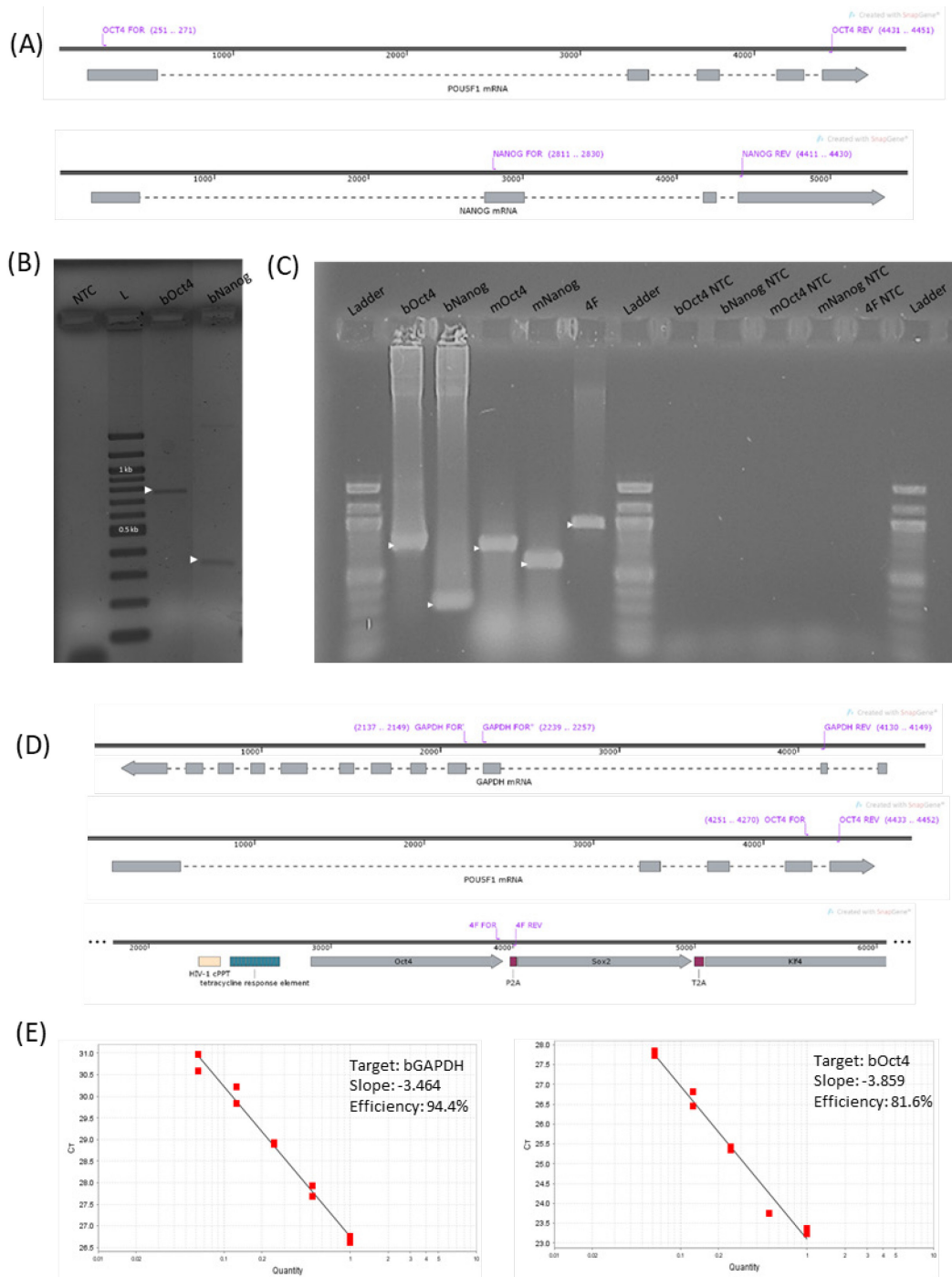
### 3.4.2 PRIMERS FOR END POINT AND REAL TIME RT-PCR

We designed end-point PCR primer sets to specifically screen for endogenous bOCT4 and bNANOG. Primers were designed to span across introns ([Figure 3.2A](#)) and were tested using cDNA obtained from 15 *in vitro* derived day-10 bovine blastocysts. We observed bands of the expected size and no presence of secondary bands ([Figure 3.2B](#)). Due to the limited availability of blastocyst cDNA, subsequent PCRs used a plasmid encoding the cDNA sequences of bOCT4 and bNANOG as a positive control ([Figure 3.2C](#)). The plasmid with the bOct4 and bNanog cDNA sequences was synthesized by an external lab (Genscript) and was not present in our lab until after the samples had been analyzed, thus eliminating the risk of contaminating the cDNA samples with plasmid containing bovine cDNA. Efficiency of qPCR primers for bGAPDH and bOCT4 ([Figure 3.2D](#)) was calculated by plotting the Ct versus the Log of the DNA dilution. Usually, an efficiency rate between 90% - 105% is considered acceptable. Primers for bGAPDH presented 94% efficiency, whereas bOCT4 primers had 81% efficiency ([Figure 3.2E](#)). However, since initially we were only interested in determining presence or absence of transcript, we used this primer set for future experiments. Both bOCT4 primer sets were also tested for non-specific reaction using the 4F vector. No expression was observed in either case.

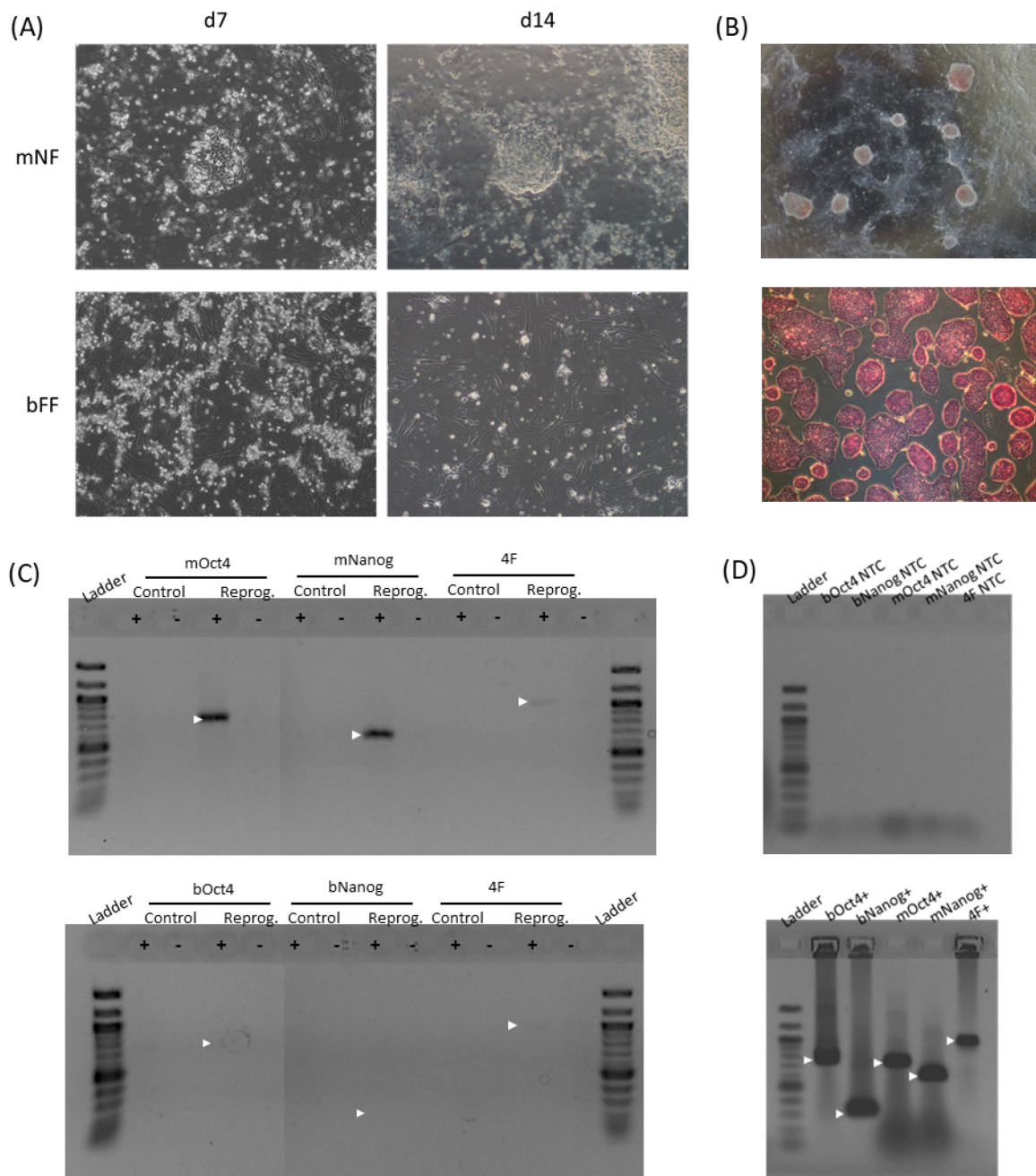
### 3.4.3 REPROGRAMMING OF MOUSE AND BOVINE FIBROBLASTS WITH DOX INDUCIBLE VECTOR AND MOUSE CONDITIONS DOES NOT REPROGRAM BOVINE CELLS

We reprogrammed mNFs and bFFs side by side using mouse reprogramming conditions ([Table 3.1](#)) on Matrigel-coated plates. Consistent with our previous results, we observed MET in mNFs around day-5 after Dox induction, and early colonies started to form around day-7. None of these features were observed in bFFs ([Figure 3.3A](#)).

Regardless of the treatment used, bFFs formed colony-like structures ~3 days after they reached full confluence. These colony-like structures were non-proliferative when individually selected and passaged with either Trypsin or by manual disaggregation with a pipette. We performed alkaline phosphatase staining of the control bFF monolayer at day-23 after Dox induction, and observed faint positive signal from this colony-like structures ([Figure 3.3B](#)). Because of this characteristic of bFFs, any future reprogramming experiment attempted in the bovine included a non-transduced control. Fully confluent bFF cultures usually formed clumps and detached from the dish, with minimal growth afterwards. At that point the cultures were discarded.



**Figure 3.2 Primer pairs for end-point RT-PCR and qRT-PCR. (A)** Representation of RT-PCR primers for bovine Oct4 (bOct4) and bovine Nanog (bNanog) in genomic DNA context. Primers were designed to span across introns. **(B)** Bovine primers were tested using cDNA from 15 day-10 bovine blastocyst. **(C)** Primers for bOct4 and bNanog were also tested using a plasmid that contains the cDNA sequence for bOct4 and bNanog; primers for mouse Oct4 (mOct4) and mouse Nanog (mNanog) were tested using mESC cDNA; and primers for the 4F plasmid were evaluated using the 4F plasmid as template. **(D)** Representation of qRT-PCR primers for bovine Gapdh (bGapdh) and bOct4 in genomic DNA context. **(E)** Amplification efficiency of bGapdh and bOct4 primer pairs was assessed using 5-fold serial dilutions of bovine blastocyst cDNA. Primer pairs for the 4F vectors have been previously evaluated in our lab.



**Figure 3.3 Reprogramming of mNFs and bFFs with a mouse-optimized reprogramming protocol. (A)** Colonies appeared as early as day-7 in mNFs, whereas no colonies were observed in bFFs. **(B)** Top image: alkaline phosphatase (AP) staining of colony-like structures in bFFs 23 days after Dox induction. Bottom image: mESCs stained for AP with the same kit and conditions is included as reference for color intensity. **(C)** End point RT-PCR for mOct4, mNanog, bOct4, bNanog, and the 4F vector. Reverse transcription reaction was performed with (+) and without (-) reverse transcriptase. **(D)** Negative control without template and positive control reactions were also included.

We harvested mNF and bFF monolayers at day-20 after dox induction and performed end point RT-PCR for mouse and bovine Oct4, Nanog, and the exogenous 4F vector. We observed presence of m*Oct4*, m*Nanog*, and very faint expression of the 4F vector in mNFs. No bands matching with b*OCT4*, b*NANOG*, or 4F vector were found in the bovine samples ([Figure 3.3C](#)). Negative and positive controls were also ran in parallel ([Figure 3.3D](#)). This experimental setting was repeated twice, with similar results. We concluded that standard mouse reprogramming conditions are not sufficient to reprogram bFFs to pluripotency.

### 3.4.3 MODIFIED CULTURE MEDIA INDUCES EARLY TRANSIENT MORPHOLOGICAL CHANGES IN REPROGRAMMING BFFS, BAFS, AND BMSCS, BUT IS NOT CONDUCTIVE TO STABLE EXPRESSION OF BOVINE PLURIPOTENCY MARKERS. THIS IS ACCOMPANIED BY EARLY LOSS OF EXOGENOUS GENE EXPRESSION IN BOVINE REPROGRAMMING CELLS.

We tested several reprogramming conditions previously described for mice, humans, and cattle. Criterion of success of a certain condition was based on the appearance of colony-shaped structures in bFFs, bAFs, and/or bMSCs growing on a Matrigel-coated tissue culture plates, for as long as the cells remained viable. mNF were always reprogrammed in parallel. Media composition is described in [table 3.1](#). Reprogramming of every cell type in the described conditions was replicated at least twice. Only treatments that induced morphological changes in bovine cells were further analyzed.

#### 3.4.3.1 biPSC medium #1

No visible morphological changes were observed in either bFFs, bAFs or bMSCs after 15 days in culture. After 15 days in culture, cells lost viability and were discarded. We observed some early MET in mNFs, but these cells did not persist and were not visible after day 5. Inability of mNFs to form colonies was likely due to the presence of bFGF in the medium.

#### 3.4.3.2 biPSC medium #2

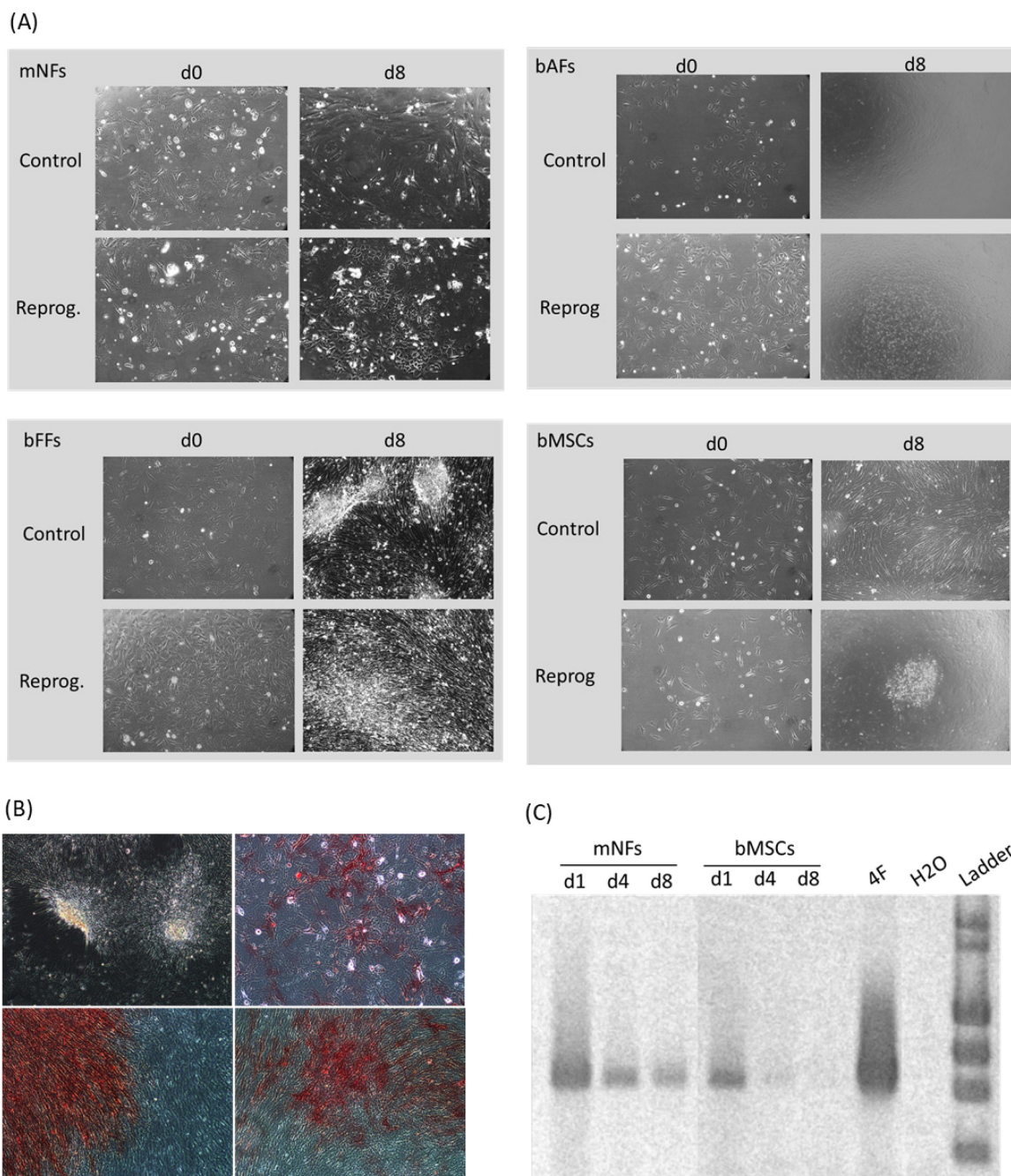
We observed early colony formation in bAFs and bMSCs around day 8 after Dox induction, with the morphological change being more noticeable in bMSCs ([Figure 3.4A](#)). These early colonies did not persist past day 10 after Dox induction. Manual passaging of early colonies to IRR-MEF coated plates was unsuccessful in maintaining this phenotype. We did not observe any early morphological changes in bFFs, possibly due to bFFs growing at a faster rate than bAFs and bMSCs and therefore reaching full confluence before day 8. Passaging bFFs either enzymatically or manually before confluence did not solve this issue. Similar to bFFs in section 3.4.2, we observed colony-like structures after cells reached full confluence in bEFs, bAFs, and bMSCs. When reprogrammed in this media, mNFs formed small cells that failed to form tightly packed colonies. Since the early morphological changes were more noticeable in bMSCs, we focused on this cell type for further characterization. We stained fully confluent day 12 bMSCs monolayers for AP; colony like-structures were not AP positive, however, seemingly random areas of the plate were very positive ([Figure 3.4B](#)). Because morphological changes appear early after Dox induction, we harvested the cell monolayer at days 1, 4 and 8 after Dox induction and screened for expression of the 4F vector. End point RT-PCR showed sustained exogenous vector expression in the mouse from day 1 to day 8, whereas in bMSCs exogenous vector expression was absent in days 4 and 8 ([Figure 3.4C](#)).

#### 3.4.3.3 biPSC medium #3

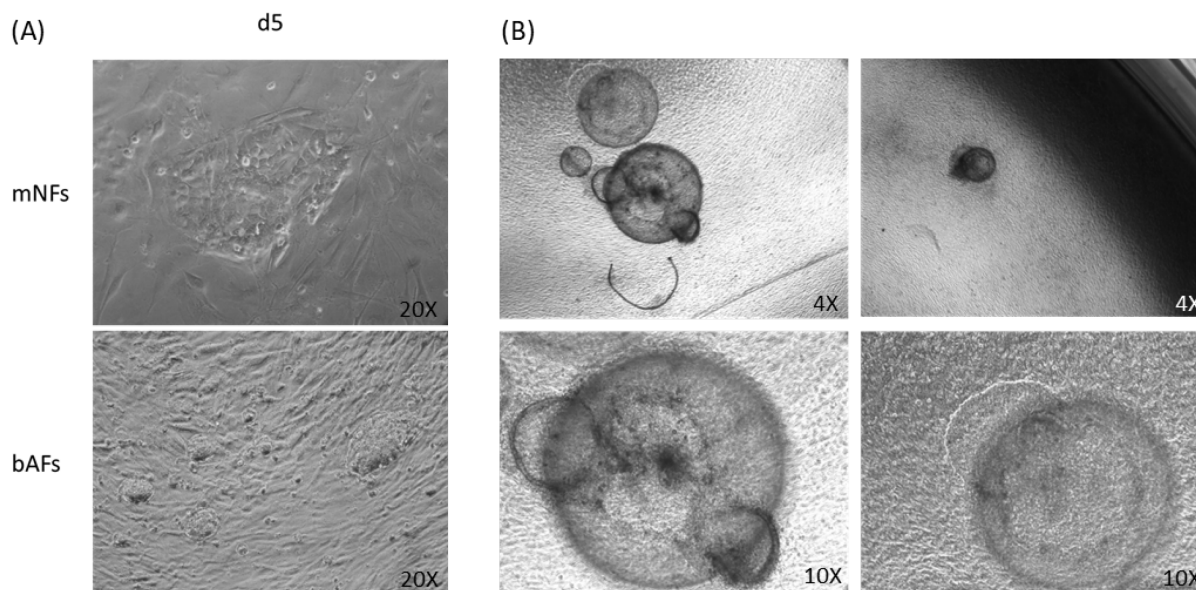
When mNFs, bFFs, bAFs, and bMSCs were reprogrammed in medium #3, we observed small colonies in bAFs as early as day 5 ([Figure 3.5A](#)). These colonies had a flat morphology, persisted in culture but were not amenable to passage with Trypsin. In some cases, some of these colonies formed spherical, trophoblast-like structures ([Figure 3.5B](#)). We did not analyze these colonies and spheres any further.



Control mNFs reprogrammed in media #3 showed MET as early as day 2, and were able to form iPSC colonies.



**Figure 3.4. Reprogramming of bovine somatic cells in biPSC medium #2.** (A) Control was not transduced with reprogramming vector but cultured in medium #3. (B) Representative images of AP stain of d12 bMSC in medium #3. Images of different areas of the plate. (C) mNFs and bMSCs were reprogrammed in parallel using medium #3, and analyzed for expression of 4F vector using RT-PCR.



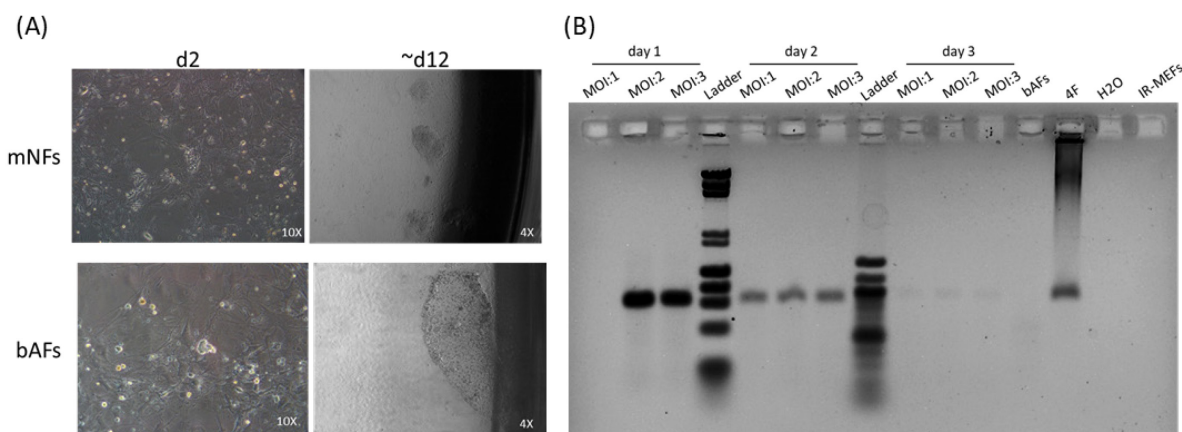
**Figure 3.5. Reprogramming of bovine somatic cells in biPSC medium #3. (A)** Colony-like structures in both mNFs and bAFs around day 5. **(B)** Ball-like structures forming over flat bAF colonies around day 14.

#### 3.4.3.4 biPSC medium #4

We reprogrammed mNFs and bAFs with medium #4, and observed appearance of MET in mNFs as early as day 2. Interestingly, bAFs formed flat colonies but no globous structures were observed. Control mNFs formed colonies as expected ([Figure 3.6A](#)). We transduced bAFs with three incremental doses of reprogramming vectors (MOI: 1, 2, and 3), and reprogrammed cells in biPSC medium #4. Cell monolayers were harvested at days 1, 2, and 3 after Dox induction. Again, we observed significant decrease in 4F vector expression in bAFs as early as 48h after Dox was added to the media. This happened regardless of the MOI used ([Figure 3.6B](#)).

#### 3.4.4 CT1 CONDITIONED MEDIUM AFFECTS MORPHOLOGY AND PERSISTENCE OF EARLY COLONIES. HOWEVER, EXPRESSION OF *BOCT4* AND 4F VECTOR IS LOST 2 DAYS AFTER DOX INDUCTION

We reprogrammed bFFs and bAFs (MOI: 5 - 10) using medium #4 supplemented with 50% CT1 conditioned medium. Two days after Dox was added to the medium, high cell mortality was observed in both bFFs and bAFs ([Figure 3.7A](#)). After 4 days, cells had recovered and we observed clusters of polygonal-shaped cells, similar to MET in the mouse, in both cell types ([Figure 3.7B](#)). These cells did not continue proliferating in bAFs. However, in bFFs, MET-like cells proliferated as compact groups of flat cells, but never formed colony-like structures. We did not attempt passage of these cells. We replicated this experiment twice. The second time, we harvested the bFF monolayers during the first 7 days after Dox induction, to screen for *bOCT4* and 4F expression with qRT-PCR. We found that *bOCT4* and the 4F vector were dramatically upregulated 24h after Dox induction, but the level of expression sharply decreased on day 2, and was not detected during later stages.



**Figure 3.6. Reprogramming of bovine somatic cells in biPSC medium #4.** (A) Colony-like structures were observed in both mNFs and bAFs around day 2 after Dox induction. Mouse cells formed dome shaped colonies, whereas bAFs formed flat looking colonies (B) bAFs transduced with different MOIs and screened for 4F expression. Sample corresponding to MOI:1 day 1 was lost.

### 3.5 DISCUSSION

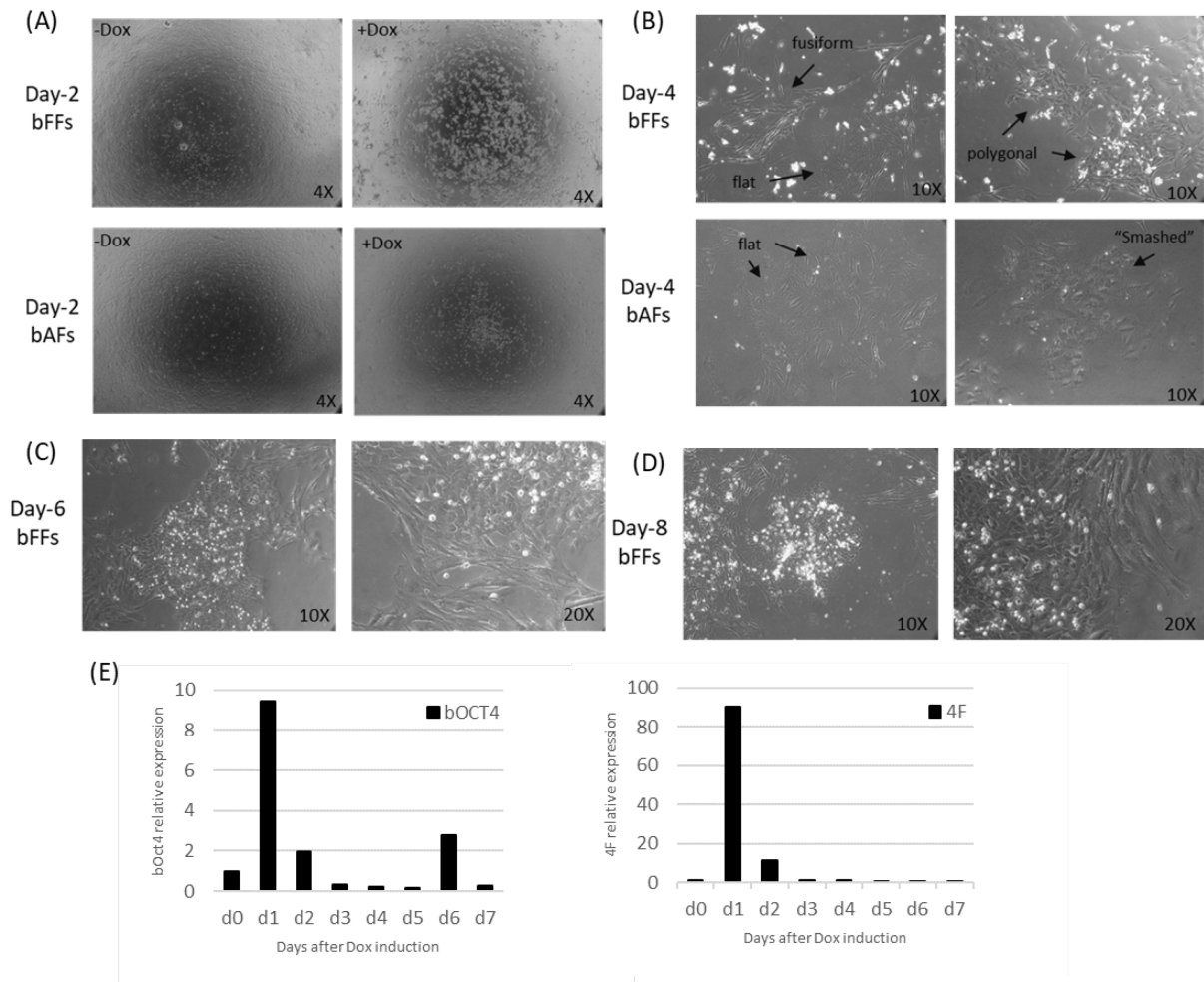
Our initial attempt to reprogram bFFs to pluripotency using standard mouse conditions did not induce any morphological change indicative of reprogramming. This is not surprising, considering that reprogramming of bovine somatic cells to pluripotency using iPSC technology has, to date, not been successfully achieved in the bovine. Therefore, we attempted reprogramming of three types of bovine somatic cells using a Dox-inducible lentiviral vector and including several additives known to increase reprogramming efficiency in other species. With some exceptions<sup>13</sup>, putative bESCs found in other work are feeder-dependent<sup>14–27</sup>. In preliminary studies, we first attempted plating bovine cells over IRR-MEFs or granulosa cells. However, bovine cells proliferated so fast, causing detachment of the monolayers in less than five days (data not shown). Matrigel is a commercially available protein mixture commonly used as a basement membrane matrix for stem cells, and is utilized frequently in culture of mouse and human ESCs or iPSCs.

During mouse reprogramming, one of the earliest morphological changes observed is the appearance of clusters of rounded cells. This process is known as mesenchymal-to-epithelial transition (MET), and is considered to be the reversal of the embryological epithelial-to-mesenchymal transition (EMT) that occurs during gastrulation and some later stages. During iPSC reprogramming, the exogenous factors activate an epithelial pattern of gene expression, shutting down mesenchymal genes and thus reverting to an epithelial state<sup>28</sup>. In the work presented here, we observed different degrees of formation of MET-like cells during reprogramming with three of the conditions tested, but these cells failed to acquire a more distinct stem cell colony morphology. These partially reprogrammed cells persisted as groups of cells that later senesced around the same time as the surrounding non-reprogrammed cells. Reprogramming is known to occur in at least two main stages or “waves” of molecular-remodeling events<sup>29</sup>. The first stage involves downregulation of differentiated-cell markers and upregulation of epithelial genes including E-cadherin, a cell adhesion molecule that regulates pluripotency, possibly by facilitating cell-cell signal exchange<sup>30</sup>. After this initial event, cells enter an intermediate stage of partial reprogramming. From here, reprogramming-competent cells will enter a second “wave” of stable upregulation of pluripotency



markers and changes in DNA methylation. Cells that fail to progress to this stage often persist as a population of partially reprogrammed cells<sup>31</sup>.

Spontaneous formation of trophoblast vesicles during incomplete reprogramming has been observed by others, and trophoblast vesicles can also form after embryoid body formation<sup>32</sup>. ROCK inhibition has been used to enhance generation of human ESCs from poor quality embryos<sup>33</sup>. In mouse blastocysts, it has been observed that inhibition of the RHO-ROCK pathway suppresses formation of the trophectoderm<sup>34</sup>. In our study, the use of ROCK inhibitor was able to prevent formation of trophoblast structures in reprogramming cells. Although our study did not pursue any further analysis of the trophoblast-like structures, our results suggest the possibility that ROCK inhibition in the bovine has a similar trophoblast inhibitory effect.



**Figure 3.7. Reprogramming of bFFs and bAFs with biPSC medium #4.** (A) Two days after Dox-induction, high cell mortality was observed in both cell types but predominantly in bFFs. Transduced cells that were not exposed to Dox had no visible cell mortality. (B) On day-4 after Dox induction, cells had recovered and cells with three distinct morphologies were observed in bFFs and bAFs. (C) Groups of small cells found in bFFs continued to proliferate. (D) Dead cells accumulate over the areas of small cells. Experiment was ended at this point, due to lack of conditioned media. (E) Relative expression (RQ) of *bOCT4* and the 4-factor vector (4F) was measured via qRT-PCR. *bOCT4* and 4F expression was normalized to *bGAPDH* and compared to untreated cells (d0). Due to limited availability of conditioned media, only 1 biological replicate with 3 technical replicate per time point was analyzed.

Last, the choice of promoter and vector seems to also affect the efficiency of reprogramming. Research in pigs show that EF1 $\alpha$  and CAG promoters were more efficient than a Dox-inducible promoter in generating iPSC-like cells in pigs, affecting the long term expression of endogenous pluripotency markers<sup>32</sup>. Although a Lentiviral Dox inducible system offer advantages such as high transduction efficiency, and the possibility to know the exact timing of transgene expression, it is possible that the bovine downregulates transgene expression regardless of the continued Dox supplementation. Based on our findings, we hypothesize that the choice of vector was an important determinant in the early downregulation of the transgenes, which could have led to insufficient time for the cells to stably turn on endogenous pluripotency gene networks, as suggested by the OCT4 expression pattern. In mice, exposure to pluripotency factors is required for at least two weeks to establish stable pluripotent cell lines<sup>35</sup>.

It has been discussed by others that transcription factors required to reprogram mouse and human cells to pluripotency, as well as the culture media conditions used, could not be appropriate or sufficient to reprogram livestock species such as the cow to pluripotency. If the promoter used also has a significant impact in the reprogramming process, this adds another level of difficulty to a process that is already inefficient in the species where it works. Therefore, due to the increasing amount of evidence suggesting against this type of reprogramming in the cow, we did not pursue in this line of research any further. For future experiments, we focused on finding alternative or additional pluripotency genes to reprogram bovine somatic cells to pluripotency. This is described in the following chapters.

As a final note, in conversations with other researchers, lack of success in reprogramming livestock species using mouse and human conditions seems to be a recurrent issue. It is very likely that lack of acceptance of negative results in scientific journals and criticism towards publication of negative results has kept this information from being widely known<sup>36</sup>.

In summary, we were unable to produce fully reprogrammed bovine iPSCs using mouse and human protocols, and the exact cause of our lack of success is unclear. From what we have learned so far, it is possible that a different method of transgene expression could play a role in reprogramming. However, these ideas would be driven by a rather empirical reasoning, extrapolating findings from other species and not contributing in our understanding of the particular differences of pluripotency in ungulates. Therefore, for the following chapters, our work focused on getting a better understanding of bovine nuclear reprogramming. This would allow us to rationally target the specific requirements of potential bovine pluripotent cells.

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### 3.7 ATTRIBUTIONS

This chapter was completed by the author, with guidance from Dr. Will Eyestone. Student interns participated during some stages of this project: veterinary student Dante Caceres assisted with qRT-PCR, and veterinary students Sebastian Rios and Nicolas Busse assisted with maintenance of cell cultures.

### 3.8 ACKNOWLEDGEMENTS

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## CHAPTER 4. FUSION OF MURINE AND BOVINE FIBROBLAST MONOLAYERS USING POLYETHYLENE GLYCOL (PEG)

### 4.1 ABSTRACT

Cell fusion is a process that involves fusion of the membrane of two or more cells to form a multinucleated cell. Fusion of cells from different lineages can be used *in vitro* to study the effect of *trans*-acting factors. It has been reported that the nucleus of a somatic cell fused to pluripotent cells expresses pluripotency genes. However, cell fusion only happens in a small proportion of the cells exposed to fusogenic conditions. In this chapter, we tested several polyethylene glycol (PEG)-based fusion protocols to obtain a method to efficiently fuse both mouse fibroblast NIH/3T3 cells and bovine fetal fibroblast (bFF) monolayers. Initially, we obtained 7.28% of multinucleated NIH/3T3 cells when using 50% PEG 1500. Addition of 10% of DMSO to the PEG solution increased the percentage of multinucleated NIH/3T3 cells to 11.71%. The treatment caused loss of 52% of the cell monolayer, and mortality was 5.4% compared to 0.5% in control cells. In bFFs, treatment with 50% PEG 1500 plus 10% DMSO produced 11.05% of multinucleated cells. Only 7.6% of bFFs were lost after treatment, and mortality was 4.9%, contrasting with 0.7% in control cells. In summary, PEG 1500 at 50% plus 10% DMSO consistently produced the highest percentage of multinucleated cells in both NIH/3T3 cells and bFFs. This method will be used to fuse bFFs to mouse embryonic stem cells (mESCs), to study the role of mESC *trans*-acting factors over the bovine somatic nucleus.

### 4.2 INTRODUCTION

Cell fusion is a naturally-occurring process that involves combining the extracellular membrane of two or more cells to form one single multinucleated cell. Cell fusion occurs *in vivo* during gamete fusion, development of placenta, formation of muscle and bone, tissue regeneration, inflammation, and cancer formation<sup>1-9</sup>. Cell fusion can be induced *in vitro* to generate hybridoma for monoclonal antibody production<sup>10</sup>, and has also been used to study the effect of *trans*-acting regulators in reprogramming and differentiation<sup>11</sup>.

*In vitro* generated fusion products can become hybrids or polykaryons, depending on the culture conditions provided. Hybrids proliferate and their nuclei fuse forming polyploidy cells, whereas polykaryons do not proliferate and their nuclei remain intact<sup>12</sup>. If the cells used to form a polykaryon are from the same cell type, the product is considered a homokaryon (i.e. homotypic fusion product), whereas if the originating cells are from different types and/or species it is named a heterokaryon (i.e. heterotypic fusion product). Researchers have shown for decades that the fusion of cells from different lineages and differentiation states results in changes of gene expression in the nuclei of the fused cells<sup>13</sup>. Interestingly, fusing somatic cells with pluripotent cells such as embryonic stem cells (ESCs)<sup>14-16</sup>, embryonal germ cells (EGCs)<sup>17</sup>, or embryonal carcinoma cells (ECCs) induces reprogramming of the somatic nucleus, suggesting that the reprogramming activity of the pluripotent cell is dominant over the gene expression pattern of the somatic cell. Changes in gene expression in heterokaryons happen in the absence of cell division, which makes cell fusion a powerful tool to study early modifications in gene expression. This approach is currently very relevant in understanding reprogramming<sup>18</sup> and differentiation<sup>19</sup>, both processes that constitute one of the fundamentals of regenerative medicine that are, to date, inefficient and poorly understood<sup>20</sup>.

Due to its simplicity and low cost, polyethylene glycol (PEG) is usually the method of choice to induce fusion in a laboratory setting. Davidson et al. (1976) compared the efficiency of hybrid formation when fusing cell monolayers with PEG of different molecular weights at different concentrations. They observed that PEG with an average molecular weight of 1000, diluted at 50% in medium (50% weight/volume (w/v)), was the optimal combination of molecular weight and concentration<sup>21</sup>. Since then, little has changed in terms of type of PEG and concentration used, and most published work on mammalian cell fusion still uses a combination of PEG in the molecular weight range of 1000 – 3700<sup>22–27</sup>. Fusion can only be induced in a small proportion of the cells, and different cell types might require modifications of the method used in terms of PEG molecular weight, concentration, and/or time<sup>28</sup>.

We have previously attempted reprogramming of bovine fibroblasts to pluripotency by overexpression of genes known to work in mice and humans with little to no success (see [chapter 3](#)), suggesting that the bovine might require expression of additional/different sets of reprogramming genes. It is therefore fundamental to understand the process of nuclear reprogramming in the bovine before meaningful attempts to reprogram somatic cells to pluripotency are made. Most cell fusion work is focused in fusing murine and/or human cells. Therefore, in this chapter we tested previously described protocols to fuse mouse fibroblasts growing on a monolayer, and used the most efficient method to fuse bovine primary fibroblasts. This study provides the foundation to later use this method to generate heterokaryons from fusion of mouse ESCs and bovine fibroblasts as a tool to study the effects of the mouse ESC transcriptome on the bovine somatic nucleus.

## 4.3 MATERIAL AND METHODS

### 4.3.1 CELL CULTURE

The mouse fibroblast cell line NIH/3T3 was purchased from ATCC (CRL-1658); primary bovine fetal fibroblasts (bFFs) were derived from a male fetus of unknown genetic background obtained at an abattoir at gestation day 60. Cells were grown in a 5% CO<sub>2</sub> in air incubator at 37°C (for NIH/3T3 cells) or 38.5°C (for bFFs) on 10 cm tissue culture dishes (Falcon) in fibroblast medium: Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; HyClone) and 50 µg/ml Gentamicin (Lonza). Alternatively, cells were also cultured in serum starvation medium<sup>16</sup>: DMEM, 0.5% FBS, 1% non-essential aminoacids (HyClone), 1% Glutamax (Gibco), and 50 µg/ml Gentamicin. Media was replaced every two to three days. Subculture was done with TrypLE Express (Gibco) before cells reached 80% confluence. NIH/3T3 cells and bFFs were passage at least twice to ensure proper growth. Primary cell lines used for all experiments were between passages 2 to 6.

### 4.3.2 CELL FUSION

One day before fusion, cells were detached with TrypLE to a monocellular suspension and counted with a hemacytometer. We plated  $0.1 \times 10^6$  (for NIH/3T3 cells) or  $0.05 \times 10^6$  (for mNFs and bFFs) in 24-well tissue culture multiwell plates (Falcon) and incubated overnight at 37°C, which generated a ~95% confluent monolayer the next day. For fusion, all media, buffers, and reagents were pre-warmed to 37°C, and media changes were performed carefully from the side of the dish to avoid detachment of the cell monolayer.

First, we tested the effect of (a) 50% (diluted 50% w/v in HEPES) PEG 1500 (Roche) (b) 25% PEG 1500 (c) 50% PEG 3000-3700 (Sigma), or (d) 25% PEG 3000-3700. Cells were washed twice with 1 ml DPBS each before addition of 50 µl of PEG for exactly 2 min at room temperature, followed by two successive washes

with DPBS and one wash with DMEM (1 ml each one). PEG solutions come as 50% w/v dilutions; to generate 25% w/v we diluted PEG by adding an equal volume of DPBS. All treatments were run in parallel with 1 to 3 wells for each one, and this was replicated four times. For every replicate, one or two wells were treated with all washing steps but no PEG. Cells were incubated for 2 h at 37°C, detached using TrypLE, stained with Hoechst, and multinucleated cells counted with a hemacytometer.

Next, we fused NIH/3T3 cells using four different PEG treatments, with every treatment replicated using three different volumes of PEG. Treatments were: (a) PEG 1500, (b) PEG 1500 additioned with 10% DMSO, (c) pre-treatment of cells with hypoosmolar buffer for 2 min, followed by PEG 1500, and (d) pre-treatment of cells with hypoosmolar buffer for 2 min, followed by PEG 1500 additioned with 10% DMSO; every treatment was replicated using three different volumes of PEG: 50 µl, 100 µl, and 200 µl. For treatments using hypoosmolar buffer, a wash step using isoosmolar buffer prior to PEG treatment was used. Isoosmolar potassium phosphate buffer (10 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM K<sub>2</sub>HPO<sub>4</sub>, 1mM MgCl<sub>2</sub>, and 250 mM sucrose, in dH<sub>2</sub>O) and hypoosmolar potassium phosphate buffer (10 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM K<sub>2</sub>HPO<sub>4</sub>, 1mM MgCl<sub>2</sub>, 75 mM sucrose, in dH<sub>2</sub>O) were prepared as previously described<sup>29</sup>, filter-sterilized, and stored at 4°C before use. Every treatment was replicated four times, with 1 to 3 wells each time. For every replicate, one or two wells were treated with all washing steps but no PEG. Cells were incubated for 2 h at 37°C, detached using TrypLE, stained with Hoechst, and binucleated cells counted with a hemacytometer.

Finally, we fused NIH/3T3 cells and bFFs with 200 µl PEG 1500 plus 10% DMSO as described above.

#### 4.3.3 TRYPAN BLUE VIABILITY ASSAY

Trypan blue (Hyclone) was filtered to remove particles and diluted 1:1 in DPBS. Cells were detached using TrypLE and resuspended in 50 µl DPBS containing trypan blue and Hoechst. Suspension was loaded on a hemacytometer and examined under an inverted microscope (Olympus) to determine the percentage of viable cells (clear cytoplasm) versus nonviable cells (blue cytoplasm); Hoechst was used to observe nuclei.

#### 4.3.4 STATISTICAL ANALYSIS

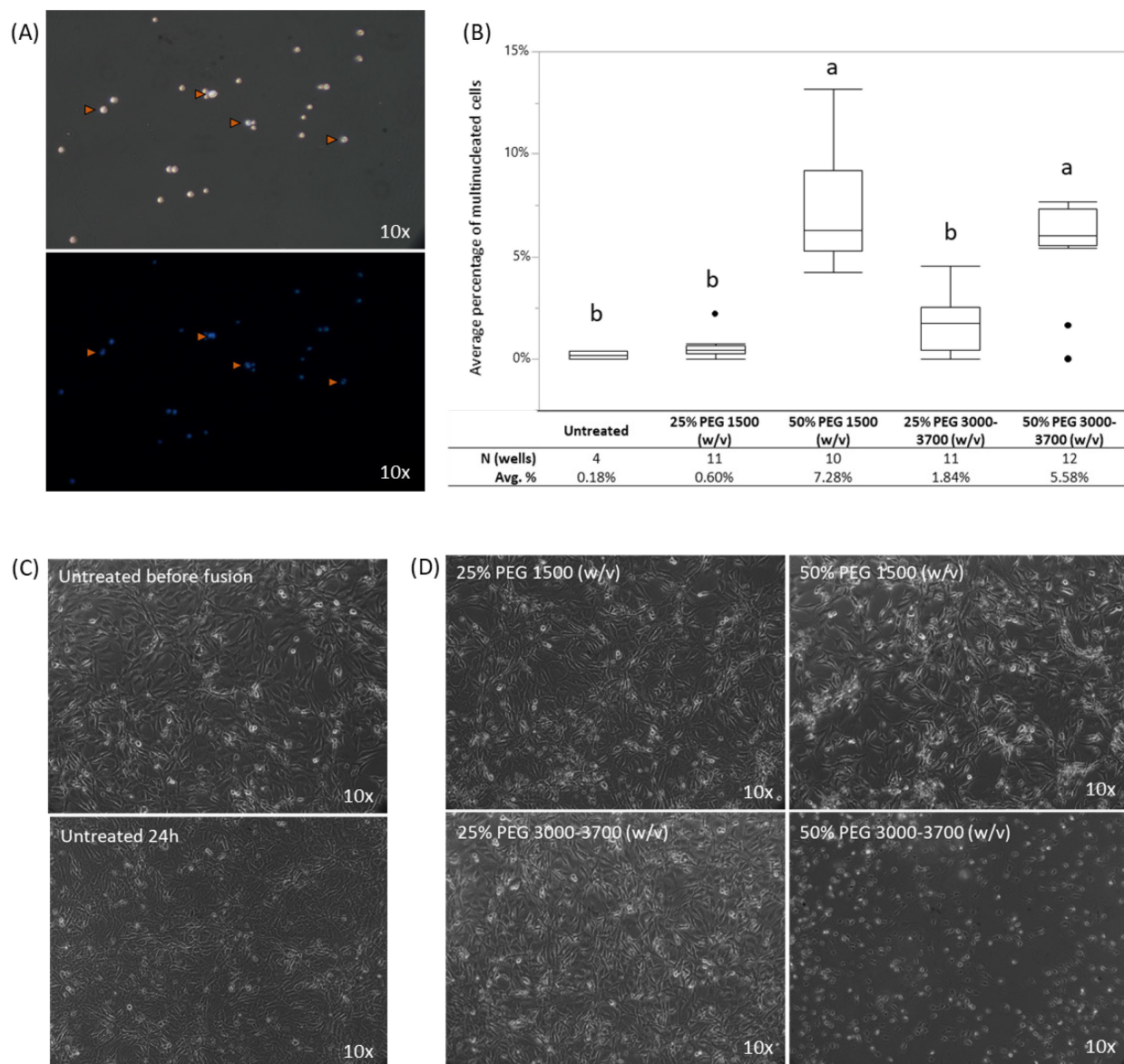
Data was organized in a Microsoft Excel 2016 spreadsheet and analyzed using JMP (version 13.2.1). We used one- or two-way ANOVA (as noted in the legend below graphs). Plates were run at different time points, and were therefore accounted as a blocking variable. When statistical significance was confirmed in the ANOVA, differences between groups were determined using t-test or the Tukey-Kramer multiple comparison post-hoc test, when appropriate. P-values <0.05 were considered statistically significant.

### 4.4 RESULTS

#### 4.4.1 NIH/3T3 MONOLAYERS CAN BE FUSED WITH 50% PEG 1500 (W/V)

We tested the effect of PEG 1500 and PEG 3000-3700 at two different concentrations (25% and 50%) and counted total and multinucleated cells 6 h after fusion treatment ([Figure 4.1A](#)). The majority of the multinucleated cells presented two or three nuclei. Occasionally, it was possible to observe cells with >3 nuclei but for consistency these were not counted ([Figure 4.1B](#)). We found that PEG 1500 at 50% w/v and PEG 3000-3700 at 50% w/v resulted in 7.28% and 5.58% of binucleated cells, respectively ([Figure 4.1C](#)).





**Figure 4.1. Fusion of NIH/3T3 cells with four PEG-based fusion treatments. (A)** Six hours after fusion, cells were detached, stained with Hoechst nuclear stain (blue), and loaded onto a hemacytometer for counting of total and multinucleated cells (orange arrowheads). **(B)** Average percentage of multinucleated cells 6h after treatment; a small number of multinucleated cells also appeared in unfused wells. One-way ANOVA followed by Tukey's HSD test. **(C)** Near confluent monolayer of NIH/3T3 cells before fusion (top) and equivalent monolayer after 24h (bottom). **(D)** Cells were left in culture for 24h after fusion treatment. Cells exposed to 50% PEG 3000-3700 (w/v) did not appear viable 24h after treatment.



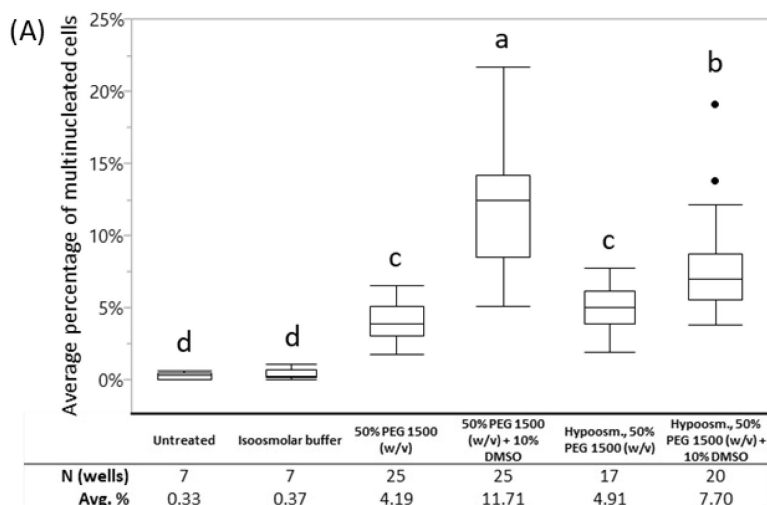
We studied the viability of the monolayers after fusion treatment, and observed that NIH/3T3 cells treated with 50% PEG 3000-3700 were not viable after 24h, whereas the remaining treatments appeared viable ([Figure 4.1D](#)). Cells were cultured for four passages; cells treated with 50% PEG 3000-3700 did not recover, whereas the remaining treatments were indistinguishable from the control group. Given that the damage on the cells was not immediately visible, for subsequent experiments the cells were left overnight in serum starvation medium before fusion percentage and viability were assessed.

#### 4.4.2 FUSION EFFICIENCY OF NIH/3T3 MONOLAYERS CAN BE INCREASED WITH THE ADDITION OF 10% OF DMSO

Next, we determined if addition of 10% DMSO to the PEG mixture, pre-treatment of monolayers with hypoosmolar buffer, and/or increase of PEG volume, had an impact on cell fusion. The incorporation of 10% DMSO to the PEG mixture has been described to increase the frequency of cell fusion in some cell types. We observed that addition of 10% DMSO had a positive effect, resulting in 11.71% of multinucleated cells ([Figure 4.2A](#)). Hypoosmolar buffer is used to increase cell volume and therefore increment the surface exposed for fusion. Pre-treatment of monolayers with hypoosmolar buffer affected the adhesion of the cells ([Figure 4.2B](#)), causing detachment of part of the monolayer in 26% and 37% of the wells exposed to this treatment ([Table 4.1](#)). PEG is a very viscous solution and small volumes tend to be difficult to pipet, with the risk of administering a suboptimal volume of PEG to the cells; because of this, we increased the volume of PEG used in the same culture well. We observed that, regardless of the treatment, PEG volume had no meaningful statistical significance over the resulting fusion percentage ([Table 4.2](#)).

**Table 4.1.** Detachment of NIH/3T3 cell monolayers after different treatments. A monolayer was considered to be detached when >40% of the cells were lost (by visual inspection).

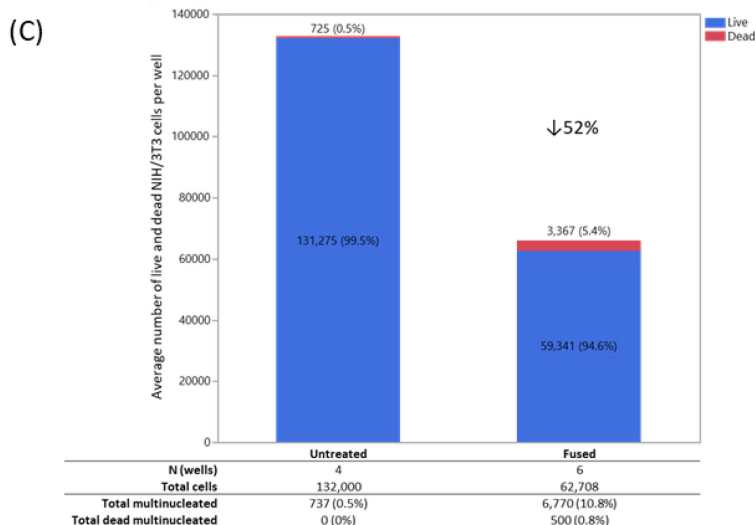
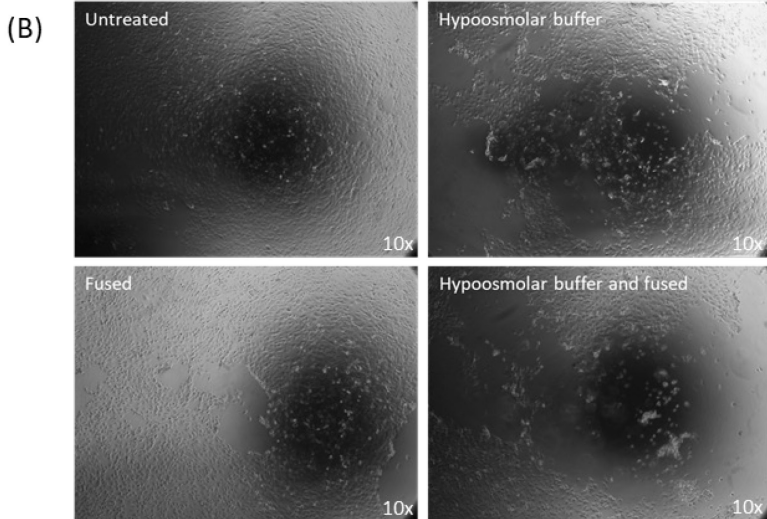
Treatment	Number of wells		Detachment percentage
	Total	Detached monolayers	
Untreated	7	0	0%
Isoosmolar buffer	7	0	0%
50% PEG 1500 (w/v)	27	2	7%
50% PEG 1500 (w/v) + 10% DMSO	27	2	7%
Hypoosmolar buffer, 50% PEG 1500 (w/v)	27	10	37%
Hypoosmolar buffer, 50% PEG 1500 (w/v) + 10% DMSO	27	7	26%



**Figure 4.2. Fusion of NIH/3T3 cells with 50% PEG 1500 and three variations of this method. (A)**

Average percentage of multinucleated cells after different fusion treatments. Controls included untreated and isoosmolar buffer treatment. Statistical significance was determined by two-way ANOVA for treatment and volume of PEG. Only treatment was statistically significant ( $P < 0.05$ ); significant groups were determined by Tukey HSD test. **(B)**

Representative images of NIH/3T3 cell monolayers 6h after treatment with hypoosmolar buffer only (top right), 50% PEG 1500 (bottom left), and hypoosmolar buffer followed by 50% PEG 1500 (bottom right); untreated control (top left) also included. **(C)** Trypan blue viability assay of NIH/3T3 cells 24h after fusion treatment. Stacked bars indicate average number of viable and dead NIH/3T3 cells per well. Cell number reduced by 52% in fused wells compared to untreated wells. The percentage of dead multinucleated cells was lower than the general percentage of dead cells.



**Table 4.2.** Average percentage of multinucleated cells after treatment with 50% PEG 1500 and three variations of this method, each one tested with three different volumes. Data was analyzed with two-way ANOVA for treatment and volume of PEG. Neither volume ( $P=0.84$ ) or the interaction between treatment and volume ( $P=0.26$ ) were statistically significant. Treatment was significant and is shown in Figure 4.2A.

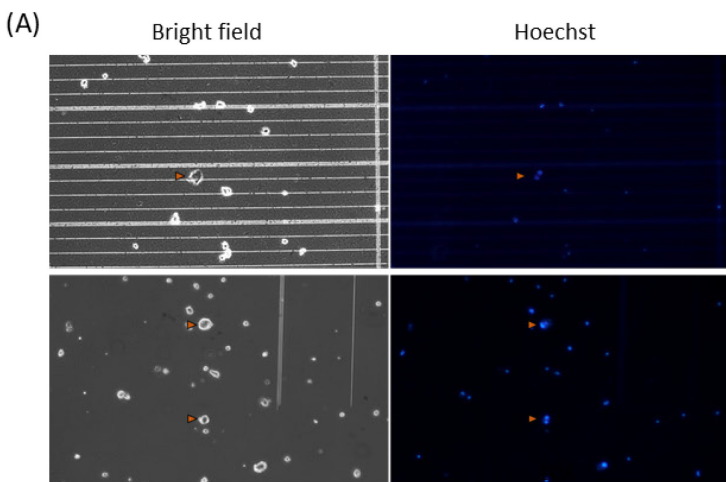
Pre-treatment	PEG	Additive	PEG solution final volume	N (wells)	Mean fusion %
	50% PEG 1500 (w/v)		50 $\mu$ l	8	4.12%
			100 $\mu$ l	9	4.14%
			200 $\mu$ l	8	4.29%
	50% PEG 1500 (w/v)	10% DMSO	50 $\mu$ l	8	10.34%
			100 $\mu$ l	9	13.71%
			200 $\mu$ l	8	11.08%
Hypoosmolar buffer	50% PEG 1500 (w/v)		50 $\mu$ l	5	4.96%
			100 $\mu$ l	5	4.31%
			200 $\mu$ l	7	5.45%
Hypoosmolar buffer	50% PEG 1500 (w/v)	10% DMSO	50 $\mu$ l	7	9.04%
			100 $\mu$ l	7	7.30%
			200 $\mu$ l	6	6.75%
Untreated				7	0.29%
Isoosmolar buffer wash step				7	0.37%

Cells exposed to 50% PEG 1500 plus 10% DMSO appeared viable 24 h after treatment. Monolayers were harvested and viability determined using Trypan blue. Cell mortality was higher in the fused group compared to control (5.4% and 0.5%, respectively). Nevertheless, mortality was not increased in multinucleated versus single nucleated cells (Figure 4.2C). Both fused and control cells proliferated similarly for three passages, at which point the cultures were ended.

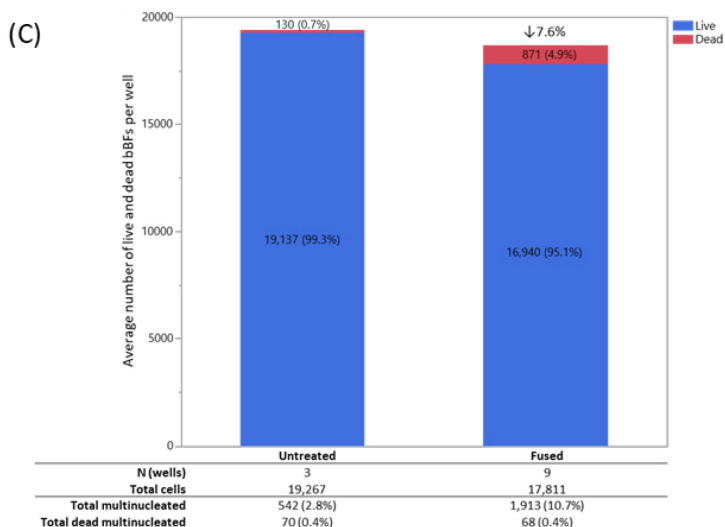
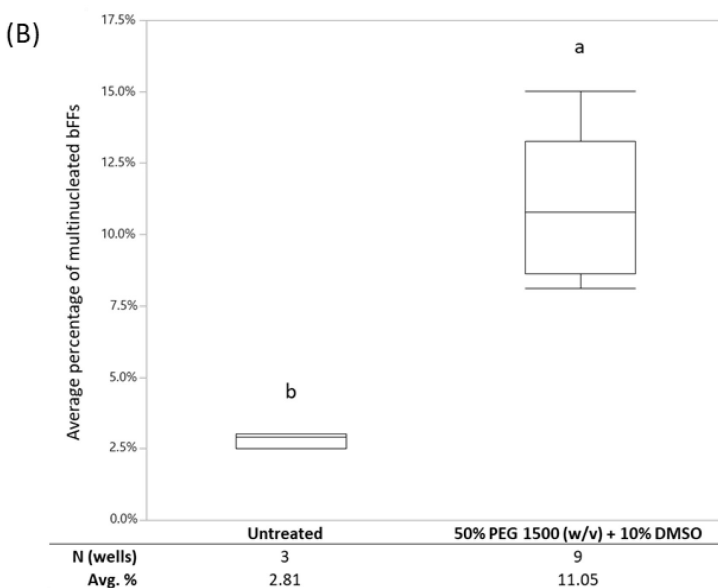
Due to its higher efficiency and easiness to pipette, subsequent experiments were performed using 200  $\mu$ l of 50% PEG 1500 with 10% DMSO, for every individual well of a 24 well plate.

#### 4.4.3 FUSION OF BOVINE EMBRYONIC FIBROBLASTS WITH 50% PEG 1500 PLUS 10% DMSO PRODUCES A SIMILAR PERCENTAGE OF MULTINUCLEATED CELLS AS IN THE MOUSE

We then fused near confluent bFF monolayers with 50% PEG 1500 plus 10% DMSO and counted total and multinucleated cells 24h after treatment (Figure 4.3A). Fusion treatment resulted in 11.05% of multinucleated cells, in contrast to the 2.81% found in the control group (Figure 4.3B). Cell mortality was higher in the fused group compared to control (4.9% and 0.7%, respectively). Nevertheless, mortality was not increased in multinucleated versus single nucleated cells (Figure 4.3C). Both fused and control cells proliferated similarly for three passages.



**Figure 4.3. Characterization of bFFs after fusion with 50% PEG 1500 plus 10% DMSO. (A)** Following fusion treatment, cells were left overnight in serum starvation medium; cells were then detached, stained with Hoechst, and loaded onto a hemacytometer to count for total and multinucleated cells (orange arrowheads). **(B)** Percentage of multinucleated bFFs after fusion treatment. Statistical significance was determined by one-way ANOVA followed by t-test. **(C)** Trypan blue viability assay of bFFs 24h after fusion. Stacked bars indicate average number of viable and dead bFFs per well. Cell number reduced by 7.6% in fused wells compared to untreated wells. The percentage of dead multinucleated cells was lower than the general percentage of dead cells.



## 4.5 DISCUSSION

Methods to induce cell fusion include PEG, electrofusion, and Sendai virus. Unlike other methods, PEG is a relatively inexpensive reagent, requires no additional equipment, and the protocol can be completed in less than one hour. Although cell fusion has been used for decades, data on the proportion of fused cells obtained after treatment is not always available. Due to technological limitations in the past, early cell fusion work studied hybrid colonies, which form days after the initial fusion event<sup>30</sup>. It is also known that not all fusion products form hybrids, making it difficult to estimate how many cells initially fused. Efficiency of cell fusion also varies depending on the cell type(s), the fusogen, and the method used to determine fusion. For example, Blau *et al.* (1983) fused human amniocytes and mouse myotubes with PEG, and observed an average of 73% of heterokaryon formation by visual inspection of multinucleated cells<sup>22</sup>. In contrast, Brady *et al.* (2013) used PEG to fuse GFP<sup>+</sup> mouse ESCs and dsRed<sup>+</sup> human primary fibroblasts, and used FACS to select for double positive heterokaryons, obtaining 1.16% of heterokaryons on a first sort, which was later increased to 51.6% after a second sort and 77.8% after enrichment<sup>26</sup>. Due to the heterogeneity of the available data, as well as the different methods used to determine fusion efficiency, in this chapter we sought to establish a method to consistently produce a high percentage of multinucleated cells. Direct microscopical visualization of multinucleated cells was preferred over indirect methods, to avoid the possibility of instrumental error.

Most published studies use mouse or human derived cell lines. Due to cost and availability in our lab, we used the immortalized mouse fibroblasts cell line NIH/3T3 as a substitute for mouse primary fibroblasts. Using PEG alone, the highest percentage of multinucleated NIH/3T3 cells was obtained using either 50  $\mu$ l of 50% PEG 1500 or 50% PEG 3000-3700 per well in a 24-well plate for 2 min. Work in amphibians by Broyles *et al.* (2006) found that PEG-mediated fusion is a gradual process, with peak number of heterokaryons found between 4 – 6 hours<sup>31</sup>. Initially, we counted the number of multinucleated cells 6h after fusion treatment. However, cells treated with PEG 3000-3700 were not viable 24h after treatment, indicating that cell mortality might not be visible in the short term. Therefore, for the remainder of the study we incubated the cells for 24h before counting multinucleated cells. To prevent nuclear fusion during incubation we cultured the cells in low serum conditions, which prevents the majority of the cells from resuming cell cycle. PEG has been associated with toxicity in several studies. We therefore recommend to test the sensitivity of the cell types being used.

Next, we tested if the addition of 10% DMSO and/or the pre-treatment of the cells with hypoosmolar buffer, increased cell fusion in NIH/3T3 cells. Depending on the cell types being fused, the addition of 10% DMSO to the PEG mixture increases the frequency of cell fusion, as has been described for mouse spleen cells and mouse ESCs<sup>32</sup>. However, the addition of DMSO had no effect over frequency of fusion nor viability when fusing mouse C2C12 muscle cells with human amniocytes<sup>22</sup>. For NIH/3T3 cells, we found that addition of 10% DMSO to the PEG solution had a positive effect over the number of multinucleated cells, resulting in a 62% increase of fusion. Volume ranging from 50  $\mu$ l to 200  $\mu$ l had no significant impact over the proportion of fused cells. We also tested the effect of pre-treating the cells with hypoosmolar buffer, a procedure commonly used to increase cell volume and therefore increment the surface exposed for fusion, when inducing fusion in cell suspensions by electrofusion. We found that treatment with hypoosmolar buffer before fusion had no beneficial effect when fusing cells growing on a culture plate. Moreover, it caused detachment of cellular monolayer, indicating that this treatment may not be adequate to fuse cells growing on monolayers.

It has been described that different cell types can have different fusing potentials when exposed to PEG<sup>28</sup>, Therefore, toxicity, molecular weight, and concentration of the PEG solution, as well as the length of the treatment, should be assessed for every cell type. Nevertheless, when we fused bFFs with 200 µl of 50% PEG 1500 plus 10% DMSO, we obtained a similar percentage of multinucleated cells when this treatment was used to fuse bFFs (11.05%). Mortality was similar for both cell types. For all treatments, we used the same volume and dish size. Although the treatment can in theory be scaled, plate size-dependent events such as meniscus effect<sup>33</sup> or uneven cell densities caused by swirling of the media due to manipulation or vibrations inside the incubator or biosafety cabinet, can potentially have an effect over the effectivity of the treatment. Therefore, we recommend characterizing the efficiency when adapting this protocol to a different culture vessel.

In summary, we produced a method to consistently produce mouse and bovine homokaryons using 200 µl PEG plus 10% DMSO. This protocol worked similarly in both mouse NIH/3T3 fibroblasts and bovine fetal fibroblasts growing on monolayers, where it produced an equivalent percentage of multinucleated cells, with low cell mortality. Next chapter extrapolates this method to produce mouse ESC and bovine fibroblast heterokaryons, as a tool to study somatic nuclear reprogramming.

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#### 4.7 ATTRIBUTIONS

This chapter was completed by the author, with guidance from Dr. Will Eystone.

#### 4.8 ACKNOWLEDGEMENTS

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## CHAPTER 5. BOVINE SOMATIC NUCLEI FUSED TO MOUSE EMBRYONIC STEM CELLS EXPRESS PLURIPOTENCY MARKERS SIMILAR TO EARLY REPROGRAMMING EVENTS

### 5.1 ABSTRACT

The mechanisms that direct nuclear reprogramming to a pluripotent state are still not fully understood. For species in which the derivation of true induced pluripotent stem cells (iPSCs) has not yet been successful, insights into the particular mechanisms that govern pluripotency can potentially help in determining which are the appropriate conditions to stimulate reprogramming in somatic cells. Cell fusion of a somatic cell to a pluripotent cell is known to induce expression of pluripotency markers in the somatic nucleus. Here, we hypothesized that fusion of bovine fetal fibroblasts (bFFs) to mouse embryonic stem cells (mESCs) would induce expression of pluripotency markers in the fibroblast nucleus. We first established a method to specifically select for multinucleated cells originated from both mESC and bFF (heterokaryon) using indirect immunofluorescence. With this in place, flow cytometry was used to select heterokaryons which were analyzed using RNA-seq. We found a pronounced change in bovine gene expression patterns between bFFs and heterokaryons obtained 24h after fusion. Upregulation of early pluripotency markers *OCT4* and *KLF4*, as well as hypoxia response genes, contrasted with downregulation of cell cycle inhibitors such as *SST*. The cytokine *IL6*, known to increase survival of early embryos *in vitro*, was upregulated in heterokaryons, although its role and mechanism of action is still unclear. The cell fusion model presented here can be used to characterize early changes in bovine somatic nuclear reprogramming, and to study the effect of different conditions during reprogramming.

### 5.2 INTRODUCTION

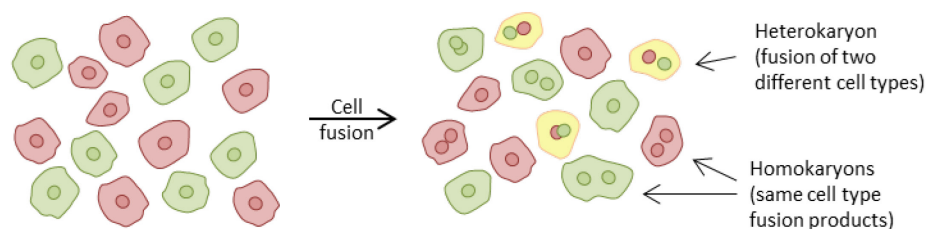
Pluripotent cells are defined as cells with unrestricted developmental potential. In nature, they are found during a very short time in the inner cell mass (ICM) of blastocysts. In certain strains of mice, rats, humans, and some non-human primates, it is possible to culture them *in vitro* as lines of embryonic stem cells (ESCs), while keeping their full developmental potential. An equivalent cell type has also been produced by over-expression of transcription factors in adult cells; these induced pluripotent stem cells (iPSCs) share the morphological and functional characteristics of ESCs. So far, conditions utilized to isolate ESCs or to induce pluripotency in somatic cells other than mouse, rat, human, and non-human primates have not been sufficient to adequately maintain a pluripotent gene expression pattern. The lack of pluripotent cell lines in large animal models such as the cow, has limited the use of this species not only for agricultural purposes but also in the biomedical field, where large animal models have long been considered fundamental to clinical trials, as well as their use as bioreactors. Several labs have attempted to improve reprogramming in the bovine by modifying the known reprogramming cocktail: adding or replacing genes, or using small molecules that inhibit or promote signaling pathways. However, this approach is rather empirical and does not address the underlying lack of knowledge. If conditions are to be adapted for the bovine (or any other species), it is essential to better understand the pluripotent state in this species. Even in species for which iPSC technology works, a better understanding of factor-based reprogramming could result in a more efficient reprogramming process, which would alleviate one of the main drawbacks of the current iPSC technology.

Cell fusion is a process in which two or more cells merge their membranes to form a multinucleated cell, and happens naturally during muscle development and trophoblast formation. In a laboratory setting, cell fusion is used to form of hybridoma cells for antibody production. In a different context, cell fusion has been used as a means to study the specific molecular mechanisms underlying cell reprogramming and differentiation. As reviewed by Helen M. Blau (1989), early cell fusion experiments using mammalian somatic cell hybrids demonstrated that gene expression could be modified by diffusible *trans*-acting regulators<sup>1</sup>. Due to technical limitations of the time, these reports were based on proliferating cell hybrids, which were analyzed after several passages and the separate contributions of the two nuclei could not be determined. Hybrids tend to be unstable and suffer from chromosomal loss and rearrangements. When fused cells are kept under low serum, non-proliferative, culture conditions, cells remain quiescent and their nuclei remain intact. This fusion product is known as a homokaryon if the cells are from the same species and cell type, or a heterokaryon if the cells are from different origins.

Heterokaryons are short-term, non-dividing, fusion products that can be used as an ideal model to study early changes in gene expression. Some key aspects that make heterokaryons a good model are: (1) nuclear fusion and mixing of genetic material does not occur because the heterokaryon nuclei do not enter S phase or mitosis in the culture conditions used, (2) controlling the ratio of nuclei provided by each cell type can provide an excess of cytoplasmic factors to drive cell type specific gene expression in a desired direction and, (3) when the cell types used are from different species (interspecific heterokaryons), it is possible to differentiate transcripts from each species based on species-specific nucleotide differences.

When fusing cells of different lineages, researchers have described for decades that if one of the fused cells is a mouse or human ESC, embryonic germ cell (EGC) or embryonal carcinoma (EC) cell, the resulting phenotype and pattern of gene expression is favored towards the pluripotent state, indicating that fusion of somatic cells with embryonic stem cells can induce pluripotency in the somatic nucleus<sup>2-4</sup>. Early studies using hybrids assessed the changes in expression of a small number of genes, using either species-specific RT-PCR or immunohistochemistry (IHC). Tada *et al.* (2001) fused mouse adult thymocytes with mouse ESCs (mESCs), and observed evidence of pluripotency in the thymocyte nucleus based on X chromosome activity, *Xist* accumulation, Oct4-GFP transgene expression, developmental potential, and DNA imprinting<sup>2</sup>. Palermo *et al.* (2009) have described that nuclear reprogramming can be induced when human keratinocytes and mouse muscle cells are fused to form a non-proliferative heterokaryon. In these bi-species heterokaryons, the direction of the nuclear reprogramming is dictated by a positive balance of regulators of one of the cells. Gene expression changes were observed within hours of fusion, and morphological changes were observed within 4 days<sup>5</sup>. Based on this evidence, we hypothesize that interspecific heterokaryons obtained by fusing mESCs and bFFs will induce expression of stemness genes in the bovine nucleus.

Due to the low efficiency of the fusion process (11.05% of multinucleated cells; [chapter 4](#)), a first step in this project was to obtain a method to efficiently identify heterokaryons (formed by fusion of mESCs with bFFs) from homokaryons. We tested four methods to label cells: lipophilic tracers, quantum dots, lentiviral vector-delivered fluorescent reporter genes, and indirect immunofluorescence. The overall goal for all cell labeling procedures was to specifically label bFFs and mESCs with two different fluorophores, and later collect heterokaryons that present both ([Figure 5.1](#)). This technical procedure proved to be more complicated than we anticipated: lipophilic tracers and quantum dots exhibited “leakiness” of the dyes, and the use of reporter genes negatively affected bovine cell survival. Indirect immunostaining proved to be the most specific method to identify fusion products originated from two different cell types. Once we were successful, we then selected and analyzed 200 heterokaryons, as well as mESC and bFF control cells for RNA-seq analysis.



**Figure 5.1** Schematic representation of the double-staining approach used to distinguish heterokaryons from homokaryons after cell fusion.

Because of differences in gene sequence between species, it was possible to discriminate between transcripts originating from the mouse and bovine nuclei. We observed significant changes in gene expression patterns predominantly in the bovine nucleus 24h after fusion. Changes involved co-regulation of early pluripotency markers *OCT4*, *KLF4*, *CCL2* and hypoxia markers, as well as downregulation of somatic cell markers such as *SST*. This indicates that the heterokaryon cell fusion model recapitulates several of the events of early reprogramming, and can therefore be used for further study of pluripotency in the bovine.

## 5.3 MATERIAL AND METHODS

### 5.3.1 CELL CULTURE

Bovine fetal fibroblasts (bFFs) were isolated from an abattoir-obtained male fetus of unknown genetic background at approximately gestation day 60. Mouse newborn fibroblasts (mNFs) were isolated from the skin of a euthanized newborn (day 1) CD1 mouse. The cell line NIH/3T3 was purchased from ATCC (CRL-1658). Packaging cells from the 293T line were purchased from ATCC (CRL-3216). bFFs, mNFs, and 293T cells were cultured in fibroblast medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; HyClone) and 50 µg/ml Gentamycin (Lonza) in a 5% CO<sub>2</sub> in air incubator at 38.5°C (for bFFs) or 37°C (for mNFs, NIH/3T3 cells, and 293T cells).

Mouse embryonic stem cells (mESCs) from the line C57BL/6 were purchased from ATCC (CRL-1002) and cultured in mESC medium: DMEM with 15% ESC-qualified FBS (Gibco), 1X Non-Essential Amino Acids (HyClone), 1X Glutamax (Gibco), 1,500 U/ml of ESGRO (Millipore), 0.55 mM beta-mercaptoethanol (Sigma), 1 µM PD0325901 (PD; Cayman chemical company), 3 µM CHIR99021 (CHIR; Cayman chemical company), and 50 µg/ml Gentamycin (Lonza); mESCs were cultured either on a monolayer of gamma irradiated mouse embryonic fibroblasts (IRR-MEFs, StemGent) or gelatin coated dishes (Sigma). After fusion, co-cultured bFFs and mESCs were incubated in basic stem cell medium: DMEM with 15% ESC-qualified FBS (Gibco), 1X Non-Essential Amino Acids (HyClone), 1X Glutamax (Gibco), 1,500 U/ml of ESGRO (Millipore), 0.55 mM beta-mercaptoethanol (Sigma), and 50 µg/ml Gentamycin (Lonza).

### 5.3.2 HETEROKARYON PRODUCTION

Heterokaryons were prepared as described previously ([chapter 4](#)). Briefly, 200,000 bFFs were seeded in one well of a 24-well plate in basic stem cell medium, and 2 h later 600,000 mESCs were seeded in the same well. Cells were co-cultured for 4h before fusion. Fusion was performed by adding 280 µl PEG with 20 µl DMSO pre-warmed to 37°C to each well during exactly 2 min and incubated at room temperature,

followed by 2 washes with DPBS and 1 wash with basic stem cell medium. Cells were cultured at 37°C in basic stem cell medium until analysis.

### 5.3.3 METHODS TO DIFFERENTIALLY STAIN FOR HETEROKARYONS

We tested several methods to distinguish heterokaryons (i.e. bFF fused to mESC) from homokaryons (i.e. bFF fused to bFF, or mESC fused to mESC).

#### 5.3.3.1 Cell labeling with lipophilic tracers Vybrant™ DiO and DiD

Staining with lipophilic tracers Vybrant™ DiO Cell-Labeling Solution (Molecular Probes) and Vybrant™ DiD Cell-Labeling Solution (Molecular Probes) was performed according to the kit's instructions. Briefly, NIH/3T3 cells were detached from the culture dish using Trypsin (HyClone) and resuspended as a monocellular suspension in serum-free fibroblast medium, at a density of  $1 \times 10^6$  cells/ml, before staining solution was applied. Upon incubation and washing steps, cells were cultured in fibroblast medium at 37°C. The loading efficiency was analyzed by FACS. Spectral characteristics of dyes are indicated in [Table 5.1](#).

**Table 5.1** Spectral properties of Vybrant™ Lipophilic Tracer dyes.

Dye	Excitation maximum (nm)	Emission maximum (nm)
Vybrant™ DiO	484	501
Vybrant™ DiD	644	665

#### 5.3.3.2 Cell labeling with Qtrackers 655 and 800

Qtracker® 655 and Qtracker 800 cell labeling kits were used according to the manufacturer's instructions. Briefly, NIH/3T3 cells were disaggregated to a monocellular suspension using Trypsin and resuspended in serum-free fibroblast medium before staining. Upon staining and washing steps, cells were placed back in culture in fibroblast medium at 37°C. The loading efficiency was analyzed by FACS. Spectral characteristics of dyes are indicated in [Table 5.2](#).

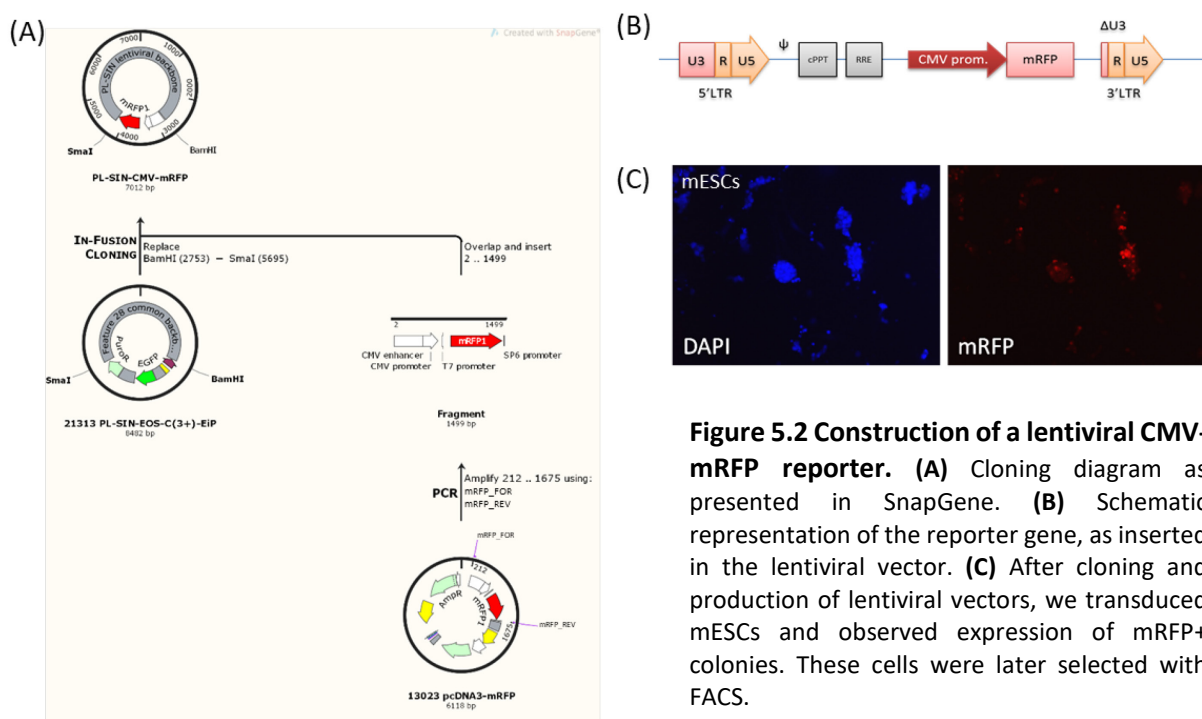
**Table 5.2** Spectral properties of Qtrackers.

Dye	Emission (nm)	Excitation (nm)
Qtracker® 655 Cell Labeling Kit	655	405–615
Qtracker® 800 Cell Labeling Kit	800	405–760

#### 5.3.3.3 Fluorescent reporter genes

We designed and built two lentiviral reporter constructs to stably transduce bFFs and mESCs. Cloning strategies and primers ([Table 5.3](#)) were designed in SnapGene and cloned using InFusion cloning (Clontech). The lentiviral plasmid vector PL-SIN-EOS-C(3+)-EiP (Addgene plasmid #21313) has been previously published<sup>6,7</sup>, and was used as the backbone for our constructs.

For mESCs, we designed a plasmid to harbor a cytomegalovirus (CMV) constitutive promoter driving expression of the monomeric red fluorescent protein (mRFP), inserted into the backbone of the PL-SIN-EOS-C(3+)-EiP vector ([Figure 5.2A](#) and [B](#)). The CMV-mRFP segment was cloned from a pcDNA3-mRFP plasmid also purchased from Addgene (#13032). The CMV-mRFP fragment was PCR amplified using high fidelity DNA polymerase and cloned in the BamHI and SmaI restriction sites of the PL-SIN-EOS-C(3+)-EiP plasmid.



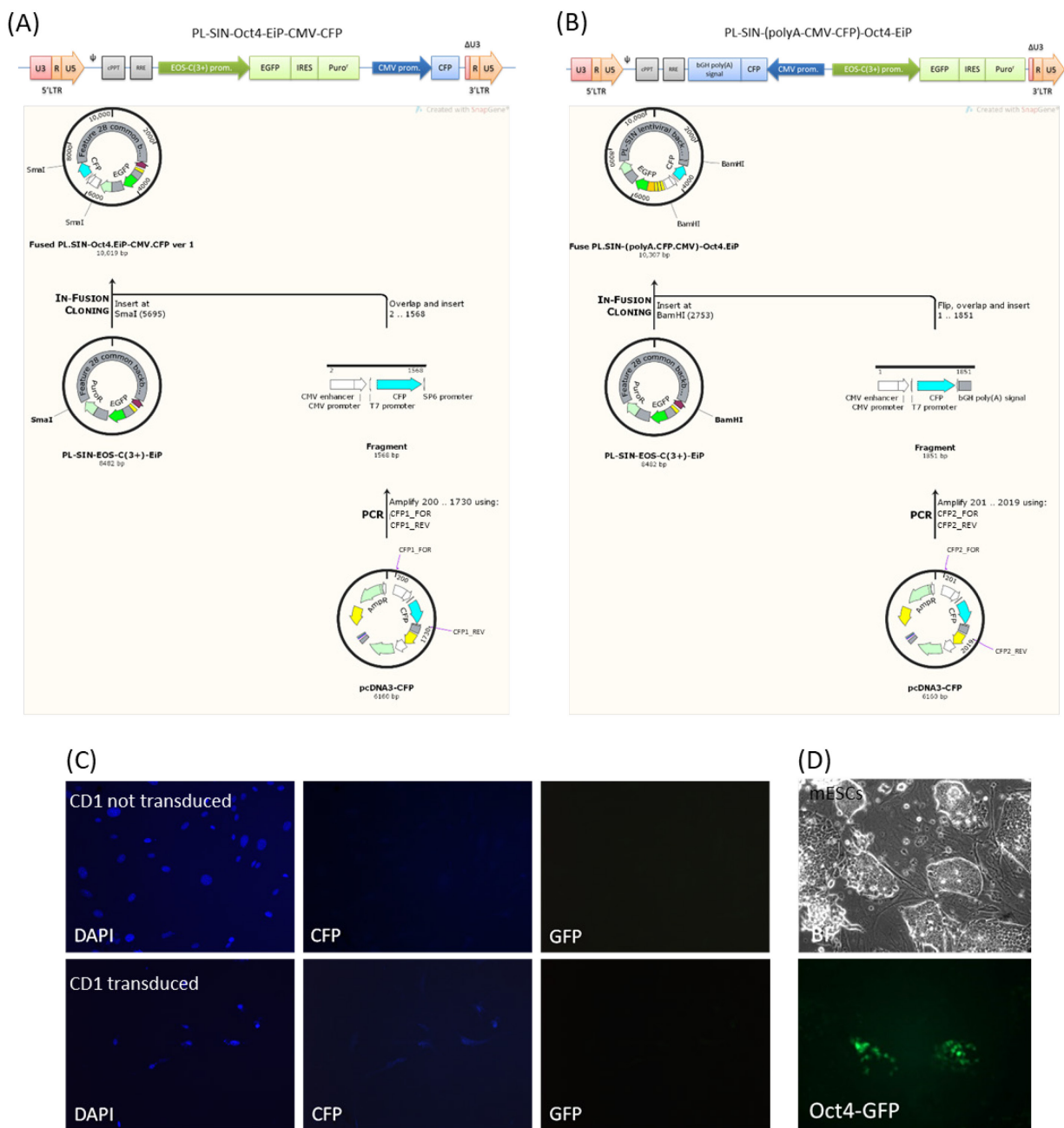
**Figure 5.2 Construction of a lentiviral CMV-mRFP reporter.** (A) Cloning diagram as presented in SnapGene. (B) Schematic representation of the reporter gene, as inserted in the lentiviral vector. (C) After cloning and production of lentiviral vectors, we transduced mESCs and observed expression of mRFP+ colonies. These cells were later selected with FACS.

Cloning reaction was transformed into Stbl3 competent cells, and several individual clones were screened using PCR. Clones that contained the insert were screened by Sanger sequencing, and a clone with no mutations was used to produce lentiviral vectors that were used to transduce mESCs. The mESCs with a positive mRFP signal (mRFP<sup>+</sup>) were sorted using FACS (Figure 5.2C).

For bFFs, we designed a polycystronic vector containing both CMV driving expression of cyan fluorescent protein (CFP) and an Oct4 promoter driving expression of EGFP. The PL-SIN-EOS-C(3+)-EiP plasmid already contains an enhanced Oct4 promoter driving expression of EGFP. Initially, we produced two versions of this construct. The first had the CMV-CFP reporter upstream of the Oct4-EGFP (PL-SIN-Oct4-EiP-CMV-CFP; Figure 5.3A). However, when transduced in mNFs or bFFs and analyzed by FACS, PL-SIN-Oct4-EiP-CMV-CFP produced expression of EGFP and was therefore no longer used. The second vector (PL-SIN-(polyA-CMV-CFP)-Oct4-EiP; Figure 5.3B) produced specific expression when tested in mNFs and mESCs (Figure 5.3C and D).

**Table 5.3** Primers for InFusion cloning.

Name	Primer 5'→3'
mRFP_FOR	ATTAGTGAACGGATCCTGTACGGGCCAGATATACGCGT
mRFP_REV	TCTTAAAGGTACCCCGGGTATAGAATAGGGCCCTCTAGATTAGGCGC
CFP1_FOR	ATCGATTTGGAACCCGGGGCTGCTTCGCGATGTACGGG
CFP1_REV	TCTTAAAGGTACCCCGGGATTTAGGTGACACTATAGAA
CFP2_FOR	GATAAAGGGAGGATCCCTGCTTCGCGATGTACGGGCC
CFP2_REV	ATTAGTGAACGGATCCACCCCTAGAGCCCCAGCTG



**Figure 5.3 Construction of a polycistronic lentiviral vector containing a constitutive CMV-CFP reporter and a specific Oct4-EGFP-reporter.** Cloning diagrams as presented in SnapGene for **(A)** PL-SIN-Oct4-EiP-CMV-CFP and **(B)** PL-SIN-(polyA-CMV-CFP)-Oct4-EiP. Only plasmid PL-SIN-(polyA-CMV-CFP)-Oct4-EiP was further used. **(C)** After cloning and production of PL-SIN-(polyA-CMV-CFP)-Oct4-EiP lentiviral vectors, we transduced mNFs and observed CFP expression only in transduced mNFs. No GFP expression was observed. **(D)** GFP was only expressed when PL-SIN-(polyA-CMV-CFP)-Oct4-EiP was transduced into mESCs.



### 5.3.3.4 Indirect immunostaining

To distinguish heterokaryons from homokaryons, cells were sequentially stained with antibodies targeting specific bFF and mESC surface antigens. As a preliminary step to find adequate antibodies, we tested several commercially available primary antibodies in fixed mESCs and BFFs, to test affinity and cross reactivity. We found that primary antibodies targeting mouse SSEA-1 and bovine CD44 did not produce cross reactivity. Therefore, further steps were performed using anti-SSEA-1 and anti-CD44. We further refined our antibody staining protocol to be used in our inverted fluorescence microscope and in our flow cytometry core facility ([Table 5.4](#)).

**Table 5.4** Antibodies used for indirect immunostaining of heterokaryons.

Cell type	Primary antibody	Dilution	Incubation time	Secondary antibody	Dilution	Incubation time
<b>Antibodies for fluorescence microscopy</b>						
bFFs	anti-mouse/human CD44 Biolegend, cat#103001	1:50	30 min	Rabbit anti-rat Alexa Fluor® 488 Jackson ImmunoResearch Inc., Cat#312545003	1:1000	20 min
mESCs	Anti-mouse SSEA1 Santa Cruz Biotechnology, sc-21702	1:2	30 min	Goat anti-mouse TxRed Santa Cruz Biotechnology, sc-2983	1:50	20 min
<b>Antibodies for ImageStream and FACS</b>						
bFFs	anti-mouse/human CD44 Biolegend, cat#103001	1:100	30 min	Rabbit anti-rat Alexa Fluor® 488 Jackson ImmunoResearch Inc., Cat#312545003	1:1000	20 min
mESCs	Anti-mouse SSEA1 Santa Cruz Biotechnology, sc-21702	1:100	30 min	Goat anti-mouse Alexa Fluor® 647 Abcam, ab150123	1:2000	20 min

### 5.3.4 MICROPIPETTE FABRICATION

To manually select heterokaryons, aspiration pipettes were crafted from glass capillary tubes (Sutter). The capillary tube was drawn using a horizontal pipette puller (Sutter Instruments P series). The pipette puller holds the capillary tubes by the edges, applying force as a heating element positioned around the center of the capillary tube heats the glass until it separates. This results in two needle-shaped half capillary tubes that have to be crafted to a desired shape and diameter. Next, we used a microforge to generate a tip of 100 µm internal diameter and a 20° angle. This gives the pipette an angulation easy enough to visualize but elevated enough from the bottom of the dish as to not cause dragging of the cells positioned on the bottom of the dish. The ends of the pipettes were briefly fire-polished by heating up the filament and slowly approximating the pipette until the glass turns dark and smooth. The tip of the pipette was coated in Sigmacote® (Sigma-Aldrich) and allowed to air dry overnight at room temperature. Sigmacote gives the pipette surface a hydrophobic coating that prevents cells from sticking to the glass surface.

### 5.3.5 MANUAL SELECTION OF HETEROKARYONS

Following fusion, cells were first dissociated to a monocellular suspension with TrypLE (Gibco) and filtered through a 100 µm cell strainer to remove cell clumps. Cells were pelleted and resuspended in DPBS with 1% FBS (HyClone) and stained according to [Table 5.4](#). All antibodies used were diluted in DPBS with 1% FBS, and cells were kept in the dark during incubation, inside the fridge at 4°C. We observed that without the cold incubation, fluorescence intensity decreased considerably. For nuclear staining, cells were incubated with Hoechst for 15 min, washed and resuspended in DPBS with 1% FBS. Droplets of 50 µl of cell suspension were placed in several 100 mm Petri dishes (Falcon), and covered with embryo grade mineral oil (Sigma) before removing from the biosafety cabinet. Droplets were screened for binucleated cells positive for both GFP and TxRed using an Olympus inverted microscope. Bicolored heterokaryons with 2 or more nuclei were aspirated with an aspiration micropipette using a Three-Axis Coarse/Fine Joystick-Type mechanical micromanipulator (Narishige) and a manual microinjector (CellTramOil, Eppendorf).

### 5.3.6 SELECTION OF HETEROKARYONS USING FLOW CYTOMETRY

Fused monolayers were first dissociated to a monocellular suspension with TrypLE (Gibco), centrifuged, resuspended in DNase solution (0.1 mg/ml deoxyribonuclease I in DMEM; Worthington biochemical corporation) and incubated for 15 min at room temperature). Following incubation, solution was filtered through a 100  $\mu$ M cell strainer. Filtered cells were centrifuged and resuspended in DPBS plus 1% FBS. Cells were stained according to [Table 5.4](#). All antibodies used were diluted in DPBS with 1% FBS, and during incubation steps cells were kept in the dark inside the fridge at 4°C. Controls (negative (unstained), GFP only, Alexa 647 only, Hoechst only, secondary antibodies only) were prepared in parallel, following all steps described for samples. For nuclear staining, cells were incubated with Hoechst for 15 min, washed and resuspended in DPBS with 1% FBS to a concentration of  $\sim 5 \times 10^6$  cells/ml for ImageStream (Amnis) and  $\sim 1 \times 10^6$  cells/ml for FACS Aria II (BD) analysis. The ImageStream is an imaging flow cytometer that permits visualization (bright field and fluorescence) of cells directly in flow; the ImageStream does not perform sorting. The FACS Aria II is a sorting cytometer. Gates and parameters identified with the ImageStream can be used to sort cells in the FACS Aria II. We first analyzed cells using ImageStream to identify location of bicolored, multinucleated heterokaryons. The obtained parameters were used to sort heterokaryons with the FACS Aria II. We sorted 200 heterokaryons directly into a 0.25 ml PCR tube (USA scientific) containing lysis buffer prepared as described in the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara; see section below).

### 5.3.7 PREPARATION OF CDNA, RNA-SEQ LIBRARIES AND SEQUENCING

RNA extraction and cDNA synthesis of bFFs, mESCs, and heterokaryons collected at 24, 48, and 72h after fusion, were performed using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing, following the manufacturer's instructions. We used 14 cycles for cDNA amplification. For samples used for qRT-PCR, the integrity and quality of cDNA was determined using an Agilent 2100 bioanalyzer, and quantity of cDNA was measured using a Qubit 3.0 Fluorometer (ThermoFisher). For the samples used for library prep and RNA-seq, we produced cDNA as above and submitted to the Biocomplexity Institute at Virginia Tech. Library prep was made using the Accel-NGS<sup>®</sup> 2S Plus DNA Library Kit (Swift biosciences), and quality of the libraries was analyzed with an Agilent 2100 Bioanalyzer. RNA-seq was run on a MiSeq (Illumina).

### 5.3.8 DATA ANALYSIS

We used Geneious to map all reads from the MiSeq fastq files to the bovine NCBI Reference Sequence Database (NCBI RefSeq; retrieved on June 2017), using the "Map to Reference" option. We used a custom setting allowing for 2% error, and the "Map Multiple Matches" option was set to "random". After alignment, data was compiled in Microsoft Access, and a final Microsoft Excel spreadsheet was used for analysis. To correct for differences in numbers of reads between different samples, read counts were normalized to reads per million mapped reads (RPM), and we only considered genes that had at least 20 reads in the 24h sample. We used the Microsoft Excel PEARSON function to identify genes that temporally co-express with a certain gene. The VLOOKUP function in Excel was used to search for specific groups of genes in the list. Assignment of Gene Ontology (GO) terms to genes of interest was obtained using the enrichment analysis tool from the Gene Ontology Consortium website<sup>3</sup>.

Two Microsoft Excel spreadsheets containing the full list of mouse and bovine mapped reads, are available as supplementary files<sup>4</sup>.

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<sup>3</sup> <http://geneontology.org/>

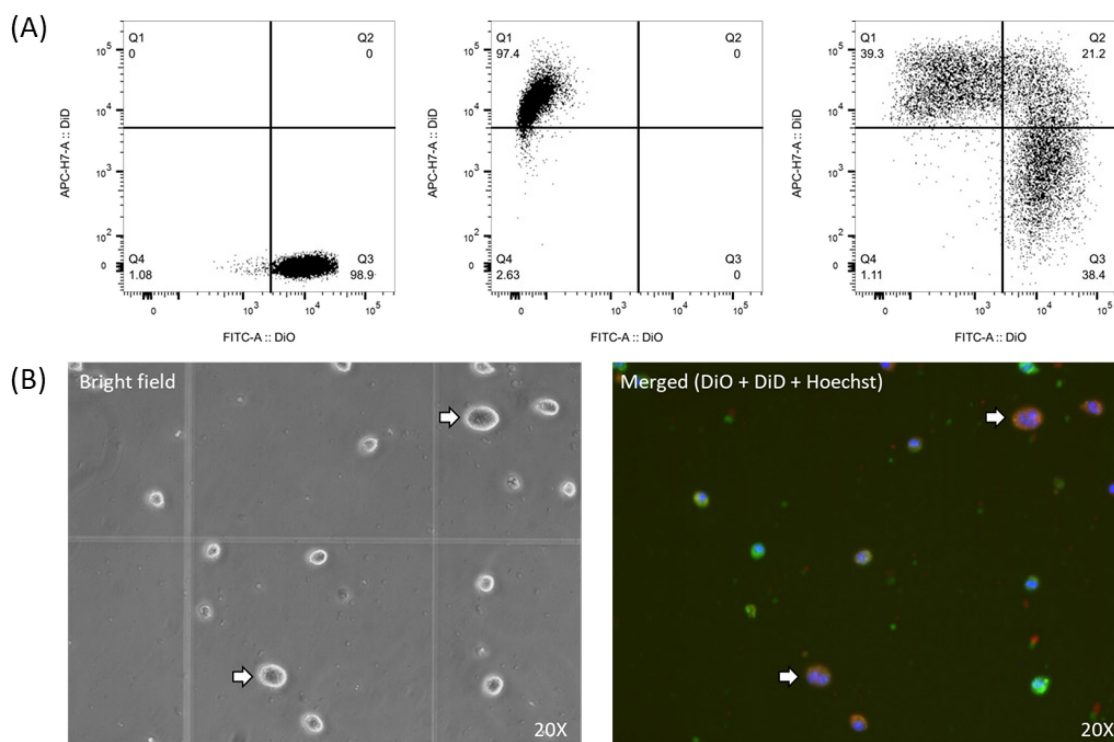
<sup>4</sup> File name: VillafrancaLocher\_MC\_D\_2018\_RNAseqData.zip



## 5.4 RESULTS

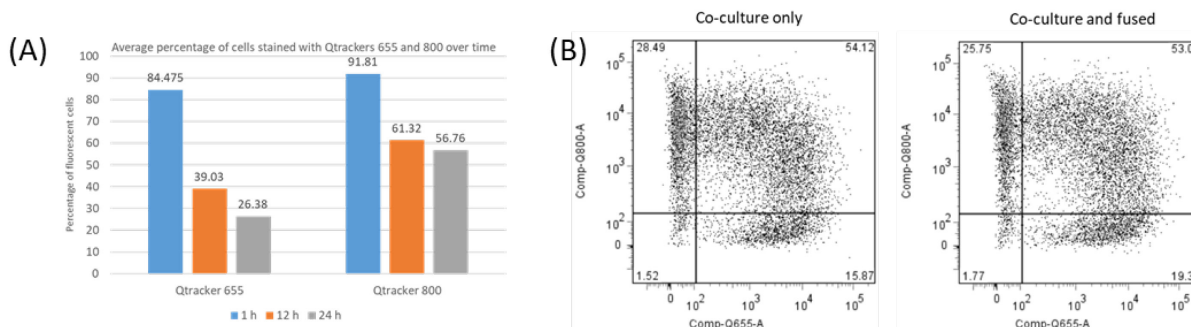
### 5.4.1 LIPOPHILIC TRACERS AND QTRACKERS DIFFUSE TO ADJACENT CELLS DURING FUSION AND CO-CULTURE

NIH/3T3 cells were stained separately with Vybrant DiO and Vybrant DiD lipophilic membrane dyes, plated in the same dish, and fused. We observed a heterogeneous population of double stained cells ([Figure 5.4A](#)). This population was sorted and analyzed under a fluorescence microscope, which showed that the majority of the cells had single nuclei ([Figure 5.4B](#)), suggesting that the dyes had transferred to adjacent cells regardless of fusion. When NIH/3T3 cells were labeled with Qtracker 655 and Qtracker 800, we first observed loss of fluorescence in the cells after 24h in culture. The decrease in fluorescence was more evident in cells stained with Qtracker 655 ([Figure 5.5A](#)). NIH/3T3 cells labeled separately with Qtracker 655 or Qtracker 800 were co-cultured, or co-cultured and fused. The total time of co-culture (including the fusion procedure) was no longer than 5h. When analyzed with FACS, we observed that >50% of the cells were positive for both Qtrackers, regardless if they were co-cultured, or co-cultured and fused ([Figure 5.5B](#)). This suggests that Qtrackers passively diffused out of the cells and into the culture media, where they could re-enter other cells. Because of the observed dye “leakiness”, we did not use these methods any further.



**Figure 5.4 Staining of NIH/3T3 fibroblasts with Lipophilic tracers Vybrant™ DiO and DiD.**

**(A)** NIH/3T3 cells were stained in suspension with either DiO only (left), DiD only (center), or DiO and DiD. Cells were plated and after 2h the cells stained with both DiO and DiD were fused. After incubation, cells were harvested and analyzed with FACS. Quadrant Q2 shows localization of double-stained cells. **(B)** Double-stained cells from top right quadrant (Q2) were sorted, stained with Hoechst for visualization of nuclei, and observed under a fluorescent microscope. We observed a heterogeneous population of double stained cells, most of them with one nucleus. Arrows indicate cells with >2 nuclei. This experiment was replicated twice.



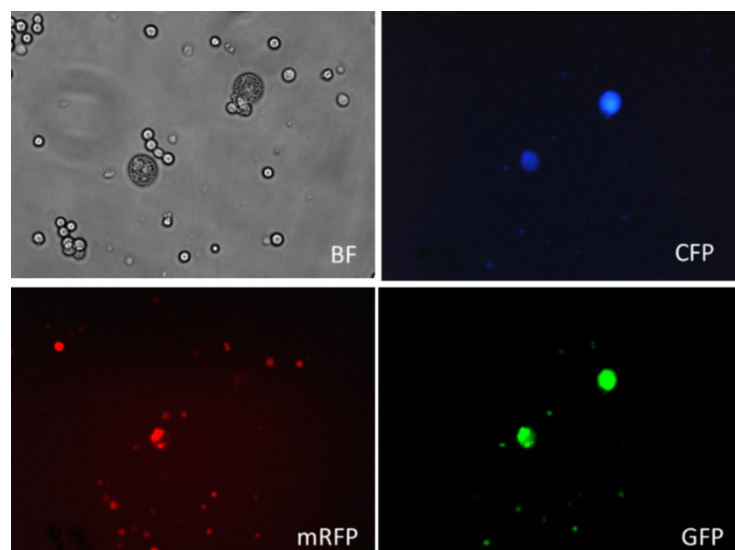
**Figure 5.5 Staining of NIH/3T3 fibroblasts with Qtrackers 655 and 800. (A)** NIH/3T3 cells were stained with either Qtracker 655 or Qtracker 800, and incubated for 1h, 12h, or 24h. Percentage of fluorescent cells was determined by FACS. N=2 replicates. **(B)** NIH/3T3 cells were stained with Qtracker 655 or Qtracker 800, and co-cultured or co-cultured and fused. Co-culture: 5h. N=2 replicates.

#### 5.4.2 LENTIVIRAL VECTOR-DELIVERED REPORTER GENES CAN CONSTITUTIVELY LABEL CELLS, BUT DECREASE VIABILITY OF BFFS.

We built several reporter constructs to label bFFs and mESCs, to later select for cells with expression of both reporters. Because the Oct4 promoter in our construct was originally derived from mouse cDNA sequence, we also transduced mNFs as a control.

For the mESCs, we cloned a CMV-mRFP reporter cassette into a lentiviral vector backbone ([Figure 5.2A](#) and [B](#)), and selected mRFP<sup>+</sup> mESCs using FACS ([Figure 5.2C](#)). For bFFs we attempted two designs of the same vector: (1) PL-SIN-Oct4-EiP-CMV-CFP, where the CMV-CFP cassette was inserted downstream the Oct4-EGFP cassette ([Figure 5.3A](#)), and (2) PL-SIN-(polyA-CMV-CFP)-Oct4-EiP, where the CMV-CFP cassette was flipped and inserted upstream of the Oct4 promoter ([Figure 5.3B](#)). Both designs were packed into lentiviral particles and transduced into mNFs, bFFs, and mESCs to evaluate functionality of the reporters. With PL-SIN-Oct4-EiP-CMV-CFP, we observed expression of both CFP and GFP in the mNFs and bFFs, indicating non-specific activation of the Oct4 promoter in fibroblasts. The PL-SIN-(polyA-CMV-CFP)-Oct4-EiP vector showed only CFP expression in both mNFs and bFFs ([Figure 5.3C](#)), and GFP was only expressed when transduced into mESCs ([Figure 5.3D](#)). We therefore used PL-SIN-(polyA-CMV-CFP)-Oct4-EiP to transduce both mNFs and bFFs, and FACS sorted them according to CFP expression. Cells were allowed to expand for 2-3 passages to increase cell number before fusion. Although mNFs expanded rapidly after sorting, bFFs appeared damaged and >90% of the cells died or remained quiescent in culture; control bFFs recovered after sorting, indicating a possible effect of the transduction over bFF cell survival after FACS sorting. We were therefore not able to use bFFs for cell fusion. Nevertheless, to test activity of the Oct4-GFP reporter in a cell fusion context, we fused CFP<sup>+</sup> mNFs with mRFP<sup>+</sup> mESCs. Expression of GFP was observed in large cells co-expressing both CFP and mRFP ([Figure 5.6](#)).

In conclusion, although our reporter constructs specifically labeled mESCs, mNFs, and bFFs, the latter cell type did not survive FACS sorting. The small population of surviving cells appeared quiescent and was therefore not considered adequate to use for cell fusion studies.



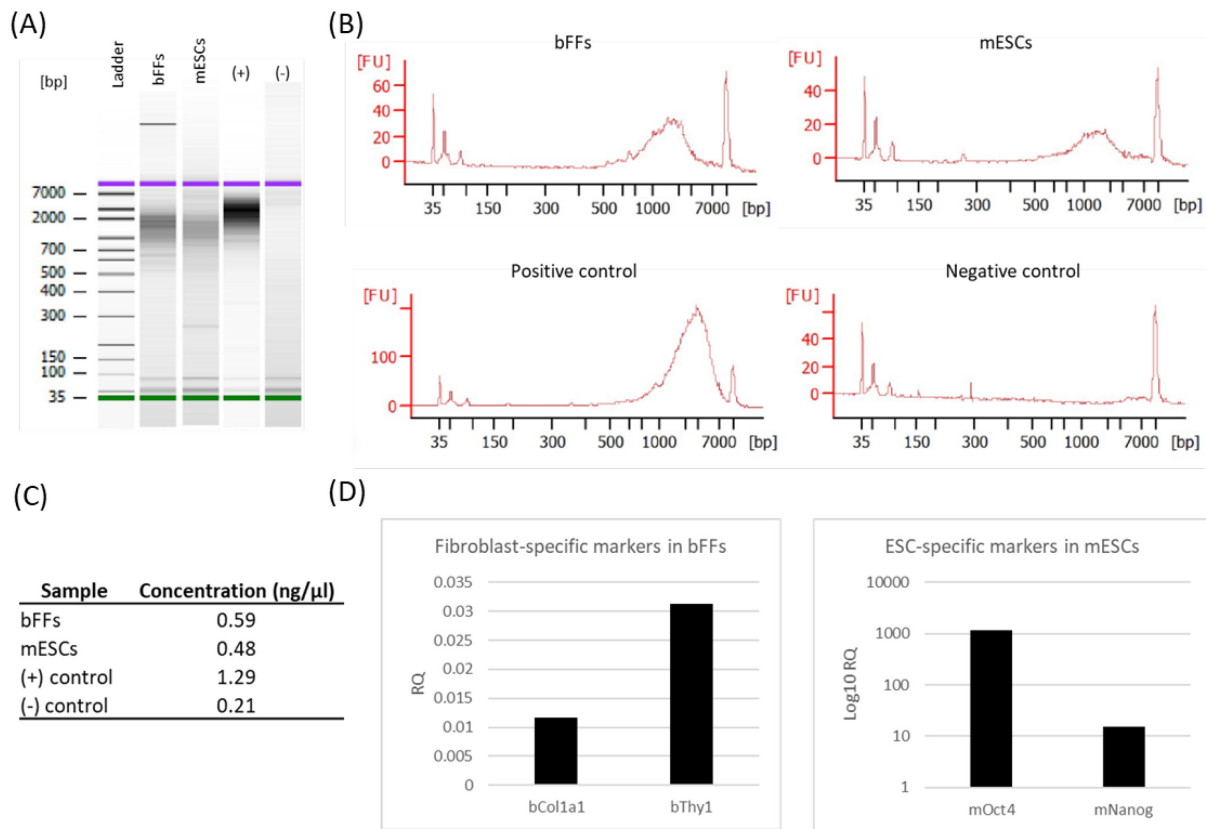
**Figure 5.6 Heterokaryons produced by fusion of CFF+ bFFs with mRFP+ mESCs.** The bFFs were also transduced with an Oct4-GFP cassette. GFP was only observed in large cells expressing both CFP and mRFP.

#### 5.4.3 SYNTHESIS OF CDNA WITH THE SMART-SEQ V4 ULTRA LOW INPUT RNA KIT

When using the SMART-Seq kit for the first time, we initially tested our ability to produce good quality cDNA, by using 1000 bFFs and 1000 mESCs as template. Cells were disaggregated to a single-cell suspension, counted, and a volume estimated to have ~1000 cells was used for RNA extraction and cDNA synthesis. A negative and positive control (included in the kit) were also included. Quality of the cDNA was evaluated using an Agilent 2100 Bioanalyzer; resulting gel-like images ([Figure 5.7A](#)) and electropherograms ([Figure 5.7B](#)) indicate high quality cDNA, especially in the bFF sample. cDNA was quantified on a Qubit fluorometer and concentration appeared in the expected range ([Figure 5.7C](#)). We used qRT-PCR to screen for expression of bFF and mESC markers, and were able to detect expression of *COL1A1* and *THY1* in bFF cDNA, and *Oct4* and *Nanog* in mESC cDNA ([Figure 5.7D](#)).

#### 5.4.4 OBTAINMENT OF DOUBLE-STAINED HETEROKARYONS BY MANUAL SELECTION PERMITS PRECISE COLLECTION OF CELLS, BUT THE LENGTH OF THE PROCEDURE CAN HAVE A DETRIMENTAL EFFECT ON CELL QUALITY

We produced heterokaryons and stained them with fluorescently labeled antibodies ([Figure 5.8A](#)). Manual selection of the cells permits exact observation and collection of the cells that are being analyzed ([Figure 5.8B-D](#)), and we were able to obtain good quality of cDNA from heterokaryons, mESCs, and bFFs selected with this method ([Figure 5.8E and F](#)). Unfortunately, the whole procedure from antibody staining to screening and manual selection of cells proved to be very time consuming, often taking up to 8 – 10h. The effect of this long manipulation time could potentially have a detrimental effect on the cells and their gene expression pattern. Moreover, when we screened for *bOCT4* and *bNANOG* expression using qRT-PCR, results were not consistent between the two biological replicates. We observed *bNANOG* expression in only one of the replicates, and *bOCT4* was expressed in both but the signal did not begin until qPCR cycle 35, with great variability in the quality of the melting curves of the technical replicates (data not shown). We decided not to use this approach to obtain cells for RNAseq.

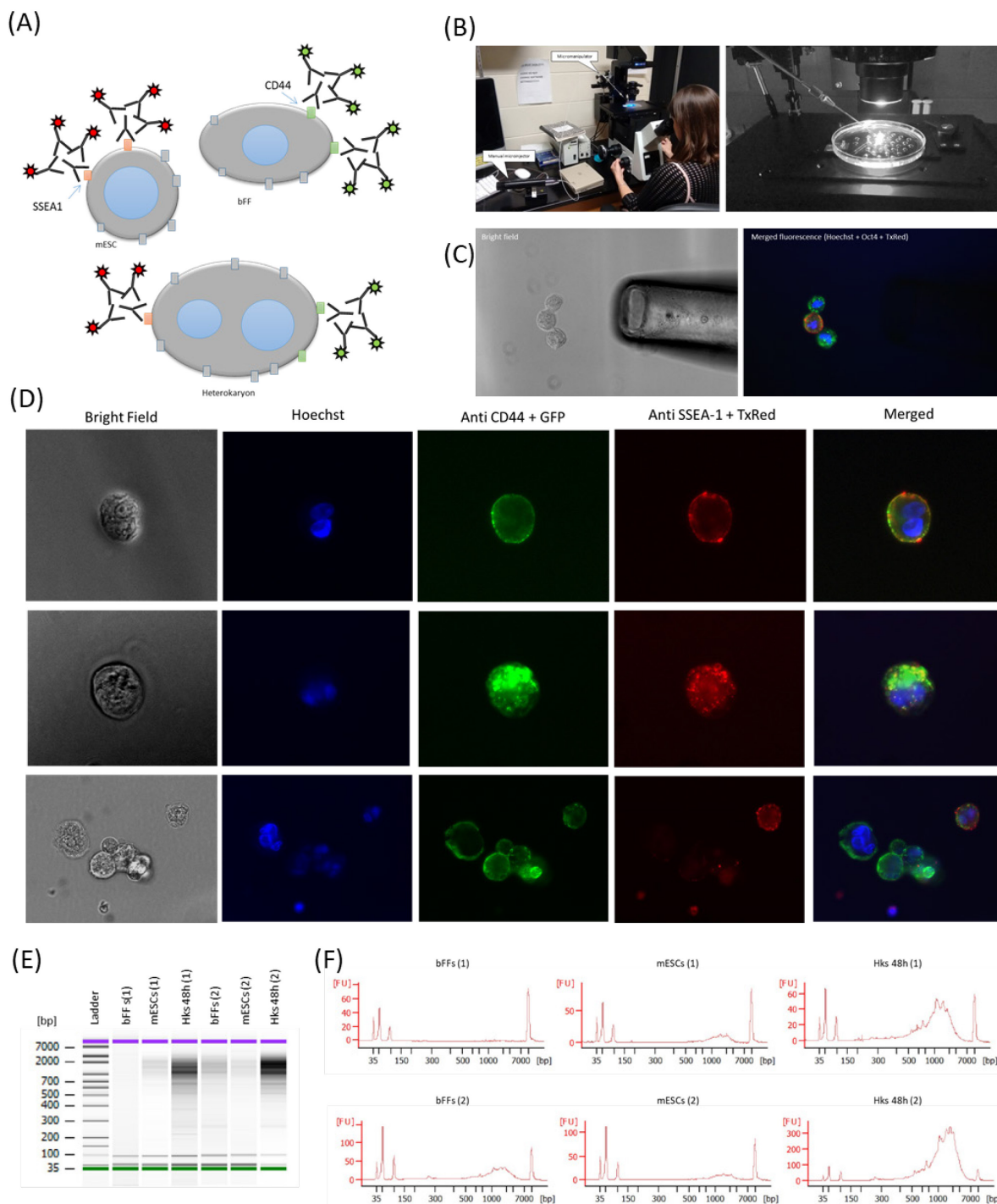


**Figure 5.7 Synthesis of cDNA with the SMART-Seq v4 Ultra Low Input RNA kit.** Quality of the cDNA was measured on an Agilent 2100 Bioanalyzer; the summary report shows (A) gel-like images of samples and (B) chromatograms. (C) Concentration of cDNA obtained was within the acceptable range. (D) Fibroblast and mESC-specific markers were detected using qRT-PCR.

#### 5.4.5 IDENTIFICATION AND SELECTION OF DOUBLE-STAINED HETEROKARYONS USING IMAGESTREAM AND FACS IS A FAST METHOD TO OBTAIN A MODERATELY PURE POPULATION OF HETEROKARYONS

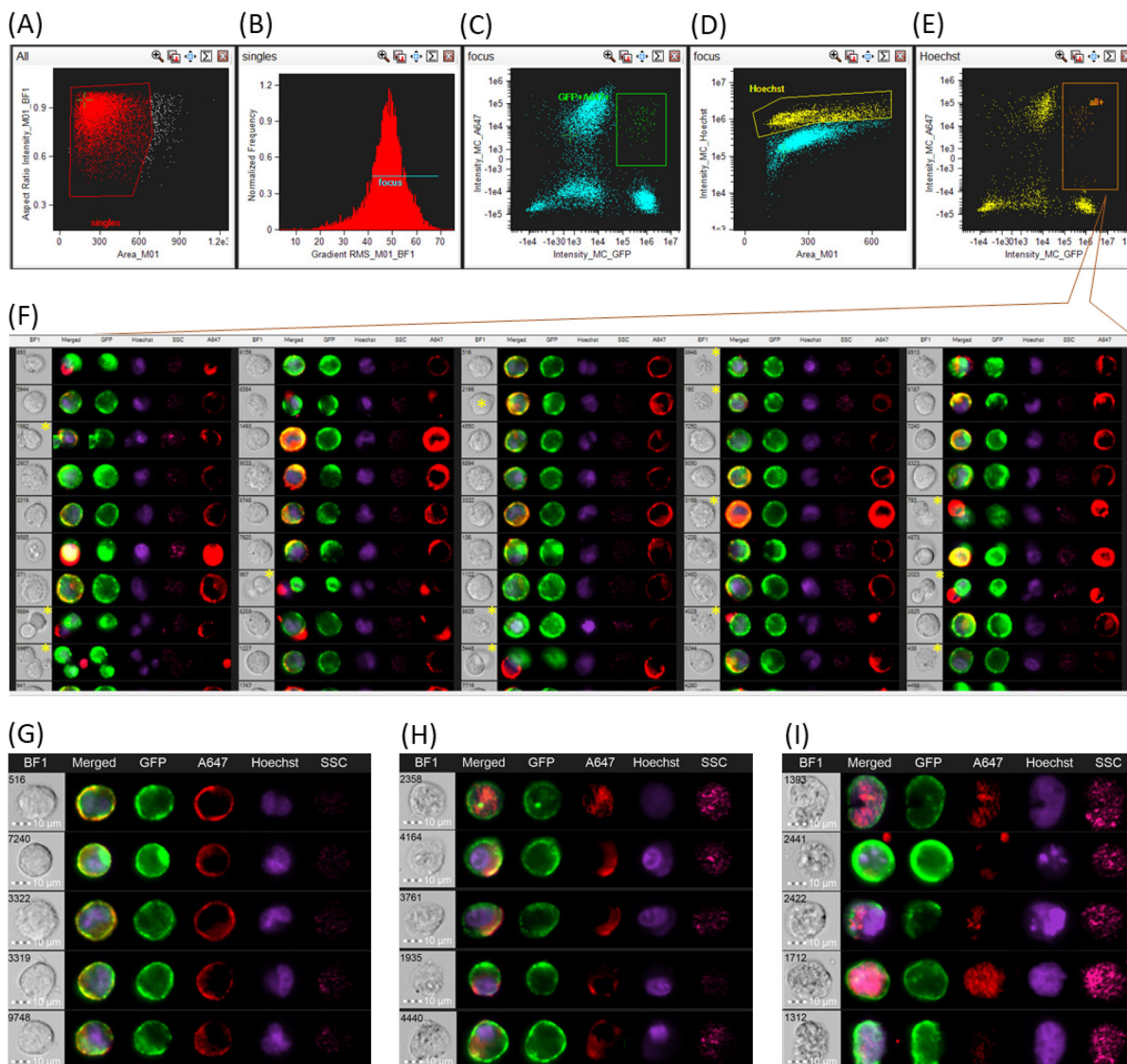
Fused cells were stained and immediately analyzed using ImageStream to identify the area in the histogram that contains heterokaryons (Figure 5.9A-E). Figure 5.9F shows a representative screenshot of the area in the histogram where the majority of the heterokaryons were identified based on gating and observation of the images. Although heterokaryons represented >50% of the cell population in this gate, false positive cells were also frequently observed. Distinct nuclei were observed in heterokaryons sampled at 24 and 48h (Figure 5.9G and H), whereas the double positive heterokaryons at 72h presented enlarged and/or fragmented nuclei (Figure 5.9I).

The parameters identified on the ImageStream were extrapolated to the FACS Aria II flow cytometer to sort 200 double-positive heterokaryons, as well as 200 bFFs and 200 mESCs, directly into a collection tube with lysis buffer. From all the labeling and cell selection methods tested, this procedure gave the best specificity in less time. Cells obtained from this method were used for RNAseq.



**Figure 5.8 Analysis of heterokaryons obtained by manual selection.** (A) Representation of the 2-color, 2-nuclei staining approach used to detect heterokaryos. (B) Heterokaryons were selected using a micromanipulator attached to a fluorescence inverted microscope. To protect cells from the environment, cell suspension was placed in droplets on a culture dish and covered with mineral oil. (C) This method permitted clear visualization of multistained cells before selection. Unfortunately, this step was very time consuming. (D) Examples of some multistained heterokaryons obtained by manual selection. We produced cDNA from 40 bFFs, mESCs, and Heterokaryons, and quality was measured on an Agilent 2100 Bioanalyzer; summary report showing (E) gel-like images of samples and (F) chromatograms. Concentration of cDNA obtained was within the acceptable range.





**Figure 5.9 Identification of heterokaryons using ImageStream imaging flow cytometer.** (A) A gate was placed around single cells, and (B) within single cells only images in focus were considered. (C) The remaining cells were plotted as intensity of GFP versus intensity of A647 dye; (D) in the resulting scatterplot, a subpopulation with high intensity GFP and A647 was subsequently gated. (E) This subpopulation was plotted as area of the image containing by Hoechst vs. intensity of the Hoechst staining. Two subpopulations emerged, and the one that contained a majority of Heterokaryons was gated. This subpopulation was, again, plotted as an intensity of GFP versus intensity of A647 scatterplot, and here a small subset of cells was found to be predominantly heterokaryons. (F) Notice that it is still possible to find false positive cells in this subpopulation (yellow asterisks in bright field image). It was not possible to gate out these events, due to their similitude with heterokaryons in terms of fluorophore presence. The parameters identified on the ImageStream were used to sort heterokaryons in the FACSria. Representative images of heterokaryons obtained at (G) 24h, (H) 48h, and (I) 72h after fusion are shown.

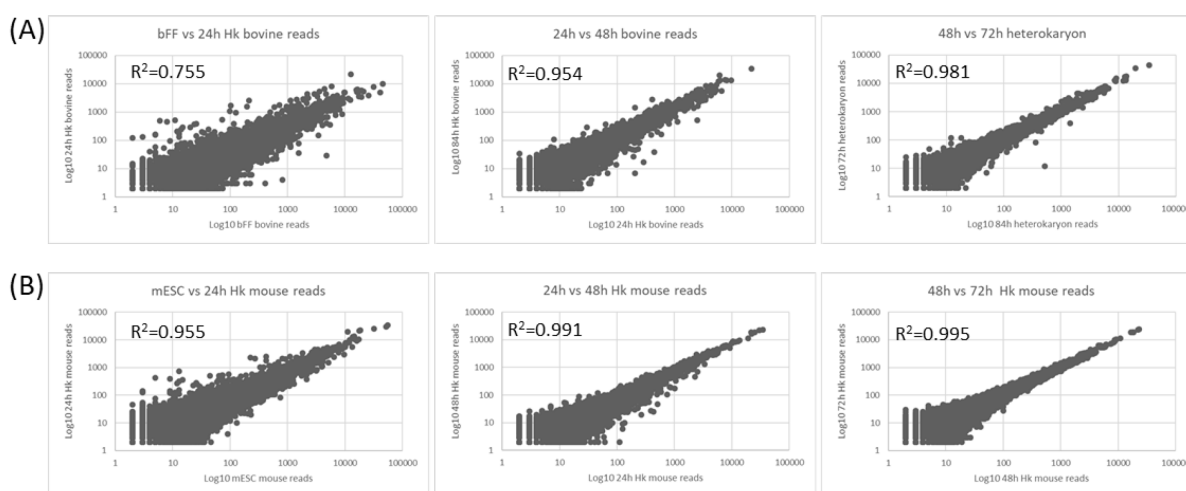
**Table 5.5** Species-specific and total reads per sample.

	Reads		
	Mouse	Bovine	Total
mESC	3,446,461		3,446,461
bFF		2,634,269	2,634,269
24h Hk	2,427,089	982,769	3,409,858
48h Hk	1,731,084	1,331,989	3,063,073
72h Hk	1,836,299	1,541,793	3,378,092

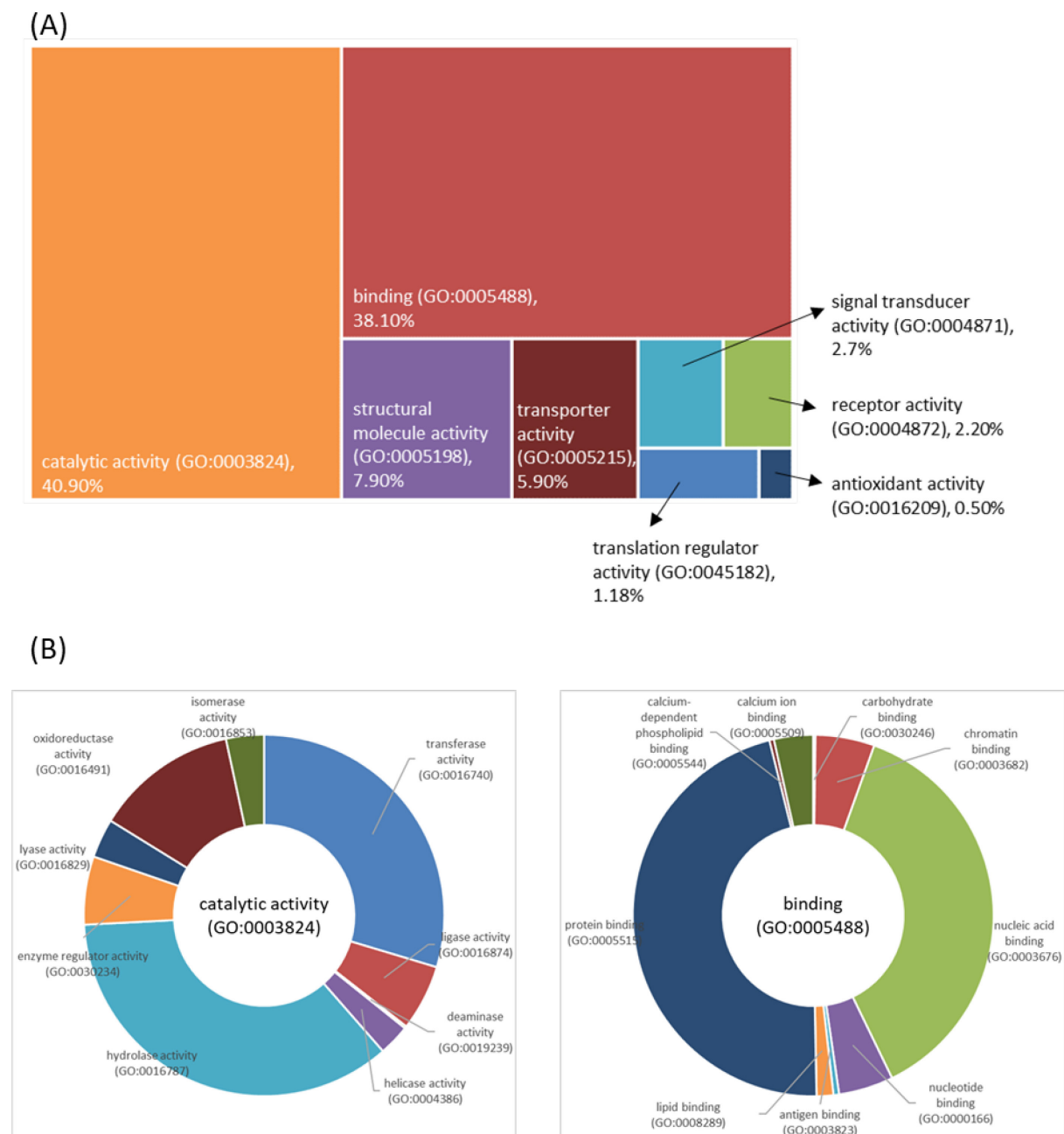
#### 5.4.6 GLOBAL CHANGES IN GENE EXPRESSION PATTERNS HAPPEN PREDOMINANTLY IN THE BOVINE NUCLEUS BETWEEN BFF AND THE 24H HETEROKARYON

Raw data obtained from MiSeq contained  $\sim 3 \times 10^6$  paired-end reads per sample. For heterokaryons, around 50% of the reads corresponded to mouse transcripts, and the other 50% to bovine transcripts (Table 5.5). For analysis, reads were separated by species into two datasets. To get a general overview of changes in gene expression trends between samples, correlation between two consecutive stages were plotted on a log scale (Figure 5.10A and B). Dispersion of points in the scatter plot indicates increased variation between compared datasets, as indicated by a smaller Pearson correlation coefficient ( $R^2$ ). The highest dispersion was found between bovine reads in bFFs and 24h heterokaryons; whereas bovine reads at later stages were highly correlated. Mouse reads were highly correlated in all stages, suggesting an overall stable gene expression level in the mESC nucleus.

We characterized bovine genes that were upregulated in 24h heterokaryons, by associating Gene Ontology (GO): molecular function terms to the gene names using Panther Classification System. Out of 2853 upregulated bovine genes, 1974 were linked to a molecular function GO term (Figure 5.11A). The most represented categories were catalytic activity and binding, and the represented subcategories for each of them are shown in (Figure 5.11B). This suggests that a great proportion of the upregulated genes are associated with protein, nucleic acid, or chromatin binding. Bovine GO terms for genes downregulated at 24h matched the terms found for upregulated genes (Figure 5.12 A and B). This suggests a remodeling going in both directions.

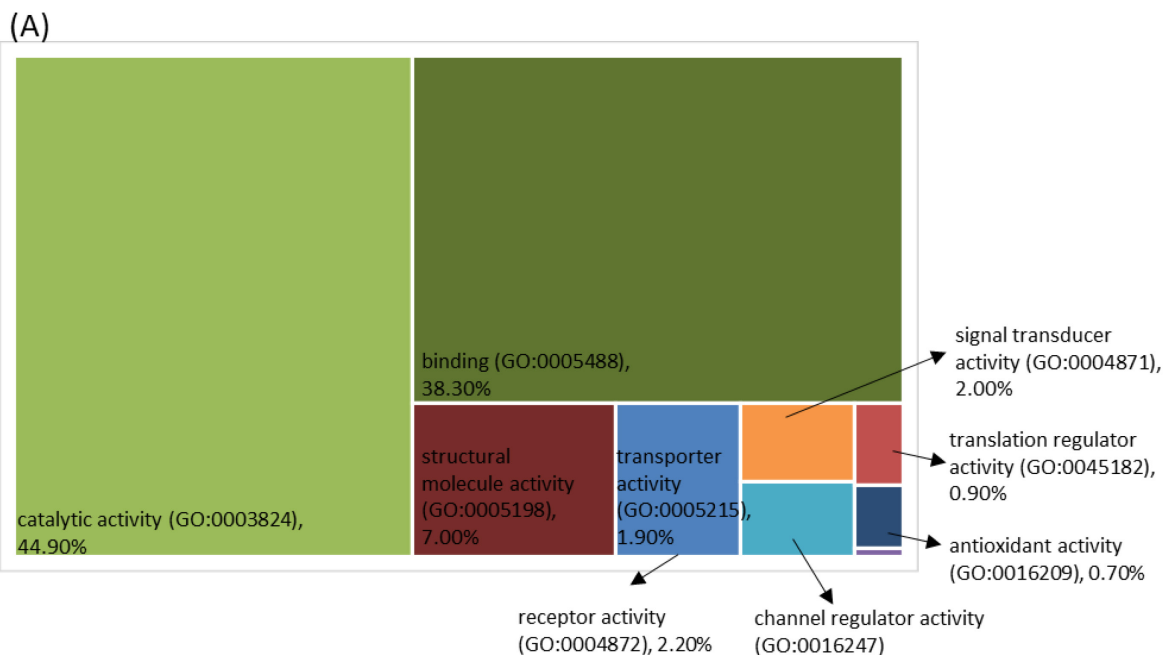


**Figure 5.10** Scatter plots of the RNAseq expression data, by species, between consecutive stages. **(A)** Bovine reads. **(B)** Mouse reads. Plots are on a log<sub>10</sub> transformed scale. Pearson correlation ( $R^2$ ) is shown on top of the plots.

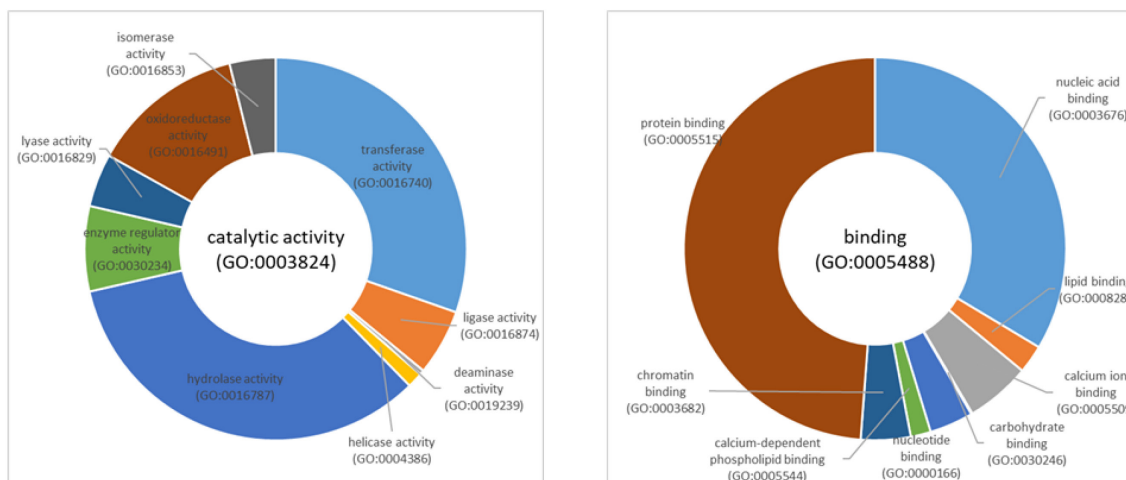


**Figure 5.11 Molecular function Gene Ontology (GO) terms for bovine genes upregulated at 24h after fusion. (A) Categories represented in 1974 genes. (B) Subcategories of the GO terms “catalytic activity” and “binding”.**

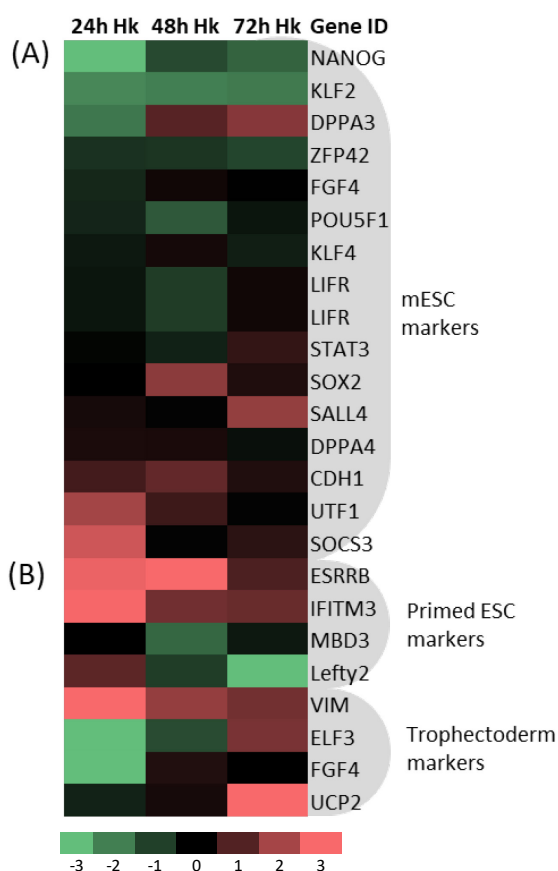




(B)



**Figure 5.12 Molecular function Gene Ontology (GO) terms for downregulated bovine genes at 24h after fusion. (A) Categories represented in 1660 genes. (B) Subcategories of the GO terms “catalytic activity” and “binding”.**



**Figure 5.13 Heat map depicting temporal gene expression changes in the mouse transcriptome. (A) stemness marker and (B) trophectoderm/primed ESC marker expression levels. Fold changes compared to mESCs are expressed in Log2.**

with *OCT4*. *CCL2* has been associated with the activation of hypoxia related genes; culture under hypoxic conditions has been used to enhance pluripotency of human iPSCs, suggesting that *CCL2* enhances pluripotency by inducing a hypoxic-like response. In line with this, we found additional 21 genes with the GO term “response to hypoxia” (GO:0001666) that also correlated with the expression pattern of *CCL2* (Figure 5.14B).

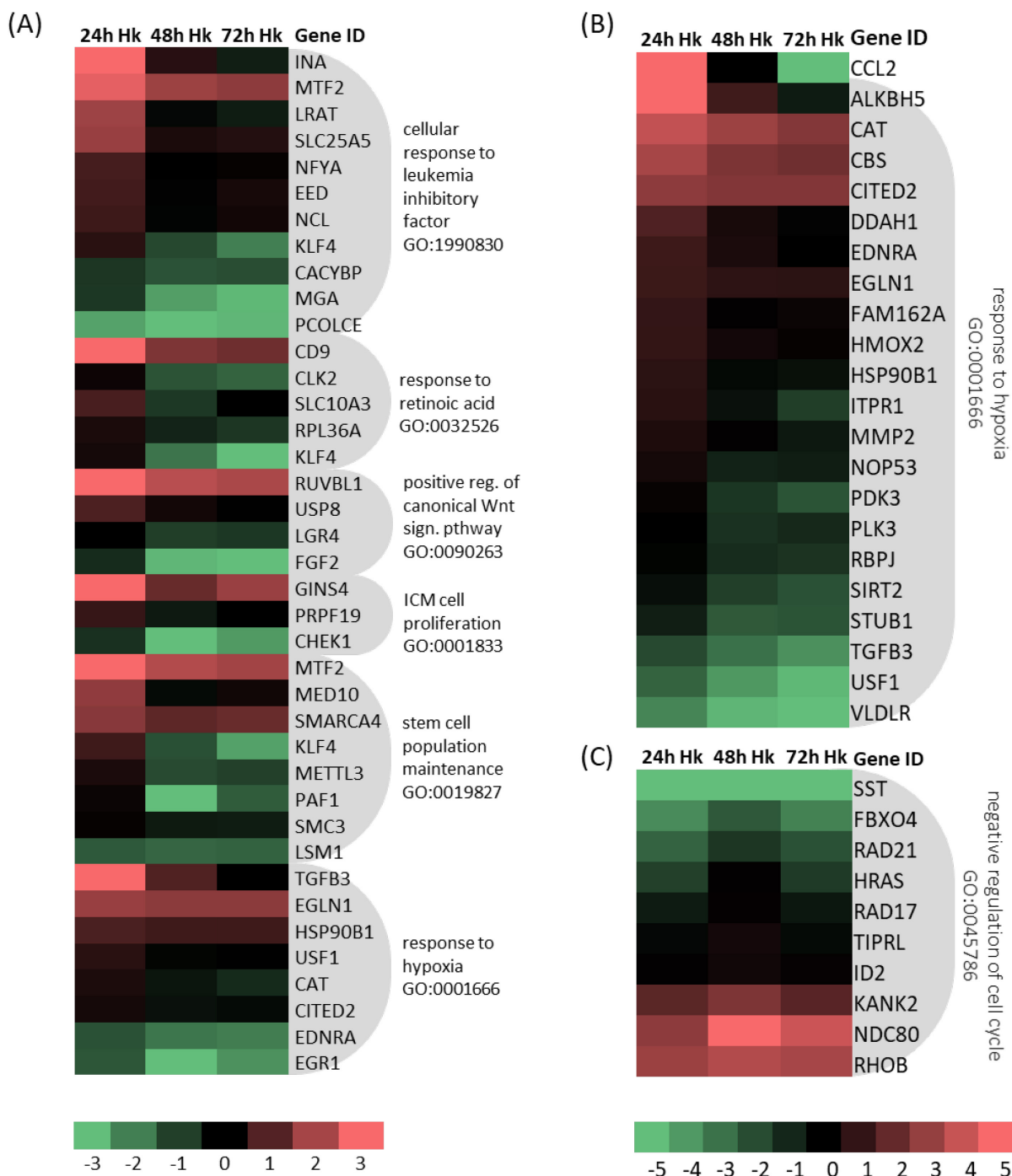
Interestingly, Methyl-CpG Binding Domain Protein 3 (*MBD3*), a component of the nucleosome remodeling and histone deacetylation (NuRD) complex, is upregulated at 24h and remains constant through 48 and 72h. *MBD3* has been associated with transcriptional silencing in a context dependent manner, and its upregulation in heterokaryons could hint a potential block in the progression of reprogramming to a more stable state.

#### 5.4.7 A SUBSET OF MOUSE PLURIPOTENCY MARKERS EXPERIENCE TEMPORARY DOWNREGULATION AT 24H, BUT REGAIN EXPRESSION LEVEL AT 48 AND 72H

We characterized expression of pluripotency and differentiation markers in expressed mouse genes. Interestingly, expression level of pluripotency markers such as *Nanog*, *Klf2*, and *Dppa3* decreased <2 fold in 24h heterokaryons, compared to mESCs. However, their gene expression level increased again over 48 and 72h (Figure 5.13A). No expression of endo- (*Sst*, *Gata6*, *Alb*, *Afp*), meso- (*Bmp4*, *Hbz*, *Connexin*, *Asm*), or ectodermal (*Nes*, *Vim*, *Gfap*, *Nes*) markers was found. The primed mESC marker *Lefty2* increased 1.3-fold at 24h, but decreased 2-fold over the next days. Trophectodermal markers *Vim*, *Elf3*, *Fgf4*, and *Ucp2* were low at 24 and 48h, and showed a slight increase at 72h (Figure 5.13B).

#### 5.4.8 THE BOVINE NUCLEUS OF 24H HETEROKARYONS CO-UPREGULATES EARLY PLURIPOTENCY MARKERS AND DOWNREGULATES CELL CYCLE INHIBITORS

The transcription factors *OCT4* (*POU5F1*) and *KLF4* are key pluripotency markers during early stages of reprogramming. Here, *OCT4* and *KLF4* co-upregulate in 24h heterokaryons, but decrease during 48 and 72h. A list of genes whose expression also correlates with *OCT4* is presented in Figure 5.14A. In addition, the C-X-C Motif Chemokine Ligand 2 (*CCL2*) is also co-upregulated



**Figure 5.14** Heat map depicting temporal gene expression changes in the bovine transcriptome. (A) Stemness markers, (B) hypoxia response associated genes, and (C) negative regulators of cell cycle. Fold changes compared to bFFs are expressed in Log2.

Interleukin 6 (*IL6*) is a cytokine (a.k.a. signaling protein) with pleiotropic effects. We found a 4.8-fold increase of *IL6* expression in 24h heterokaryons, and this expression level remained high in 48 and 72h heterokaryons. The classical mechanism of action of *IL6* is through the JAK/STAT3 signaling pathway. Here, we found that expression of *STAT3*, as well as other markers of this pathway, remained at a relatively constant expression level. However, *STAT3* becomes activated after phosphorylation, which does not necessarily correlate with a higher gene expression level of *STAT3*. The Serine/Threonine-Protein Kinase Pim-1 (*PIM1*) is a downstream target of *IL6*; *PIM1* was expressed in heterokaryons of all stages but not in bFFs, although the expression level was lower than our 20-reads threshold. In addition, it is possible that *IL6* acts through a yet unknown pathway.

Pluripotent cells are characterized by their rapid cell cycle. We found that *SST*, an inhibitor of cell proliferation, presented a 6-fold decrease between fibroblast and 24h heterokaryons, and the level of expression remained low through 48 and 72h. Expression of *SST* receptors (*SSTR1 – 5*) was not detected. Using Pearson correlation to *SST*, we compiled a list of 323 genes that presented >95% correlation with *SST*; from that list, we found five genes that are associated with the GO term “negative regulation of cell cycle” (GO:0045786) (Figure 5.14C). This suggests that proliferation inhibitors are downregulated in heterokaryons, suggesting a change in cell cycle more similar to mESCs.

## 5.5 DISCUSSION

By far, obtaining a method to reliably stain and select for heterokaryons proved to be the most challenging part of this project. For both lipophilic tracers and Qtrackers, we observed diffusion of dyes from the originally labeled cells to adjacent cells in co-culture, indicating that dyes and particles were not retained in their original cells. The use of lipophilic tracers and Qtrackers is often described in the literature; however, our experience using these methods raises concerns about the validity of those results. Although scarce, others authors have described the lack of specificity of Qtrackers. In line with our findings, Ranjbarvaziri *et al.* (2011) described a progressive decrease in fluorescent signal in cells stained with different types of Qtrackers, with the highest durability in Qtracker 800. Moreover, when cells independently stained with different Qtrackers were co-cultured for 24h, they found presence of all different types of Qtrackers in each of the co-cultured cells, suggesting that fluorescent particles were not retained in their respective cells<sup>8</sup>.

In our cell fusion model, we observed co-upregulation of bovine *POU5F1* (*OCT4*) and *KLF4*, with no detectable level of *SOX2* and *cMYC*. Previous research in mice has shown that expression and stoichiometry of exogenous OSKM affects the epigenetic state of the resulting iPSCs; in that study, a high expression of *Oct4* and *Klf4*, together with low *Sox2* and *cMyc*, was optimal for generating high quality iPSCs<sup>9</sup>. Recent studies have shown that early expression of *Oct4* and *Klf4* marks the beginning of a partially reprogrammed stage<sup>10</sup>. It has been described that reprogramming to pluripotency occurs in distinct phases, also called “waves”, during which *Oct4* and *Klf4* expression occurs early, preceding an intermediate stage that leads to the second, stable stage of pluripotency. It is possible the changes in gene expression that we observed in the bovine nucleus of 24h heterokaryons resemble those observed during early stages of reprogramming.

During reprogramming of human fibroblasts to pluripotency, it has been described that only ~20% of the cells that initiate reprogramming become stable, mature iPSCs. Stoichiometry of the transduced reprogramming factors, possibly also including posttranscriptional regulation, might be a cause for low reprogramming efficiency<sup>11</sup>. In addition, some roadblocks in iPSC formation are dependent on the

reprogramming system being used, with the consequent relative expression of pluripotency factors being a major determinant in reprogramming success<sup>12</sup>. Monitoring the relative dosage of OSKM in a transcription-factor transduction context is a difficult task. Moreover, research in mESCs has shown that the relative expression level of *Oct4* can trigger different cellular responses<sup>13</sup>. The authors found that *Oct4* expression level 50% above or below the normal diploid expression level in undifferentiated stem cells will induce differentiation. In a cell fusion context, the pluripotency factors received by the somatic nucleus from the mESC are in a level compatible with maintenance of pluripotency in mESCs, and it has been suggested in mice that reprogramming cells with a gene expression pattern similar to ESCs are more likely to become stable iPSCs<sup>11</sup>.

The cytokine *IL6* has many roles in cell signaling, and during early embryo development it acts as a paracrine factor. During *in vitro* culture of mouse embryos, supplementation with *IL6* improves embryo survival and increases the number of inner cell mass cells<sup>14</sup>. The exact mechanism through which *IL6* causes these effect is unclear. Studies using mESC-human fibroblast heterokaryons have shown that *PIM1* is a downstream target of *IL6*, and its presence enhances nuclear reprogramming in a cell fusion context<sup>15</sup>. In our study, we were not able to associate *IL6* expression to any known pathway. Since many of the downstream targets of *IL6* are unknown, it is possible that *IL6* exerts its pro-survival effect through alternative pathways.

SST is an inhibitory polypeptide hormone with a variety of functions, including inhibition of cell proliferation via activation of five different receptor subtypes<sup>16</sup>. Due to its anti-proliferative function, SST analogues have been proposed as potential anti-tumor drugs<sup>17</sup>. The effects of SST are mediated through five transmembrane receptor subtypes (SSTR1 – 5). Pluripotent cells are characterized for presenting a fast cell cycle, and it has been shown that *cMyc* can be replaced with other genes that accelerate cell cycle. The downregulation of cell cycle inhibitors in 24h heterokaryons suggests a cell cycle similar to ESCs. It is possible that promoting conditions that inhibit *SST* and other genes with similar effect could have a positive impact in isolation of bESCs or biPSCs.

Some researchers have suggested that, in a heterokaryon context, both nuclei experience changes in a bi-directional fashion<sup>5</sup>. We observed transient downregulation of mouse pluripotency markers at 24h, which recovered over 48 and 72h. No major changes in gene expression in the ESC nucleus were observed (changes were <2-fold). Overall, we observed that gene expression levels of core pluripotency genes in the mESC nucleus is not affected in heterokaryons, showing that our model is a reliable source of pluripotency factors for the bovine nucleus.

It has been described that early factor-based reprogramming initiates with a stochastic phase of gene activation; from here, some cells will enter a transient phase that can lead to a deterministic (hierarchical) phase of reprogramming, that will lead to stably reprogrammed cell<sup>18</sup>. During nuclear reprogramming in nuclear transfer and cell fusion, reprogramming happens faster than in transcription factor-based reprogramming. Therefore, it is unknown whether the phases of gene activation apply to these forms of nuclear reprogramming. Nevertheless, our findings point towards a gene expression pattern similar to first wave of reprogramming.

In summary, bovine somatic nuclei 24h after fusion present a gene expression pattern similar to early reprogramming cells, with expression of *OCT4* and *KLF4*, upregulation of hypoxia response genes, and downregulators of factors with a negative impact on cell proliferation such as *SST*. However, bovine nuclei of 48 and 72h heterokaryons did not express pluripotency genes at a level comparable to a more advanced reprogramming state. Moreover, the upregulations of cell cycle regulators, plus the steady expression of

MBD3, hints towards a block in bovine nuclear reprogramming. We hypothesize that the progression towards a more stable pluripotent state in the bovine nucleus might be inhibited by some mechanism that is not present, or has not the same relevance, in mice and humans, and removal of this roadblock could have a positive impact in the progression towards a stable pluripotent state. This hypothetical roadblock can explain the inability to isolate true bovine iPSCs by us and others, and the fact that bovine iPSCs described so far do not persist without a constant supply of exogenous pluripotency markers, which is indicative of incomplete reprogramming. The cell fusion model described here can be used further as a tool to study the effect of different reprogramming conditions on the bovine transcriptome.

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### 3.7 ATTRIBUTIONS

This chapter was completed by the author except library prep and RNAseq data manipulation in Geneious and Microsoft Access. Dr. Bill Huckle and Dr. Colin Bishop provided guidance and insights during molecular cloning. Dr. Rick Jensen assisted during RNA-seq related procedures as well as data analysis. Melissa Makris provided valuable assistance to make our ImageStream/FACS method work.

Student interns participated during some stages of this project: bachelor student Katie Morrison and veterinary student Marcelo Saldivia assisted during lentiviral plasmid cloning; Marcelo Saldivia wrote his senior DVM thesis with the work done in the lab (Full text in Spanish; English abstract available in [Appendix A](#)). Veterinary student Maria Jesus Garrido assisted with indirect immunofluorescence procedures and also wrote his senior DVM thesis with the work done in the lab (Full text in Spanish; English abstract available in [Appendix B](#)).

An illustration made by the author based on this project was published in the Silhouette Literary and Art Magazine in Fall of 2013 ([Appendix C](#)).

### 3.8 ACKNOWLEDGEMENTS

RNA-seq was made possible thanks to the collaboration of Dr. Rick V. Jensen.

## APPENDIX A

English abstract from: Saldivia Barria, M.I., Villafranca Locher, M.C., and Morera Galleguillos, F. (2014). Design and cloning of a lentiviral vector containing a monomeric red fluorescent protein (mRFP) reporter gene to label mouse embryonic stem cells. Universidad Austral de Chile.

### **DESIGN AND CLONING OF A LENTIVIRAL VECTOR CONTAINING A MONOMERIC RED FLUORESCENT PROTEIN (mRFP) REPORTER GENE TO LABEL MOUSE EMBRYONIC STEM CELLS**

Saldivia Barria, MI; Villafranca Locher, MC; Morera Galleguillos, F

Embryonic stem cells (ESCs) are pluripotent cell lines derived from pre-implantation embryos of mice, monkeys and humans. Induced pluripotent cells (iPSCs) are generated from differentiated cells by forced expression of transcription factors found in ESCs and are functionally equivalent to ESCs. Mouse and primate iPSC protocols have failed to yield iPSCs in cattle, suggesting that bovine cell reprogramming requires alternative/additional factors.

Cell fusion is a process in which cells combine to form a multinuclear cell known as heterokaryon. Mouse and human somatic nuclei can be reprogrammed to pluripotency when fused with mouse ESCs, suggesting that ESCs cytoplasm contains reprogramming factors. Cell fusion is a rather low efficient process, and techniques developed in the lab have yielded a rate of up to 16% of binucleated cells. Therefore, it is essential to specifically label the cells prior fusion, so the heterokaryons can be isolated after fusion.

Lentiviral vectors allow stable expression of transgenes in target cells over many generations. Our aim is to generate a lentiviral vector harboring a monomeric red fluorescent protein (mRFP) reporter driven by the cytomegalovirus promoter (CMV), with the ultimate goal of labeling mESCs prior to fusion with bovine somatic cells. The lentiviral plasmid vector was designed using bioinformatics software. Briefly, the CMV-mRFP cassette was ligated into the self-inactivating lentiviral backbone using InFusion cloning. The reaction was transformed into competent cells and the colonies were screened using PCR.

We obtained two positive colonies, which were expanded and sequenced. Obtained sequences were aligned and analyzed using bioinformatics software. No alterations were observed in any of the clones. Further steps will involve generation of lentiviral vectors and transduction into mESCs.

Keywords: lentiviral plasmid, reporter gene, cell fusion, mESC.



## APPENDIX B

English abstract from: Garrido Bauerle, M.J., Morera Galleguillos, F., and Villafranca Locher, M.C. (2017). Identification of interspecific mouse-bovine heterokaryons using indirect immunofluorescence. Universidad Austral de Chile.

### IDENTIFICATION OF INTERSPECIFIC MOUSE-BOVINE HETEROKARYONS USING INDIRECT IMMUNOFLUORESCENCE

Garrido Bauerle, MJ; Morera Galleguillos, F; Villafranca L, MC

Embryonic stem cells (mESCs) are pluripotent cell lines derived from preimplantational embryos of mice, humans, and non-human primates. Induced pluripotent stem cells (iPSCs) can be generated by over expression of transcription factors found normally in ESCs. It has been described that iPSCs are functionally equivalent to ESCs, and are considered a fundamental tool in regenerative medicine and biotechnology. Current mouse and human reprogramming protocols have failed to produce true iPSCs in the bovine, a species with significant agricultural and commercial value.

Cell fusion is a process in which two or more cell types combine to form a multinucleated cell. It has been described that mouse ESCs can reprogram mouse or human somatic cells upon fusion. The present body of work was part of a study which aimed to characterize the changes in the bovine somatic nucleus upon fusion with mouse ESCs. In our lab, we have developed a cell fusion method that yields up to 14% of binucleated cells. However, a key aspect of this approach is to correctly identify fusion products made of two different cell types (heterokaryons) and separate them from multinucleated cells made of two cells of the same type (homokaryons). For this, we used indirect immunofluorescence in live cells.

First, we tested the effect of two common reagents used for subculturing cells: trypsin and EDTA. Next, we tested primary antibodies targeting cell-specific surface markers for mouse ESCs (SSEA-1) and bovine fetal fibroblasts (THY-1 and CD44), followed by staining with secondary GFP- and Texas Red- conjugated antibodies. Cells subcultured with EDTA were considerably brighter after immunostaining, compared to cells passaged with trypsin. Adequate level of fluorescence was obtained when staining mouse ESCs and bovine fetal fibroblasts with anti-SSEA-1 and anti-CD44 antibodies, respectively. We were not able to obtain sufficient fluorescent signal with anti-THY-1 primary and secondary antibodies.

Labeling with anti-SSEA-1 anti-CD44 antibodies allowed the identification of heterokaryons under fluorescent microscopy. Multinucleated cells positive for both SSEA-1 and CD44 were selected with a micromanipulator and analyzed by qPCR to evaluate the presence of bovine-specific Nanog. Further steps will involve analyzing heterokaryons with RNAseq.

Keywords: indirect immunofluorescence, cell fusion, cell reprogramming, pluripotency.

## APPENDIX C

Traditional ink illustration published in the Silhouette Literary and Art Magazine. Published in Fall of 2013. Volume 36, Issue I. Pages 34-35. Reprinted with permission.

Depending on what one wants to see when looking through a microscope, it can be a moment as extraordinary as contemplating an unexplored landscape, or one that you have only dreamed of visiting. As Robert Hook wrote, "For the limits, to which our thoughts are confined, are small in respect of the vast extent of Nature itself; some parts of it are too large to be comprehended, and some too little to be perceived."

*Maria Cristina Locher*

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editor's choice

Cell Culture ✦ Maria Cristina Villafraña Locher

