FUNCTIONAL STUDIES OF OCT4, CDX2 AND SOX2 IN THE BOVINE PRE-IMPLANTATION EMBRYO

By

Marcelo Demarchi Goissis

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillments for the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Animal Science

2012

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

ABSTRACT

FUNCTIONAL STUDIES OF OCT4, CDX2 AND SOX2 IN THE BOVINE PRE-IMPLANTATION EMBRYO

By

Marcelo Demarchi Goissis

Assisted reproductive technologies (ARTs) in domestic animals are used to increase the efficiency of cattle production, especially by reducing the generation interval in breeding programs. Somatic cell nuclear transfer (SCNT) allows for the generation of identical individuals of high genetic merit and it is also used to generate transgenic animals by modification of the donor's genome. However, SCNT efficiency is still very low when compared to other ARTs such as in vitro fertilization. The use of embryonic stem cells (ESCs) as donor nucleus could enhance the outcome of SCNT as well as allowing targeted gene modification. Nevertheless, true ESCs were not derived so far in the bovine species. Thus, we focused on SCNT as an ART that can accomplish not only the horizontal propagation of a given genome but also introduce genome modifications. During SCNT, a somatic cell must be transformed into a pluripotent cell first – in a matter of hours – and then reinitiate embryonic differentiation. The first differentiation event the pre-implantation embryo is lineage specification, in which a fraction of the cells in the embryo will form the trophectoderm and the rest will form the fetus itself. This process is poorly understood in the bovine, not only in the context of SCNT but in fertilized embryos as well. We suggest that studying the mechanisms of lineage specification in bovine could help understand some of the inefficiencies observed in

bovine SCNT and it could also help us develop novel strategies to obtain true bovine ESC. We focused in three genes, OCT4, CDX2 and SOX2 that are key regulatory factors in most pre-implantation mammalian embryos. First we tested the hypothesis that expression of *OCT4* in donor cells would improve the efficiency of SCNT. Subsequently we tested the hypothesis that *CDX2* is not required for trophectoderm establishment, but important in maintaining its integrity in bovine embryos. To further comprehend lineage specification in bovine, we tested the hypothesis that *SOX2* is required for inner cell mass formation. We found that indeed preconditioning the somatic cells with OCT4 expression prior to SCNT has a distinct effect on the phenotype of the cloned blastocysts. We also found, contrary to our expectations, that CDX2 and SOX2 are not required for the first cell differentiation event in the bovine fertilized embryo. Overall, the data presented here has direct implications in the understanding of bovine embryology. It could help improve SCNT outcome and further understand lineage specification in the pre-implantation embryo as well as subsequent embryonic processes.

DEDICATION

To my wife Carolina,
to my parents and family,
for their unrestricted support

ACKNOWLEDGEMENTS

I would like to thank:

Dr.Jose Cibelli, for giving me the opportunity to come to Michigan State, for providing the resources for executing the experiments and for all the advice given throughout the program;

All the evaluation committee members, **Dr.Jason Knott, Dr.Gloria Perez, Dr.Gary D. Smith and Dr.Vilma Yuzbasiyan-Gurkan**, for their assistance throughout the years and for invaluable lessons and advice;

Dr.Steve Suhr, for all the assistance with molecular biology and for every scientific (or non-scientific) discussion we had;

Dr.Pablo Ross, for teaching me the SCNT technique and for helping me during the transition when moving from a different country;

All the current and past members of the **Cellular Reprogramming Laboratory**, for the contributions, big and small, they all had on my development as a scientist;

Tak Ko and Gabriela Saldana, lab managers during the time I was here, which allowed proper execution of this work;

João Paulo Martins, for all assistance in work or personal matters;

Dr.George Smith and Dr. Richard Pursley, for their collaborative interactions;

Dr. Joe Folger, for organizing the ovary collection and all **undergrad students** that went to Kalamazoo every week to collect the ovaries;

Ken Metz, Dr.John Gunther, Mike and Gerald Green for all the work done in the farm, which while not used in this dissertation, contributed to my overall knowledge of farming and veterinary medicine;

The **Department of Animal Science**, especially **Kathy Tatro and Dr.Steve Bursian**, for all their assistance during my PhD program;

The **Ministry of Education of Brazil (through CAPES)** and the **Fulbright Program** for providing financial support throughout my PhD program.

My wife **Carolina**, for moving here and putting a hold on her career to support me in every way possible. And my parents **João Carlos e Marcia** for encouraging me to be here during every moment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO SYMBOLS OR ABBREVIATIONS

3i	Combination of three small molecules to maintain pluripotency
5-aza	5-aza-2'-deoxycytidine
ART	Assisted reproductive technologies
BAF	Bovine adult fibroblast
BFF	Bovine fetal fibroblast
bFGF	Basic fibroblast growth factor
CTRL	Control group
DMAP	6-Dimethylaminopurine
DMEM	Dulbecco’s modified eagle media
EGA	Embryonic genome activation
EpiSC	Epiblast stem cells
ESCs	Pluripotent embryonic stem cells
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
H3K27me3	Histone 3 lysine 27 trimethylation
H3K9me3	Histone 3 lysine 9 trimethylation
HDACi	Histone deacetylase inhibitor
hESCs	Human embryonic stem cells
HEPES	Hydroxyethylpiperazineethanesulfonic acid
HH	HECM – HEPES medium
ICM	Inner cell mass

iPSCs	induced pluripotent stem cells
IVF	*In vitro* fertilization
IVP	*In vitro* production of embryos
KSOM	Potassium simplex optimized medium
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
mESCs	Mouse embryonic stem cells
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT PCR	Reverse transcriptase PCR
SCNT	Somatic cell nuclear transfer
SCR	Scramble
siRNA	Small interfering RNA
TALP	Tyrode’s albumin lactate pyruvate medium
TE	Trophectoderm
TRITC	Rhodamine isothiocyanate
TSA	Trichostatin A
VPA	Valproic acid
YFP	Yellow fluorescent protein

CHAPTER 1

INTRODUCTION: FROM SOMATIC CELL NUCLEAR TRANSFER TO EMBRYO LINEAGE SPECIFICATION: THE INVERSE ROUTE TO IMPROVE BOVINE ASSISTED REPRODUCTIVE BIOTECHNOLOGIES

Livestock used for food production is of great social and economic importance. The constant rise in human inhabitants of the planet requires increase of food supply, including animal products. However, as Thomas Malthus proposed, population grows in a geometric scale while food production grows in an arithmetic trend. Thus, refinements in livestock production have been necessary throughout the years to meet the demand. Reproductive technologies are some of the tools developed to help increase the effectiveness of livestock production.

Reproductive technologies can be used to simply increase the efficiency of the reproductive rates of a herd. Furthermore, it can be combined to increase efficiency of breeding programs and enhance the genetic merit of a herd. Techniques such as artificial insemination and embryo transfer allow the maximum use of genetically superior individuals, reducing the interval between generations, especially bovine, which have larger interval than other livestock species.

The development of in vitro embryo production (IVP) technologies permitted even more advancements in breeding programs, especially when combined with sperm freezing technologies. Moreover, IVP allowed progress of other techniques, such as somatic cell nuclear transfer (SCNT –Wilmut et al. 1997). SCNT can be used to increase the number of proven animals used in breeding programs (Smith, 1989) and also, to generate transgenic animals by genetically modifying the donor nucleus (Schnieke et al. 1997, Cibelli et al. 1998a).

The introduction of different genes into livestock could be used to increase the productivity or create animal products with higher nutritional or economic value. However, this technique has been hampered by low efficiencies of SCNT and the lack of available embryonic stem cell (ESC) lines from livestock. ESCs could be used to both increase efficiency of SCNT (Rideout et al. 2001) and generate transgenic animals by homologous recombination (Rossant et al. 1993), which might facilitate its production and future approval for human consumption. While true ESCs are not available for livestock, the study of the developmental mechanisms in livestock species is required to understand the discrepancy between embryos of different species.

In this review, we will discuss events occurring during bovine SCNT and relate them to the problems observed during gestation and strategies to overcome the low efficiencies observed. Also, we will discuss the isolation of ESC in different species altogether with the generation of induced pluripotent stem cells (iPSCs). At the end, we will review the mechanisms of lineage specification and how it could help generating cloned cows more efficiently - transgenic or otherwise - and perhaps, ESCs from livestock.

1.1 Somatic cell nuclear transfer

The technique of SCNT consists of the introduction of a differentiated cell into an oocyte, which will reprogram the donor cell nucleus to an extent that allows development into a new organism. Epigenetic changes in the chromatin, which alter gene expression without changing the DNA sequence, occur during reprogramming of

the donor nucleus. These changes silence somatic genes and allow the expression of embryonic genes (Latham, 2005). Blastocysts derived from SCNT have similar global gene expression when compared to in vitro fertilized (IVF) counterparts (Smith et al. 2005, Beyhan et al. 2007), demonstrating that successful reprogramming is already observed at this stage. However, reprogramming does not occur instantly, as different patterns of gene expression are observed in SCNT mouse 2-cell embryos compared to IVF ones (Vassena et al. 2007).

Despite successful reestablishment of gene expression in the SCNT blastocyst, transfer of these embryos only yields an average of 9% of full term development (Panarace et al., 2007), which is very low when compared to 40-60% of IVF-derived embryos (Yang et al. 2007). Alterations in placental phenotype are related to the majority of SCNT-pregnancy losses, which happen in the first trimester of gestation (Hill et al. 2000). Moreover, most losses in the third trimester are also related to placental problems, such as hydroallantois and placentomegaly (Constant et al. 2006).

Several pathologies that occur in bovine gestation are similar to what is observed when there are mutations or deletions on imprinted genes (Chavatte-Palmer et al. 2012), which are mostly regulated by DNA methylation, an epigenetic mechanism (Reik and Walter, 2001). Bovine SCNT derived embryos were reported to display aberrant methylation patterns of imprinted genes, such as H19 (Suzuki et al. 2011) and SNRPN (Lucifero et al. 2006, Suzuki et al. 2009). Furthermore, placenta from calves deceased during perinatal period shows altered patterns in differentially methylated regions of imprinted genes, while tissue from animals that survived for longer periods had no such alterations (Su et al. 2011a).

Besides changes in DNA methylation of imprinted genes, histone methylation is also referred to be altered in SCNT-derived embryos. Fertilized bovine embryos display an asymmetric pattern of histone 3 trimethylation of lysine 9 (H3K9me3) in the inner cell mass (ICM) and the trophectoderm (TE), while SCNT embryos lose this asymmetry (Santos et al. 2003). The same asymmetry was observed for H3K27me3 in mouse embryos (Zheng et al. 2009). Thus, these errors in histone methylation patterns may account for some of the losses observed during SCNT gestation. Another epigenetic mark of interest is histone acetylation, which is related with active gene transcription sites (Shahbazian and Grunstein, 2007).

In order to improve the development of SCNT embryos, scientists have been trying the use of chemicals that can modulate epigenetic changes. The DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza) was first used in bovine donor cells but had no significant effect on embryo development (Enright et al. 2003, Enright et al. 2005). The histone deacetylase inhibitor (HDACi) trichostatin A (TSA) was also used to treat donor cells and increased blastocyst production (Enright et al. 2003, Wee et al. 2007). Use of TSA in bovine SCNT embryo culture yielded similar blastocyst rates and histone acetylation when compared to IVF ones (Iager et al. 2008). Combining 5-aza and TSA to treat both donor cells and embryos increased bovine blastocyst rates (Ding et al. 2008) and slightly increased development to term from 2.7% to 13.4% (Wang et al. 2011a), which is still low when compared to IVF. Other HDACi, such as Oxamflatin (Su et al. 2011b), Scriptaid (Wang et al. 2011b) and valproic acid (VPA -Xu et al. 2012) were tested and showed similar results, including increased histone acetylation; however, no development to term was assessed in these studies and

combination of 5-aza and VPA did not improve development to term of bovine SCNT-transferred embryos (Sangalli et al. 2012).

In the light of the small advances observed with chromatin modifiers, we believe that other alternatives should be tested and might be more fruitful. *XIST* is the gene responsible for inactivation of one of the X chromosomes in female embryos. *XIST* can be considered an imprinted gene as it is only expressed from one of the two inherited chromosomes, maternal or paternal (Navarro and Avner, 2009). It was shown that *XIST* expression is increased in mouse female and male SCNT-derived embryos, as well as in bovine SCNT embryos (Nolen et al. 2005; Inoue et al. 2010). Knockout of one XIST allele increased the outcome of mouse SCNT (Inoue et al. 2010). The lack of ESCs in bovine hampers the possibility of *XIST* knockout; however, use of shRNA approaches might allow reduction in *XIST* expression. Recently, macroH2A was shown to confer resistance of NT mediated reprogramming, especially by stabilizing the inactive X chromosome (Pasque et al. 2011) and could also be a target to improve SCNT outcome.

ESCs were reported to be more efficient in generating live offspring in mouse nuclear transfer than other somatic cells (Rideout et al. 2001), which makes sense in light of epigenetics, as these cells are less differentiated. However, there are no reports of stable culture of ESCs in cattle, which will be discussed together with the generation of induced pluripotent stem cells (iPSC) in the next section of this review. These iPSC are generated by the introduction of a combination of four transcription factors into differentiated cells (Takahashi and Yamanaka, 2006; Yu et al. 2007). One of these factors is OCT4, which is encoded by the POU5F1 gene (Scholer et al., 1990).

OCT4 is expressed in embryonic stem cells, primordial germ cells and oocytes (Scholer et al., 1990, Kurosaka et al. 2004, Kocabas et al. 2006). Mouse ESCs depleted of OCT4 had reduced expression levels of chromatin remodeling genes (Sharov et al. 2008) and knockout of OCT4 caused more compact chromatin in pluripotent epiblast stem cells in mouse embryos (Ahmed et al. 2010). We suggest that the expression of OCT4 in donor cells could improve reprogramming and the outcome of bovine SCNT.

1.2 Pluripotent stem cells

Mouse ESC (mESC) lines were first obtained in 1981 and the success of the isolation was credited to three factors: the developmental stage of the embryo at the time of isolation; the retrieval of enough number of cells and; culture conditions that allow self-renewal instead of differentiation (Evans and Kaufman, 1981). Non-human primate ES cells were obtained in 1995 after immunosurgery of rhesus monkey blastocysts (Thomson et al., 1995) and human ES (hESCs) cells were obtained using the same method in 1998 (Thomson et al., 1998). The interest for ES cell lines of livestock increased with the accomplishment of transgenic animals (Gordon and Ruddle, 1981) and with the reprogramming of an adult nucleus to generate another individual by SCNT (Wilmut et al., 1997). These cells could be used, alone or in combination with SCNT, as tools to produce transgenic livestock.

Despite several attempts, establishment of bona fide bovine ESC has not been achieved (Talbot et al. 1995; Cibelli et al. 1998b; Mitalipova et al. 2001; Saito et al. 2003; Wang et al. 2005; Keefer et al. 2007; Telugu et al. 2010). Bona fide ESCs retain

the abilities of self-renewal and differentiation into the three germ layers: endoderm, mesoderm and ectoderm. There are standard tests to prove stemness, including proliferation, gene or protein expression and in vitro and in vivo differentiation assays, such as embryoid body or teratoma formation. Bovine putative ESCs succeeded in some tests and failed others.

Mouse ESC can also be tested to form chimeras or to generate whole individuals by tetraploid complementation. Due to ethical issues, hESCs have not been tested at these levels. However, there are reasons to believe that they would fail. Mouse ESC pluripotency is maintained in vitro by action of leukemia inhibitor factor (LIF) that leads to JAK-STAT signaling (Smith et al., 1988, Niwa et al. 1998), while hESC are maintained by bFGF signaling (Xu et al., 2005). Colony and cell morphology are also slightly different. These facts lead to suspicions that mESC and hESC were derived from different stages of embryo development. This uncertainty was clarified when cells obtained from mouse expanded blastocysts yielded bFGF dependent cells, called epiblast pluripotent cells (EpiES – Brons et al. 2007, Tesar et al. 2007). These EpiES are the equivalent to hESC and importantly to the interest of livestock ESC researchers, EpiES are not able of undergoing homologous recombination, which would be one of the main reasons of isolating ESC in cattle or other species.

Forced expression of defined transcription factors is able to reprogram the cells to a pluripotent state and mouse and human iPSC retain the characteristics of their embryonic counterparts (Takahashi and Yamanaka, 2006; Yu et al. 2007; Okita et al. 2007; Wernig et al., 2007; Takahashi et al. 2007). Addition of small molecules that inhibit specific signaling pathways were used to obtain iPSC and mESC from a mouse

strain recalcitrant to ESCs derivation(Hanna et al. 2009). Small molecules were also able of reverting mouse EpiSC into mESC (Hanna et al. 2009, Zhou et al. 2010) and allowing the derivation of rat ESC (Buehr et al. 2008) and rat iPSC (Li et al. 2008).

This molecularly defined intracellular signaling pathway allow mouse ESCs to grow and self-renew without exogenous signaling. This was determined after exogenously blocking mitogen-activated protein kinase, fibroblast growth factor receptor, tyrosine kinases and glycogen synthase kinase-3 (Ying et al., 2008). We thought that these small molecules would also facilitate the generation of bovine ES or iPSC using OCT4, SOX2, KLF4 and C-MYC. However, despite several attempts and different media condition, we were not able to obtain bovine ESCs or iPSCs (Table 1.1). Recently, bovine iPSCs generation was described with the addition of NANOG only (Sumer et al. 2011) or NANOG and LIN28 (Han et al. 2011) to the other four factors mentioned above; however, these cells required bFGF in the culture medium, which may indicate that they are closer to EpiSCs.

The reasons for failure in derivation of true bovine ESCs are not clear. Several factors could be pointed, including timing of ICM isolation, method of ICM isolation, appropriate media conditions and the intrinsic differences of the bovine embryo development when compared to mouse embryos. The first noticeable difference is the implantation process. Mouse embryos undergo an invasive implantation process at blastocyst stage while bovine embryos elongate before attaching to the endometrium around day 18 after fertilization (Peippo et al. 2011). Moreover, mouse embryos express pluripotency markers in the ICM, such as OCT4 and NANOG, while in bovine these

markers are also expressed in the TE (Kirchhof et al. 2000, Muñoz et al., 2008, Cao et al. 2009), what makes the characterization of true bovine ESCs more difficult.

These differences suggest that methods for derivation of bona fide bovine ES should be different than mouse. In order to figure out how the methodology should be changed, it is first required to understand the biology of the bovine blastocyst, especially how ICM and TE specification occurs and also, patterns of gene and protein expression. In the next section we will discuss the known differences between mouse and bovine.

Table 1.1 – Summary of bovine ESC and iPSC derivation attemps.

Origin	Basal Media	Knockout Serum %	FGF (ng/ml)	hLIF (ng/ml)	mLIF (UI/ml)	3i
Embryo	DMEM/F12	15	1	1		Yes
Embryo	DMEM/F12	15	1	1		
Embryo	KO-DMEM	20			1000	Yes
Embryo	KO-DMEM	20			1000	
Embryo	KO-DMEM	20	4		1000	Yes
Embryo	KO-DMEM	20	4		1000	
BAF	DMEM/F12	15	1	1		Yes
BAF	DMEM/F12	15	1	1		
BAF	KO-DMEM	20			1000	Yes
BAF	KO-DMEM	20			1000	
BAF	KO-DMEM	20	4		1000	Yes
BAF	KO-DMEM	20	4		1000	
BFF	DMEM/F12	15	1	1		Yes
BFF	DMEM/F12	20	4			
BFF	KO-DMEM	10		1		
BFF	KO-DMEM	2	4	1		
BFF	KO-DMEM	2		1		

BAF = Bovine adult fibroblast, BFF = bovine fetal fibroblast, KO-DMEM = knockout DMEM, FGF = basic fibroblast growth factor, hLIF = human leukemia inhibitor factor, mLIF= mouse leukemia inhibitor factor, 3i = supplementation with small molecules.

1.3 Lineage specification in the pre-implantation embryo

Trophectoderm (TE) and inner cell mass (ICM) specification is widely studied in the mouse, in which cell specification is thought to start at the morula stage, when the outside cells would become polarized as an epithelium and differentiate into TE; while the inside cells would be apolar and turn into ICM (Sasaki, 2010). However, recent findings suggest that specification starts in the mouse 4-cell embryo, as more OCT4 protein starts to be retained in the nucleus, cells undergo asymmetrical cell division that will generate an inner cell and an outside cell (Plachta et al. 2011). Outside cells then express CDX2 while inside cells do not, and OCT4 or Nanog expression will be restricted only to the ICM at blastocyst stage (Ralston and Rossant, 2005).

In mouse embryos, CDX2 was found responsible for silencing OCT4 expression in the TE (Strumpf et al. 2005); however, CDX2 knockdown does not impede TE formation although it impairs its function and differentiation (Strumpf et al. 2005, Meissner and Jaenisch, 2006, Wu et al 2010). It was found that Tead4 acts upstream of CDX2 as embryos lacking Tead4 are unable to make TE cells and blastocoel formation is lethally impaired. (Yagi et al. 2007; Nishioka et al. 2007). Tead4 is expressed in all cells of the morula, but Hippo signaling components negatively regulate it in inside cells, inhibiting activation of CDX2 gene (Nishioka et al. 2009).

The precise mechanism of TE and ICM specification is not very well described in cattle. A recent paper has proposed the cow as a suitable model for studying mammalian early development. Bovine has an OCT4 promoter region CR4 that do not allow CDX2-mediated repression and it is shared with other species as human, horse, dog and rabbit; however, the same CR4 promoter region in the mouse has a different

binding site that allow CDX2-mediated repression of OCT4 (Berg et al. 2011). This result provides evidence that this specification mechanism is not as conserved among mammals as previously thought.

Expression patterns of genes related to TE establishment are not characterized in bovine early development. Also, as mentioned above, proteins commonly used as pluripotency markers in the mouse are expressed in both ICM and TE of bovine embryos. It is possible that alterations of spatial and temporal gene expression patterns during earlier stages negatively affect development to term. For example, dysfunctional placenta observed in SCNT might stem from aberrant expression of TE specification genes, due to incorrect reprogramming in some of the cells, leading to early differentiation and functional problems.

Identification of genes and signaling pathways involved in bovine ICM and TE differentiation will provide a better understanding of the pre-implantation metabolic needs and in turn, facilitate the optimization of culture media conditions for embryos, ESCs and iPSCs derivation. Supplements added to the media, such as growth factors or specific pathways inhibitors or agonists, could be defined specifically for bovine cells requirements. Also, novel genes could be included in the reprogramming strategy, allowing generation of bovine iPSCs that are amenable to homologous recombination. Eventually, these cells can be used for improving SCNT rates and generating transgenic cloned animals.

CHAPTER 2

EFFECTS OF DONOR FIBROBLASTS EXPRESSING OCT4 ON BOVINE EMBRYOS GENERATED BY SOMATIC CELL NUCLEAR TRANSFER

2.1 Abstract

The production of healthy, live cloned animals by somatic cell nuclear transfer (SCNT), in all species cloned to date, has been hampered by low efficiencies. Significant epigenetic changes must take place in order to ensure proper chromatin remodeling in SCNT. We hypothesized that exogenous expression of *OCT4* in the donor fibroblast prior to its fusion with an enucleated oocyte would facilitate SCNT reprogramming by the oocyte. We infected fibroblasts of adult cows with retroviral vectors containing yellow fluorescent protein (YFP) only, or *OCT4* gene fused to YFP (YO). We used immunocytochemistry to confirm exogenous OCT4 protein expression. We found that development to blastocyst was not different between NT-YFP and NT-YO groups. NT-YFP embryos had the fewest trophoblast cells, measured by numbers of CDX2-positive cells, while NT-YO- and IVF-derived embryos had higher numbers of trophoblast cells. Fibroblasts expressing *OCT4* had reduced levels of histone 3 lysine 9 or 27 trimethylation (H3K9me3 and H3K27me3 respectively). NT-YO blastocysts displayed higher H3K9me3 levels than IVF and NT-YFP embryos; however, they did not have different H3K27me3 levels. The levels of *XIST* mRNA expression in NT-YO and NT-YF were higher when compared to IVF blastocysts. We observed no differences in the expression of *SOX2*, *NANOG*, and *CDX2*. Although overexpression of *OCT4* in

donor cells increased H3K9me3 and did not reduce *XIST* gene expression in bovine SCNT-derived embryos (both of which markers have previously been associated with poor development to term), we show that a single transcription factor can affect the number of trophectoderm cells in bovine SCNT embryos. Its effect on the efficiency of production of healthy cloned animals remains to be determined.

2.2 Introduction

After the first reported use of somatic cell nuclear transfer (SCNT) to produce offspring from an adult donor cell (Wilmut et al. 1997), researchers have successfully employed this technique in several mammalian species (reviewed by Cibelli, 2007). Bovine SCNT is considered well-established; however, the transfer of SCNT embryos results in an average of 9 percent of live offspring (Panarace et al., 2007), a much lower percentage than *in vivo*-derived or *in vitro* fertilized (IVF) embryos (reviewed by Yang et al., 2007). The loss of embryos in the first trimester of SCNT gestations reaches 50 percent after the first pregnancy check at 30 days — fivefold higher than the losses observed for IVF embryos and tenfold higher than natural conceptions (Hill and Chavatte-Palmer, 2002). These losses and later pregnancy issues most likely arise from failures of the oocyte to reprogram the somatic nucleus.

Cellular reprogramming in SCNT involves changes in gene expression that are associated with epigenetic modifications (Latham, 2005). Chromatin modifications — including DNA methylation, histone lysine methylation, and histone acetylation — are components of the epigenetic mechanism that ultimately determine the expression level of most genes (Santos and Dean, 2004). Histone 3 trimethylation of lysine 9 (H3K9me3)

and lysine 27 (H3K27me3) are associated with transcriptional repression, and its changes are catalyzed by specific histone methylases or demethylases (Hublitz et al. 2009). With the exception of imprinted genes, both paternal and maternal genomes are demethylated in fertilized embryos, and *de novo* methylation occurs, leading to an asymmetrical pattern that characterizes the inner cell mass (ICM) and the trophectoderm (Santos and Dean, 2004). This pattern is not well observed in bovine embryos derived from SCNT, as seen by homogeneous staining of the ICM and trophectoderm (TE) with antibodies against DNA methylation or H3K9me3 (Santos et al., 2003). In the mouse, H3K27me3 staining was more intense in the ICM of control IVF blastocysts, while asymmetry was not seen in SCNT embryos (Zhang et al., 2009). These results suggested that the lower number of live births from SCNT might stem from errors in the reestablishment of histone methylation patterns.

In an attempt to improve bovine SCNT results, a series of studies have tried to induce epigenetic changes either before or after the somatic cells fuse to the oocyte. Using the histone deacetylase inhibitor trichostatin A (TSA) in bovine donor cells increased the blastocyst rate when compared to nontreated cells (Enright et al., 2005). When used in embryos, TSA led to SCNT embryos having similar levels of *in vitro* development and histone H4 acetylation at lysine 5 to IVF embryos (Iager et al., 2008). The combined use of DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza) and TSA increased both blastocyst rates (Ding et al. 2008) and development to term (Wang et al. 2011). The use of oocyte extract to induce changes in the donor cell population *in vitro* has also reportedly reduced histone acetylation, increasing blastocyst

formation in cows (Tang et al. 2009) and increasing live birth rates in sheep (Rathbone et al. 2010).

Oocytes contain significant levels of mRNA for the pluripotency-related gene *OCT4* (Scholer et al., 1990, Kurosaka et al. 2004, Kocabas et al. 2006). *OCT4,* part of the POU transcription factor family, binds an octameric nucleotide motif within promoters or enhancer regions (Pesce and Scholer, 2001). In addition to being expressed in oocytes, *OCT4* is also expressed in mouse pluripotent embryonic stem cells (ESCs) and primordial germ cells (Scholer et al., 1990). The use of mouse ESCs as donor cells in nuclear transfer increases the number of live births when compared to somatic donor cells (Rideout et al. 2001). *OCT4* is generally considered the most important of the reprogramming factors used to produce induced pluripotent stem cells (iPSCs; Takahashi and Yamanaka, 2006; Yu et al., 2007). Generation of iPSCs from neuronal stem cells has been achieved using *OCT4* alone (Kim et al., 2009). In addition, suppression of *OCT4* in mouse ESCs reduced the expression levels of several chromatin remodeling genes (Sharov et al. 2008), and *OCT4*-null embryos displayed more compact chromatin in pluripotent epiblast stem cells than wild-type embryos (Ahmed et al. 2010). Based on these facts, we hypothesized that the exogenous expression of *OCT4* in the donor fibroblast might facilitate SCNT reprogramming by the oocyte.

Our goal was to evaluate the epigenetic and gene expression changes in SCNT-derived bovine embryos using donor fibroblasts ectopically expressing *OCT4*. In this study, we used a retroviral vector to introduce the human *OCT4* gene into the bovine adult fibroblasts being used for SCNT. We characterized epigenetic changes in these

fibroblasts and generated SCNT blastocysts. We assessed development rates and analyzed these embryos for ICM and TE cell allocation, for the degree of methylation at H3K9 and H3K27 residues using specific antibodies for trimethylation, and to quantify gene expression of embryonic transcription factors and epigenetic modifying enzymes.

Our results show that *OCT4* expression in donor fibroblasts could reduce the global levels of histone trimethylation and increase the expression of demethylases. The SCNT embryos produced using these cells had more TE cells than the control SCNT-derived embryos. Expressing *OCT4* in donor cells increased H3K9me3 global levels but did not induce changes in H3K27me3 levels. We found an increase in gene expression of *XIST* and a reduction in endogenous *OCT4* in both SCNT groups as compared to their *in vitro* fertilized counterparts.

2.3 Material and methods

Unless otherwise stated, we purchased all reagents from Sigma (St. Louis, MO).

2.3.1 Production of transgenic cells

Human *OCT4* open reading frame was cloned from human ESCs, fused to YFP genes, and cloned into a MMLV retroviral vector containing Neomycin selection cassette and TetOff regulation, all flanked by LTR promoter (Figure 1A). We produced viral vectors containing *YFP* only (*YFP*) for control studies or *YFP* fused to *OCT4* (YO), using HEK cells cultured in fibroblast culture media consisting of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10 percent fetal bovine serum, 1 percent NEAA, and

1 percent Pen/Strep. We filtered the supernatant of infected HEK cells and then used it to transduce bovine adult fibroblasts (BAFs) from a female Jersey heifer. We thawed the BAFs at the fifth passage and cultured them at low confluency with fibroblast culture media. We transduced cells using three eight-hour rounds of viral medium and then cultured them on DMEM, changing the media every five days. We selected positive cells by adding 500 µg/ml of G418 to the culture for ten days. Cells were then passaged and cultured with G418 for five more days before freezing. Cells were thawed, expanded for two more passages, and frozen again in working aliquots. We checked for the presence of *YFP* using an inverted fluorescence microscope (Nikon TE-2000, Tokyo, Japan).

2.3.2 Immunocytochemistry of cultured cells

We used PBS in all washes and solutions. Fibroblasts at passage 10 were cultured for four days, washed three times, and then fixed with a 4 percent paraformaldehyde solution for 20 minutes. We washed the cells three times before blocking with 3 percent BSA and 0.05 percent Triton X-100 solution for 90 minutes. We removed the blocking solution and added primary antibody solution, supplemented with 1 percent BSA, for one hour at room temperature. We then washed the cells three times and added secondary antibody solution with 1 percent BSA for one hour. Cells were washed again three times and incubated with 0.01 mg/ml of Hoechst stain. Finally, we washed the cells and observed them in an inverted fluorescence microscope. We used the following primary antibodies: anti-OCT4 (sc8628; Santa Cruz Biotechnology, Santa Cruz, CA); anti-H3K9me3 (07-442; Millipore, Merck KGaA, Darmstadt, Germany); and

anti-H3K27me3 (C36B11; Cell Signaling, Beverly, MA). We used the secondary antibody Alexa Fluor 568 donkey anti-goat or donkey anti-rabbit IgG (Invitrogen).

2.3.3 *In vitro* maturation of oocytes

We harvested bovine oocytes from slaughterhouse-derived ovaries, as described previously (Ross and Cibelli, 2010). We placed the oocytes in TCM 199 medium containing 10 percent FBS, 3 µg/ml LH (Sioux Biochemical, Sioux Center, IA), 3 µg/ml FSH (Sioux Biochemical), 22 µg/ml sodium pyruvate, and 25 µg/ml gentamycin (Invitrogen) for 16 hours for SCNT or 22 hours for *in vitro* fertilization.

2.3.4 SCNT

We used a bovine SCNT protocol similar to the one previously described (Ross and Cibelli, 2010), with the modifications described below. We cultured YFP or YO donor nucleus fibroblasts in serum starvation (0.5 percent FBS in DMEM) for 24 hours and collected them using a 10 UI/ml pronase solution. Cells and enucleated oocytes were placed in drops of HH medium. Using a glass pipette with 20 µm, we verified YFP-positive cells by brief exposure to fluorescence and individually placed them in the perivitelline space of each oocyte. After reconstruction, we fused, activated, and cultured these oocyte-cell couplets as described in the protocol. After seven days in culture, blastocysts rates were recorded and used for protein or RNA expression analysis.

2.3.5 *In vitro* fertilization

We transferred mature oocytes into TALP-based fertilization media (Parrish et. al 1986) supplemented with 20 µg/ml heparin. Frozen-thawed semen was fractionated in a Percoll gradient and $1x10^6$ sperm cells/ml were added to the medium containing the oocytes. Fertilization was carried out for 18 hours at 38.5 °C and 5 percent CO_2 in high humidity. Presumptive zygotes were then denuded and cleaned from excessive sperm using HH media and cultured *in vitro* as described above for seven days.

2.3.6 Immunocytochemistry of blastocysts

All solutions were prepared with PBS. We washed embryos three times in PBS and fixed them with 4 percent paraformaldehyde solution for 20 minutes. Then, we washed day seven (D7) blastocysts three times in 0.1 percent Triton X-100 solution and permeabilized them for 15 minutes with 0.5 percent Triton X-100 solution. We performed the blocking of unspecific binding sites using 0.1 percent Triton X-100, 1 percent BSA, and 10 percent normal donkey serum solution for one hour at room temperature. We incubated primary antibody for two hours in the dark at room temperature in 0.1 percent Triton X-100 and 1 percent BSA solution. We washed embryos three times for 15 minutes in 0.1 percent Triton X-100 solution, followed by incubation with secondary antibody for one hour at room temperature in the dark. We then washed the embryos three times for 15 minutes in the dark and mounted them on slides with Prolong Gold Antifade Reagent with DAPI (Invitrogen). We visualized the blastocysts using an inverted spinning-disk confocal microscope and obtained stacks of

pictures using Metamorph software (Molecular Devices, Sunnyvale, CA). We used the same primary antibodies as above and anti-CDX2 (sc166830, Santa Cruz Biotechnology). We used the same secondary antibodies as above and Alexa Fluor 568 donkey anti-mouse immunoglobulin G (Invitrogen).

2.3.7 Intensity quantification and differential cell counting

Using Metamorph software for image analysis, we performed intensity quantification of fibroblasts by delineating each nucleus; the software calculated average pixel intensity. Data was normalized by an average of two background regions for each projection. We randomly selected and quantified three different image fields for each group (YFP and YO). For H3K9me3 quantification, 63 YFP cells and 100 YO cells were quantified. For H3K27me3 quantification, 57 YFP cells and 111 YO cells were quantified.

We performed differential cell counting by counting CDX2-positive cells and DAPI-stained nuclei on all planes of images obtained from a single embryo. We considered CDX2-positive cells as TE (Kuijk et al. 2008) and CDX2-negative cells as ICM. We determined the total cell number by DAPI staining. A total of 63 embryos were used: 18 for IVF, 21 for NT-YFP and 24 for NT-YO.

We performed the intensity quantification for embryos as described previously (Ross et al. 2008a). Briefly, all planes were combined in a maximum projection, and each nucleus was assessed individually for its average pixel intensity. Data was normalized, as mentioned earlier for fibroblasts. Each staining used a total of ten

embryos, developed from two different batches of oocytes, for each group (IVF, YFP, and YO).

2.3.8 RNA extraction, reverse transcription and quantitative PCR

We pooled five to ten blastocysts per group from each oocyte batch in a total of five replicates. Embryos were placed in 20 µl of extraction buffer from the PicoPure RNA Extraction Kit (Applied Biosystems, Foster City, CA), incubated for 30 minutes at 42 °C and stored at -80 °C until further processing. We thawed samples on ice and extracted RNA according to the PicoPure manufacturer's protocol, including a step for incubation with DNase (Qiagen, Hilden, Germany). Isolated RNA was used for reverse transcription reaction using the Superscript II Reverse Transcriptase Kit (Invitrogen) with 250 ng of random primers (Promega, Madison, WI) following the manufacturer instructions. We quantified the complementary DNA that was synthesized using the NanoDrop Spectrophotometer (Thermo Scientific, Rochester, NY), and we diluted all samples to 2 µg/ml. We performed duplicate quantitative PCR reactions for each sample, using SYBR Green 2X PCR Master Mix (Applied Biosystems) and 2 µl of cDNA with the ABI 7000 Detection System. Thermal cycle settings were 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. We used the histone 2A (*H2A*) housekeeping gene to normalize the expression of target genes, using the ΔΔCt method (Pfaffl, 2001). Table 2.1 describes the primers used.

2.3.9 Statistical Analysis

We used SAS software (SAS Institute, Cary, NC) to analyze our data. We performed ANOVA using PROC GLM, considering treatment as an independent variable and using Tukey's adjustment as a post hoc test to compare means. Quantitative PCR data was analyzed using PROC MIXED of SAS as described previously (Steibel et al. 2009). Data from qPCR is presented in graphs with Log2 distribution and for ease of understanding the fold change is used in the text. We set the statistical significance at $p<0.05$.

Table 2.1 Bovine-specific primers used throughout this chapter.

Primer Name	Primer Sequence (5'to 3')	Reference
H2A	fwd GCAAATGACCCTGATGCTACC rev CGGCCCTCCATAACATCAA	NM_001078089
XIST	fwd AATAATGCGACAGGCAAAGG rev TCCCGCTCATTTTCCATTAG	Inoue et al. 2010
CDX2	fwdTGGGCAGCCAAGTGAAAACCAGG- rev GCGGCCAGTTCGGCTTTCCT	NM_001206299
IFN-T	fwd ACAGTGACTGCGCCTGGGA rev GGTGATGTGGCATCTTAGTCAGCG	ENSBTAT00000048583
SOX2	fwd AGCGCATGGACAGCTACGCG rev ATGGGCTGCATCTGAGCGGC	NM_001105463
NANOG	fwd TCCAGCAAATGCAAGAACTTTC rev TTACATTTCATTCTCTGGTTCTGGAA	NM_001025344
5'OCT4	fwd GCTGGAAGTGAAGGCCCGCA rev TGGTGGCGGTGGTGTCTGGA	ENSBTAT00000028122
EHMT1	fwd CTGGATTCCGAGAAACCCAAG rev TCAACCAACATGAGCAGCACC	NM_001099041
KDM4C	fwd CCTGAGGATATTGTGAGCCGAG rev TTGATCCGAGATACTTGGCCC	XM_002689595
JARID2	fwd TCGGCTCAGGACTTACGGAAA rev AAGGTCTGACTTCGCGCATCT	XM_002697579.

2.4 Results

2.4.1 Characterization of transgenic cells

In the present study, we generated transgenic bovine adult fibroblasts (BAFs) expressing *OCT4* fused to YFP (BAF-YOs) or YFP only (BAF-YFPs) for use as donor cells for SCNT. After viral transduction by means of retroviral vectors (Figure 2.1A), selection with neomycin, and freezing, we thawed the BAFs and, detected YFP fluorescence in the cytoplasm of the BAF-YFP cells and only in the nuclei of the BAF-YO cells (Figure 2.1B). Immunocytochemistry revealed that OCT4 protein was not expressed by BAF-YFP cells, but, as expected, it was present and properly localized in BAF-YO cells (Figure 2.1C).

To determine if *OCT4* ectopic expression affected epigenetic markers in fibroblasts prior to nuclear transfer, we performed immunocytochemistry for H3K9me3 and H3K27me3 (Figure 2.2A). Analyzing both markers for the intensity of immunofluorescence showed that BAF-YO had significantly less intensity than BAF-YFP cells (Figure 2.2B). To further investigate the possible causes, we assessed gene expression of histone modification enzymes known to be regulated by OCT4 in mouse ESCs (Loh et al. 2006). Quantitative RT-PCR showed that the amount of RNA for the enzymes responsible for H3K9 and H3K27 methylation (*EHMT1)* and demethylation (*JARID2)*, were not statistically different between the two cell lines; however, H3K9 demethylase *KDM4C* was upregulated in BAF-YO (Figure 2.2C), suggesting that active demethylation likely accounts for the diminished signal.

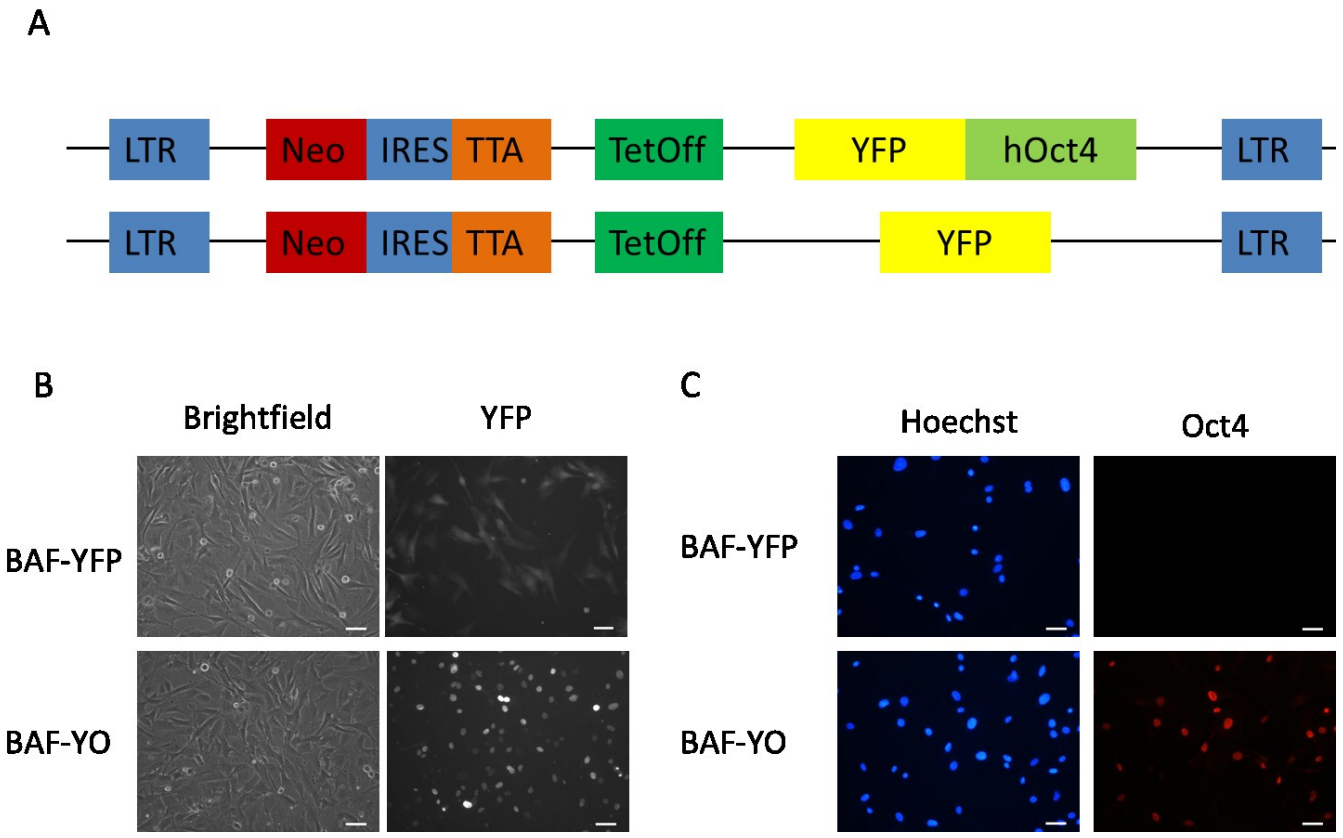

Figure 2.1 - **Generation and characterization of transgenic fibroblasts** A) Schematic representation of the retroviruses constructed for transfection of fibroblasts. B) Brightfield and fluorescence microscopy of BAF-YFP or BAF-YO showing that YFP was localized to the nucleus when fused to hOCT4. C) Fluorescence images of BAF-YFP and BAF-YO nuclei and OCT4 immunocytochemistry. Scale bar is equal to 40μm. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

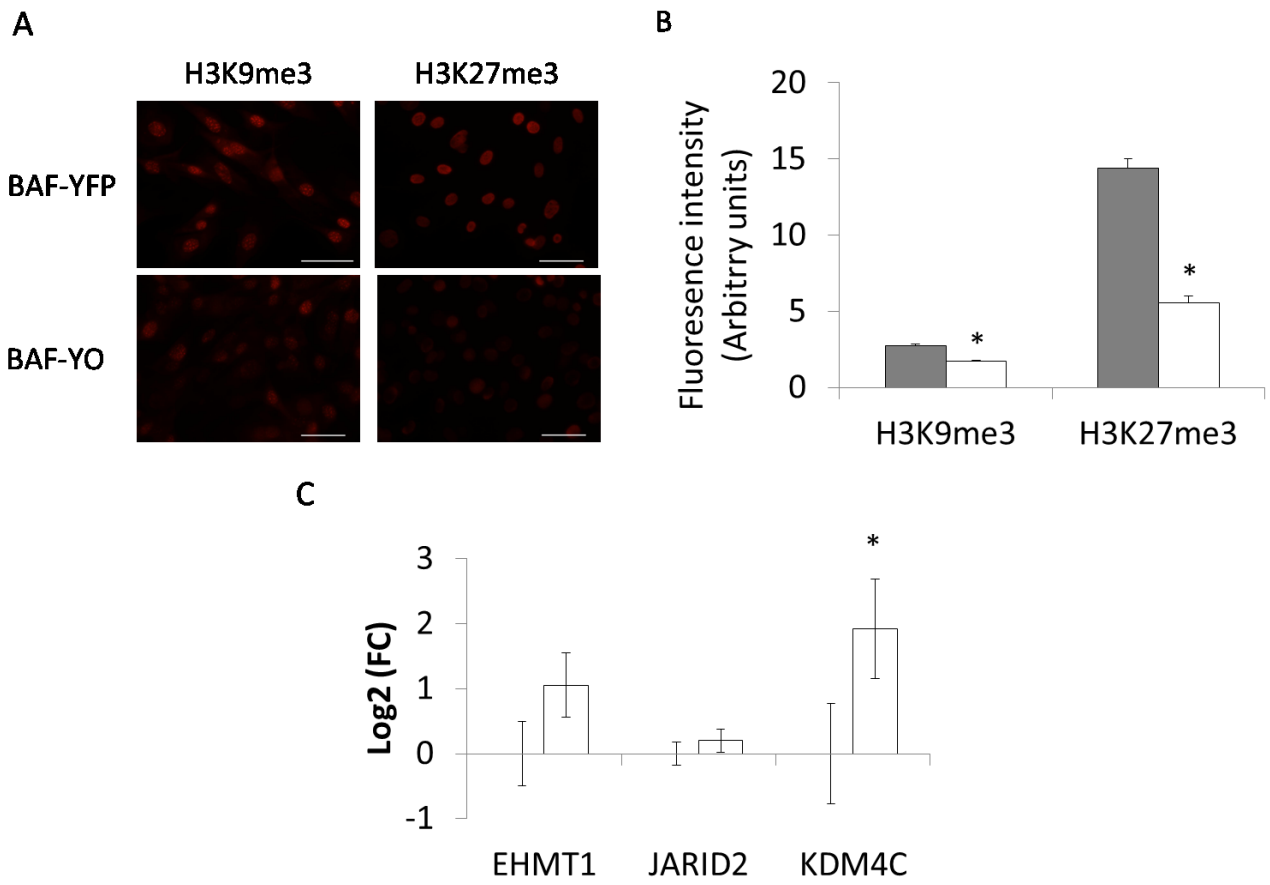

Figure 2.2 - Characterization of transgenic fibroblasts. A) H3K9me3 and H3K27me3 immunocytochemistry of BAF-YFP and BAF-YO; B) Semiquantitative analysis of fluorescence intensity for images of H3K9me3 and H3K27me3 in BAF-YFP (grey bars) and BAF-YO (open bars). C) Quantification of gene expression of histone modification enzymes in BAF-YFP (set to zero) and BAF-YO (open bars). Asterisk indicate significant statistical difference ($p \leq 0.05$); n = 3. Scale bar is equal to 40µm.

2.4.2 Embryo development rates and differential cell counting

We performed a total of 12 SCNT manipulations in which we assessed fusion, cleavage, and blastocyst rates; we observed no differences between BAF-YFP- or BAF-YO-derived embryos (Figure 2.3). Although YFP was clearly visible in the donor cells, we could observe no YFP fluorescence 72 hours after SCNT activation in either BAF-YFP- or BAF-YO-derived embryos (henceforth referred to as NT-YFP and NT-YO, respectively). In order to verify cell allocation to ICM or TE, we performed immunocytochemistry against the CDX2 protein in a total of 63 IVF- and SCNT-derived day seven blastocysts. Confocal images showed that CDX2 staining was limited to the TE of all IVF or SCNT embryos (Figure 2.4). Both SCNT groups had a lower number of ICM cells than the IVF control, although the number of TE cells was higher in both IVF and NT-YO than in NT-YFP embryos (Table 2.2). Consequently, the total number of cells was higher in IVF blastocysts than in NT-YFP embryos, while NT-YO embryos did not statistically differ from either group (Table 2.2).

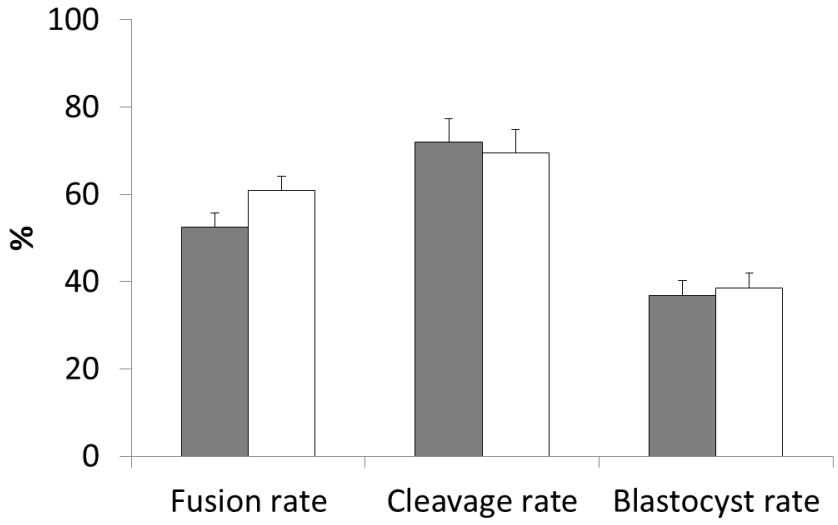

Figure 2.3 - **Fusion, cleavage and blastocyst rates** – as a proportion of the total fused structures – of somatic cell nuclear transfer using BAF-YFP (grey bars) or BAF-YO (open bars) as donor cells.

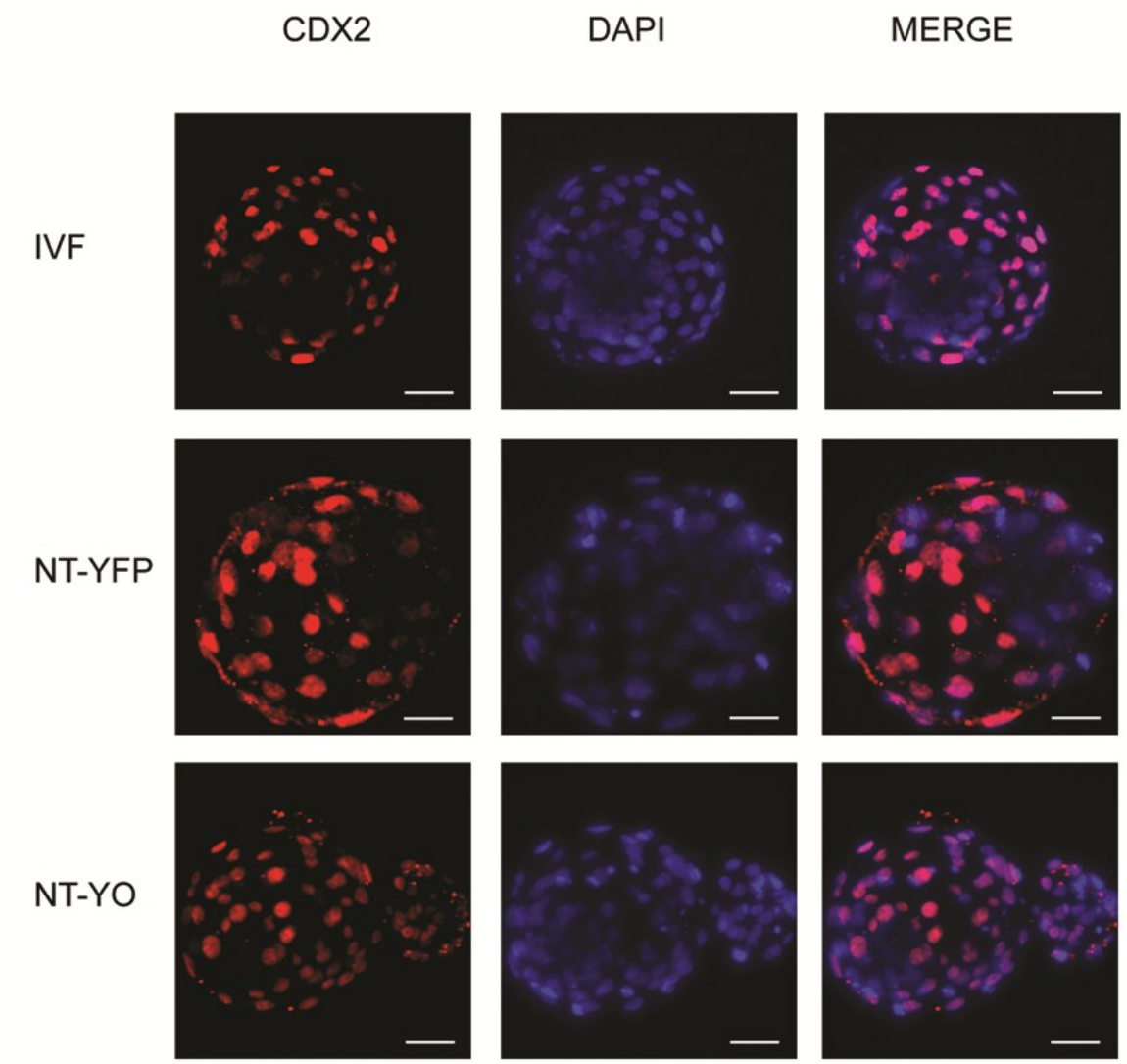

Figure 2.4 - CDX2 immunostaining in IVF, NT-YFP and NT-YO blastocysts. CDX2 protein is present in the nucleus of trophoblast and absent in ICM cells. Scale bar is equal to 40μm.

Table 2.2. Allocation of bovine nuclear transfer blastocyst cells to the TE or ICM at D7, as determined by CDX2 staining. We considered positive cells as trophoblast cells and negative cells as ICM cells. ICM = inner cell mass; TE = trophectoderm.

	ICM cells (CDX2 -)	TE cells (CDX2 +)	Total cells
IVF	42.27 ± 2.64^{b}	58.66 ± 4.22^{b}	100.94 ± 5.59^{b}
NT-YFP	33.71 ± 2.44^{a}	45.04 ± 3.91^{a}	78.76 ± 5.17^{a}
NT-YO	31.91 ± 2.28^{a}	54.29 ± 3.65^{b}	86.20 ± 4.84^{ab}

Different superscript letters indicate significant statistical differences (P≤0.05).

2.4.3 Histone trimethylation quantification

We performed immunocytochemistry against H3K9me3 and H3K27me3 in IVF, NT-YFP, and NT-YO day seven blastocysts to study modifications in the chromatin that could be induced by expressing *OCT4* in donor cells. We quantified the average intensity using Metamorph software. We observed no obvious difference of intensity in the ICM and TE of H3K9me3-stained embryos (Figure 2.5A); however, NT-YO embryos displayed a higher intensity of H3K9me3 staining than IVF or NT-YFP embryos (Figure 2.5B). H3K27me3 was uniform in all cells at the blastocyst stage (Figure 2.6A) and did not differ among treatment groups. The average fluorescence intensity of H3K27me3 was the same among all three groups (Figure 2.6B).

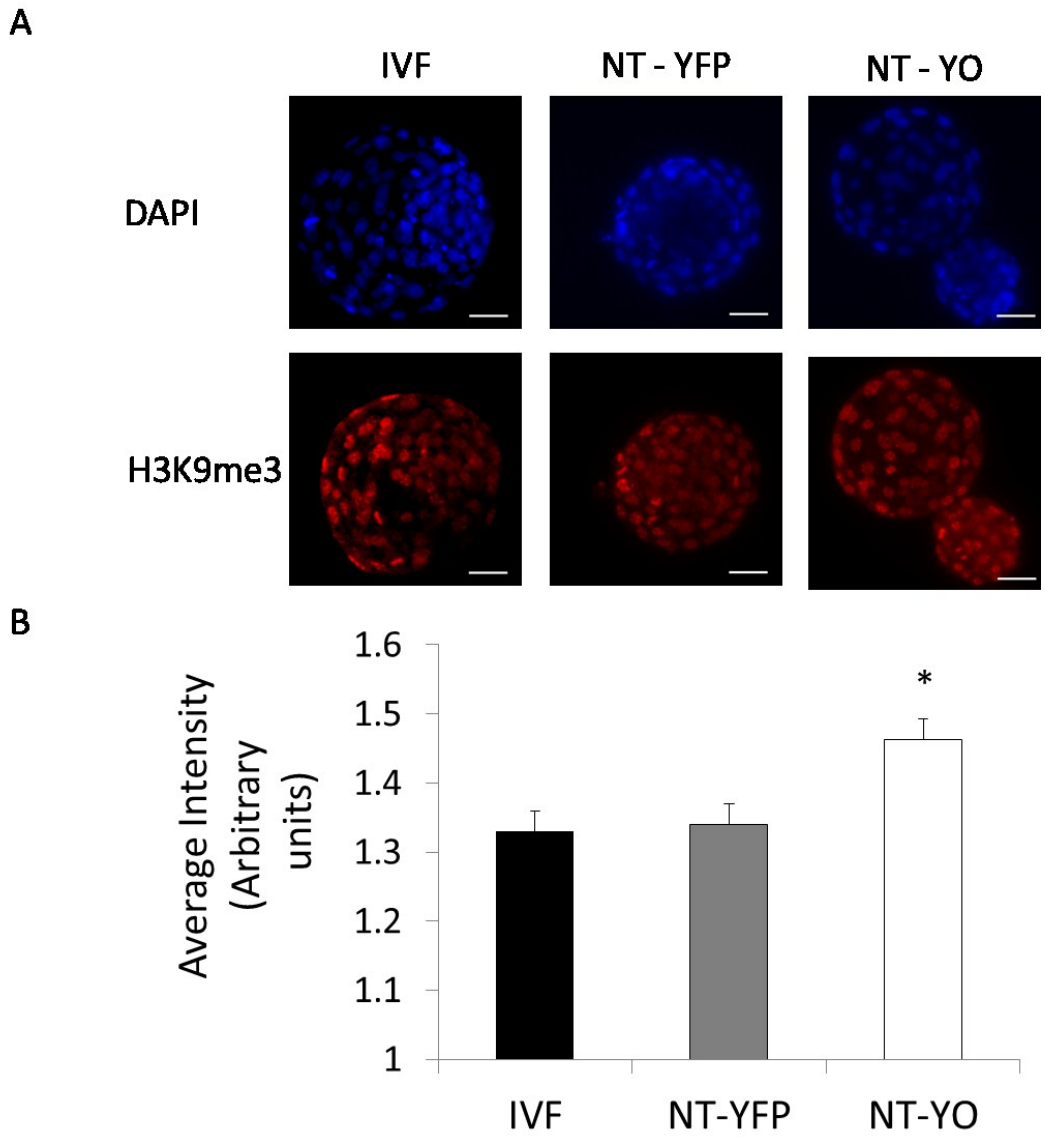

Figure 2.5 - H3K9me3 detection in day 7 blastocysts of IVF, NT-YFP and NT-YO groups. A) Representative fluorescence images of DAPI and H3K9me3 immunostaining. B) Semiquantitative analysis of average fluorescence intensity. The nucleus of NT-YO blastocysts displayed stronger H3K9me3 staining when compared to IVF and NT-YFP produced blastocysts. Asterisk indicates significant statistical difference ($p \leq 0.05$); n = 10. Scale bar is equal to 40μm.

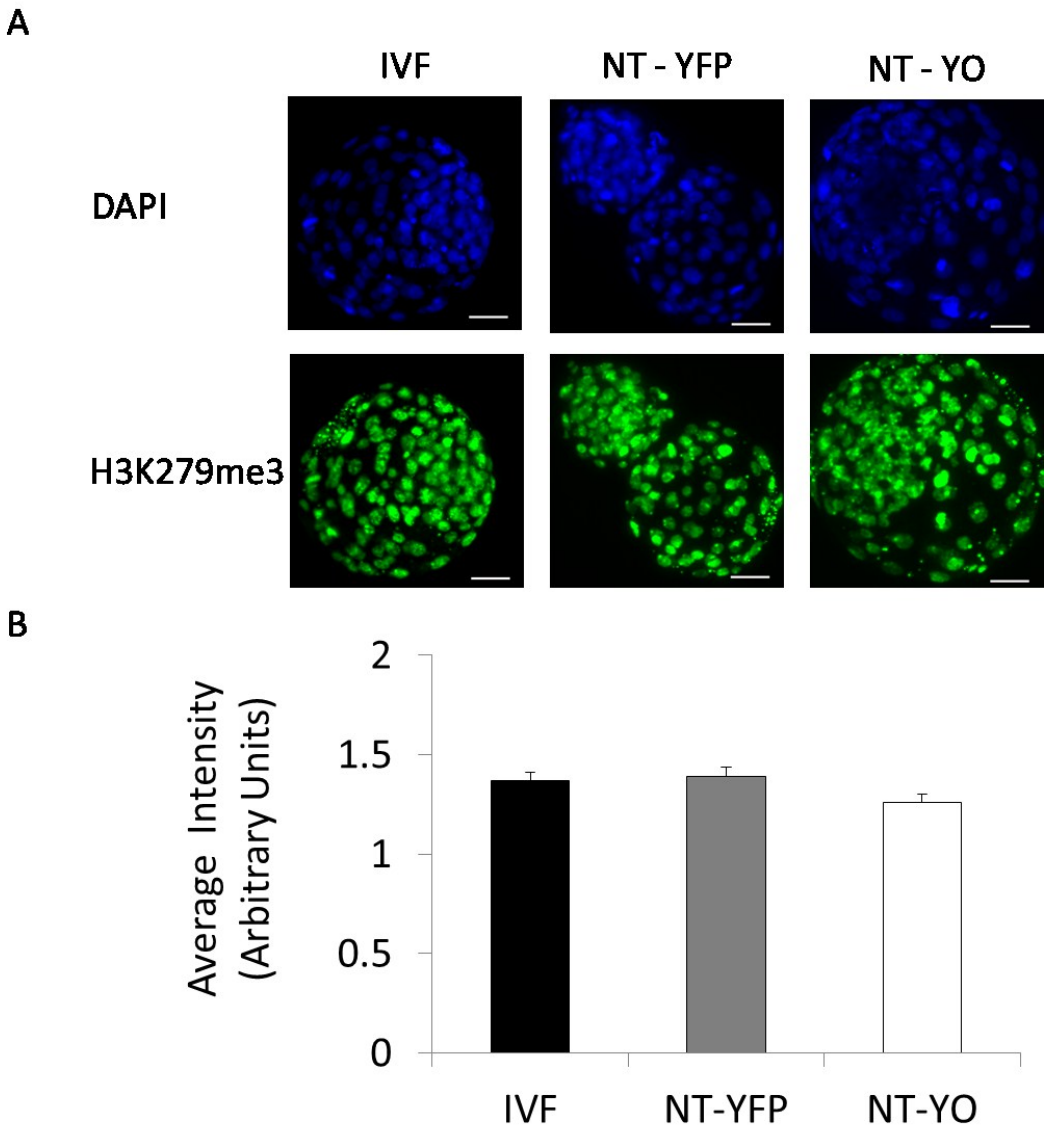

Figure 2.6 - H3K27me3 immunostaining in blastocysts of IVF, NT-YFP and NT-YO groups. A) Representative fluorescence images of DAPI and H3K27me3 immunostaining. B) Semiquantitative analysis of average fluorescence intensity. No difference was observed in the levels of H3K27me3 staining between all 3 treatment groups. Asterisk indicates significant statistical difference ($p \leq 0.05$); n=10. Scale bar is equal to 40 µm.

2.4.4 Quantitative PCR gene expression analysis

We performed quantitative RT-PCR analysis to verify gene expression changes in pools of IVF, NT-YFP, and NT-YO day seven blastocysts. We selected genes known for their role during pre-implantation development, including genes associated with the H3K9 methylation mark. For *EHMT1* and *KDM4C*, we observed no significant differences in mRNA levels (Figure 2.7A). We found that *XIST*, recently shown to be upregulated in SCNT bovine embryos (Inoue et al. 2010), was upregulated in both the NT-YFP and NT-YO groups compared to the IVF group (Figure 2.7B). We assessed endogenous *OCT4* expression using primers for the 5' untranslated region (5'UTR), and we observed a downregulation in the NT-YFP and NT-YO groups compared to the IVF group (Figure 2.7B). Expression of other important genes, such as *CDX2, SOX2* and *NANOG* did not differ among the three groups (Figure 2.7B).

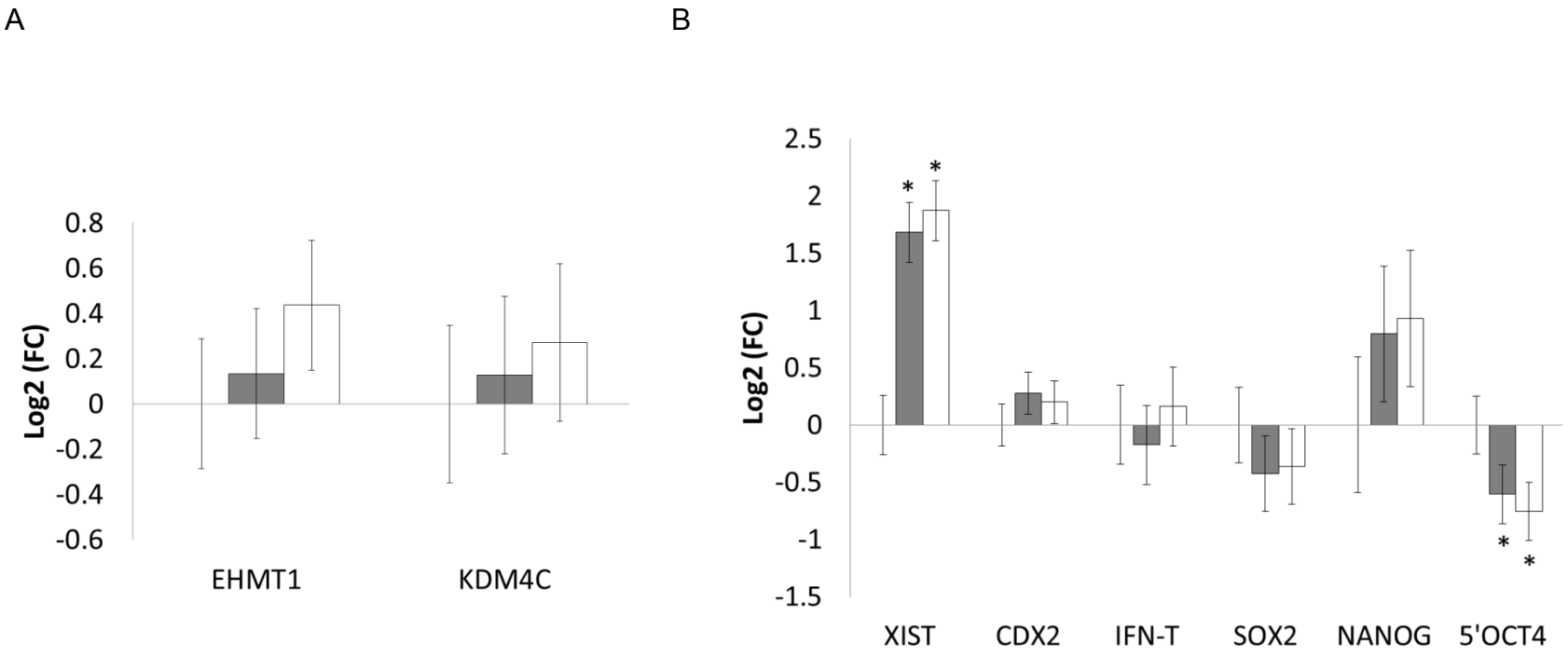

Figure 2.7 - Gene expression analysis in D7 blastocysts. A) Quantification of gene expression of histone modification enzymes EHMT1 and KDM4C of IVF (set to zero), NT-YFP (grey bars) and NT-YO (open bars) groups; p=0.11 and p=0.08 respectively. B) Gene expression of developmentally important genes *XIST, CDX2, IFNT, SOX2, NANOG and OCT4* of IVF (set to zero), NT-YFP (grey bars) and NT-YO (open bars) groups. *XIST* expression was significantly higher and *OCT4* expression was significantly lower in the NT-YFP and NT-YO when compared to IVF blastocysts. Asterisk indicates significant statistical difference ($p \leq 0.05$); n = 5, pools of 5 to 10 embryos.

2.5 Discussion

The SCNT technique has proven itself capable of producing viable cloned offspring; however, its efficiency remains very low. Problems, including low pregnancy rates and high pregnancy losses, are thought to be related to failure in the epigenetic reprogramming of the somatic donor nucleus (Campbell et al. 2007). The use of ESCs in mouse nuclear transfer yields better results than the use of somatic cells (Rideout et al. 2001), arguing in favor of the notion that a pluripotent nucleus is more easily reprogrammed into a live animal by SCNT. *OCT4* maintains pluripotency in ESCs (Pesce and Scholer, 2001) and keeps an open chromatin state in embryos, as measured by electron spectroscopic imaging (Ahmed et al. 2010). We hypothesized that we could facilitate the oocyte's task of reprogramming a somatic cell's chromatin by expressing *OCT4* in the donor fibroblast prior to SCNT. To test our hypothesis, we generated donor fibroblasts that ectopically expressed YFP and OCT4-YFP fusion protein. We used immunohistochemistry to confirm proper nuclear localization of OCT4.

Before using these fibroblasts as donor cells, we wanted to determine whether *OCT4* overexpression could induce changes in the epigenome. We observed a decrease in H3K9me3 and H3K27me3 after quantification of fluorescence immunocytochemistry. In order to understand how these changes occurred, we verified the gene expression of the histone methylation enzyme *EHMT1* and the demethylation enzymes *JARID2* and *KDM4C* enzymes; which promoter regions can bind directly to OCT4 (Loh et al. 2006). We found the expression of KDM4C, a demethylase that acts specifically on the H3K9me3 residue (Whetstine et al. 2006), was higher in YO than in

YFP fibroblasts, which might explain the reduction in global methylation levels of this epigenetic mark. H3K27me3 is catalyzed by the polycomb repressive complex 2 (PRC2), and JARID2 is involved in PRC2 recruitment (Li et al. 2010). However, *JARID2* is unchanged in YO fibroblasts, strongly suggesting that the reduction of H3K27me3 as a consequence of *OCT4* expression in fibroblasts occurs due to a JARID2-independent mechanism and deserves further characterization.

Expression of *OCT4* in fibroblasts prior to fusing them to MII oocytes did not affect SCNT cleavage rates or blastocyst formation. The expression of the transgene itself in both groups — control and YFP-OCT4 — was absent at 72 hours and at the blastocyst stage, the two time points we investigated. The silencing of the transgenes likely occurred due to the use of retroviral vectors that can be actively methylated and silenced during embryo development (Jähner et al. 1982).

SCNT-derived embryos reportedly have lower TE cell numbers than *in vivo* fertilized or IVF-derived embryos, and this alteration was thought to play a role in pregnancy losses (Koo et al. 2002). We observed a significant increase in the number of TE cells in embryos reconstructed with BAF overexpressing *OCT4*. The number of cells in these embryos was similar to IVF ones. Provided that one of the reasons for placental failure in the SCNT embryos is indeed the reduced TE cell numbers at the blastocyst stage, expression of OCT4 in the donor fibroblast should be explored as a strategy to increase pregnancy and birth rates in cloned bovine embryos.

We assessed the levels of histone trimethylation to verify global epigenetic changes in SCNT-derived blastocysts. The H3K9me3 mark has been shown to display an asymmetry at the bovine blastocyst with the ICM showing higher intensity than the

TE (Santos et al. 2003). Curiously, we did not observe any asymmetry in our IVF or SCNT embryos. The relatively lower levels of H3K9me3 observed in YO fibroblasts were not maintained in NT-YO blastocysts. In fact, we saw an increase in H3K9me3 compared to IVF and NT-YFP embryos and observed no changes in histone-modifying enzymes directly regulated by OCT4. Other histone methyltransferases, such as *EHMT2, ESET*, and *SUV39H1*, might have been upregulated, perhaps as a compensatory mechanism for the reduction in H3K9me3 in donor cells.

We are in the process of characterizing the dynamics of H3K27me3 during bovine pre-implantation development and its significance during SCNT. So far, we know that H3K27me3 gradually decreases from the MII oocyte stage up to the morula stage (Ross et al. 2008a). The enzyme JMJD3 actively removes methyl groups in a cell-cycle-independent manner; soon thereafter, in parallel with embryonic genome activation, a new pattern of H3K27me3 is reestablished (Canovas et al. 2012). This includes the trimethylation of the H3K27 of one of the two X chromosomes in its entirety. Despite the fact that the levels of several histone methylation marks are the same in IVF and SCNT bovine blastocysts (Wu et al. 2011), we previously reported differences in H3K27me3 levels depending upon the method of egg activation. Using phospholipase C zeta (PLCz) — the protein responsible for oocyte activation at fertilization — causes the methylation levels of SNCT embryos to resemble IVF methylation levels more closely (Ross et al. 2009). Like Wu et al., we saw no differences in the levels of H3K27me3 among all three groups in the present study. Embryos used in our previous study had a higher number of total cells at the blastocyst stage in all groups compared to the present study, reflecting a slight difference in the developmental stages used in the two

studies. We need further research to determine whether H3K27me3 can act as a reliable marker for successful reprogramming. Unfortunately, live tracing of methylation changes is not possible at this time.

Due to the epigenetic alterations we observed in the donor somatic cells when *OCT4* was overexpressed, we decided to measure gene expression in SCNT and IVF blastocysts. We saw no difference in *SOX2, CDX2*, and *IFNT* expression levels, which agreed with our previous results and those described by others (Ross et al. 2009, Yao et al. 2008, Fujii et al 2010, Wang et al. 2011). *NANOG* gene expression was slightly upregulated in SCNT groups versus IVF, as shown previously (Iager et al. 2008). We also assessed the expression of the 5' UTR region of OCT4 to differentiate from the exogenous open reading frame of human OCT4, and we observed that both SCNT groups had lower expression levels than the IVF group. This result conforms with previous observations (Ross et al. 2009, Wang et al. 2011).

Taken together, these gene expression results underscore the fact that single-gene analysis cannot be used as a marker for predicting successful SCNT reprogramming (Somers et al. 2006). Nonetheless, it was recently shown that *XIST* expression increased in SCNT-derived mouse male and female embryos (Nolen et al. 2005, Inoue et al. 2010). Moreover, *XIST* knockout significantly improved development to term in cloned embryos (Inoue et al. 2010). Our results show that both SCNT groups expressed higher levels of *XIST* than IVF embryos, which might indicate that complete reprogramming was not yet achieved. Nevertheless, it will take more studies to further validate the predictive value of *XIST* expression in cows as a marker of successful pregnancy outcome.

In summary, we exogenously expressed OCT4 in bovine donor fibroblasts and generated SCNT-derived blastocysts. Retroviral transfection of OCT4 into adult fibroblasts increased cell division and reduced H3K9 and H3K27 trimethylation levels. However, we appear to have negated our initial hypothesis, that exogenous expression of *OCT4* would facilitate reprogramming, since embryos were generally not different from control SCNT-derived blastocysts. Both SCNT groups differed in some analyzed endpoints from IVF-derived embryos, including increased XIST expression and reduced endogenous *OCT4* expression. Although our data do not suggest that overexpression of *OCT4* will improve overall reprogramming efficiency, it was notable that expression of a single pluripotency factor significantly altered the number of TE cells, moving the SCNT embryos closer to IVF embryos. This suggests that even small changes in histone methylation status and mRNA levels within donor cells might dramatically affect their reprogrammability and the outcome of SCNT. Combining *OCT4* overexpression with other approaches, such as histone deacetylase inhibitors, might further change the outcome of bovine SCNT.

CHAPTER 3

FUNCTIONAL CHARACTERIZATION OF CDX2 DURING BOVINE PRE-IMPLANTATION EMBRYO DEVELOPMENT

3.1 Abstract

Comprehension of events leading to inner cell mass (ICM) and trophectoderm (TE) specification would be of interest to help understand some of the problems observed in embryos produced in vitro. We hypothesized that *CDX2*, a TE specific marker, is not required for formation of the TE in bovine embryos, but it is important for its integrity. Protein localization of CDX2 was characterized by immunocytochemistry, from zygote to blastocyst stage and only the later one displayed CDX2. To further understand the roles of *CDX2* in bovine development we injected siRNA into zygotes. We observed an average of 78% reduction in *CDX2* mRNA expression after 7.5 days of embryo culture, without any detectable protein. However, despite a clear loss of CDX2 protein, embryos were able to form blastocysts at the same rate as non-injected embryos or injected with scramble siRNA. Knockdown of *CDX2* did not cause alteration in the number of TE, ICM or total cells in the blastocyst. Gene expression of developmentally important genes *SOX2, OCT4, NANOG*; and TE markers such as *IFN-T* and *KRT18* were not affected by the reduction in CDX2 levels. Protein localization of SOX2 and OCT4 was also unchanged. Using a functional barrier assay we observed that a higher percentage of siRNA injected embryos had reduced integrity of the TE epithelial barrier. Based on these data, our initial hypothesis was correct, indicating that *CDX2* is not required for TE formation during bovine development; nevertheless, it is important for maintaining TE integrity.

3.2 Introduction

The first observable differentiation event during mammalian embryo development is the specification of trophectoderm (TE) and inner cell mass (ICM). The TE will contribute to the formation of the placenta, while the cells of ICM will differentiate into the three germ layers and form the fetus. Unlike the mouse, trophectoderm and ICM cell specification in cattle has not been thoroughly studied.

CDX2 is a caudal type homeobox transcription factor that is detected in the TE of mouse embryos (Beck et al. 1995). The outer cells of the 16- and 32-cell mouse embryo express CDX2 while inner cells do not. At the blastocyst stage CDX2 expression is then restricted to the TE (Ralston and Rossant, 2005, Suwinska et al. 2008). Trophectoderm specification was thought to start at the 16-cell stage, in which the outer cells would be polarized as an epithelium and become TE; and the inner cells would be apolar and become ICM (reviewed by Sasaki, 2010). Apical cell polarization appears to occur prior to CDX2 expression at 8-cell stage, indicating that CDX2 is not a master regulator of TE specification (Ralston and Rossant, 2008). Recently, mechanisms for this differentiation were observed already in 4-cell embryos, as cells with low rates of nuclear import and export of OCT4 protein undergo asymmetrical cell division, which generates an inner cell and an outer cell (Plachta et al. 2011).

Embryos lacking *CDX2* are able to form TE; however, epithelial integrity of the TE is compromised as embryos displayed altered localization of protein composing tight and adherens junctions (Strumpf et al. 2005). *CDX2* knockdown does not impede TE formation, although it impairs implantation and decidua formation in mouse (Meissner

and Jaenisch, 2006). It was later found that transcription factor Tead4 is upstream of *CDX2* and its absence impairs TE and blastocoel formation (Yagi et al. 2007; Nishioka et al. 2007) This is expressed in both inside and outside cells, but negatively regulated by Hippo signaling components in inside cells, preventing the activation of *CDX2* gene (Nishioka et al. 2009).

We hypothesized that *CDX2* is not required for TE formation in bovine and that it is important for maintenance of TE integrity. Our goal was to evaluate morphological and gene or protein expression changes in bovine blastocysts after knockdown of *CDX2*. In this study, we characterized the expression of CDX2 protein in pre-implantation embryos until blastocyst stage. We microinjected bovine zygotes with siRNA targeting *CDX2* and we assessed blastocyst formation, cell allocation, gene and protein expression and TE permeability. Our results show that CDX2 protein is only present in blastocysts and that its knockdown does not interfere with blastocyst formation and gene expression, however it influences TE epithelial integrity.

3.3 Material and Methods

All reagents were purchased from Sigma Aldrich (St.Louis, MO) unless otherwise stated.

3.3.1 In vitro fertilization and embryo culture

Bovine oocytes were obtained from slaughterhouse-derived ovaries as described (Ross and Cibelli, 2010). Selected oocytes were placed in TCM 199 medium containing 10% FBS, 3 µg/ml LH (Sioux Biochemical, Sioux Center, IA), 3 µg/ml FSH (Sioux Biochemical), 22 µg/ml sodium pyruvate and 25 µg/ml gentamycin (Life Technologies) for 22h. Mature oocytes were transferred into fertilization media supplemented with 20µg/ml heparin. Frozen-thawed semen was submitted to Percoll gradient selection and $1x10^6$ sperm cells/ml were added to the medium containing the oocytes. Fertilization was carried for 18h at 38.5°C and 5%CO2 in high humidity. Presumptive zygotes had the cumulus cells removed and were cleaned from excessive sperm using HH medium. A group of uninjected control zygotes were placed directly into 90µl drops of KSOM media (Millipore, Concord Road, Billerica, MA) under oil and cultured at 38.5°C in high humidity. At day 3 of culture cleavage rates were assessed and 5% of FBS was added to the culture drops. The zygote, 2-cell, 4-cell, 8-cell, 16-cell-stage embryos were collected respectively at 16h, 30h, 40h, 52h and 72h post insemination. Morulas were collected at day 5 and blastocysts at day 7.5 post insemination, when blastocyst rates, over total oocyte number, were obtained.

3.3.2 siRNA synthesis

Target sequences for bovine *CDX2* (Gene bank accession: NM_001206299.1) were obtained using the software Target Finder from Ambion (no longer available). Sense and antisense oligonucleotides were designed using siRNA Template Design

Tool software (no longer available) from Ambion and are described in Table 3.1. We used Ambion Silencer siRNA Construction Kit (Life Technologies, Grand Island, NY) to synthesize three different siRNAs against different exons of bovine *CDX2* and a Scramble (SCR) siRNA that would not target any other bovine gene as verified by NCBI BLAST online resource.

3.3.3 Zygote Microinjection

Groups of denuded zygotes were placed in HH medium drops supplemented with 20% of FBS under mineral oil. Microinjection was performed as described previously (Ross et al. 2008b). Briefly, a microinjection needle containing either 25μM CDX2 siRNA or SCR siRNA with 2mg/ml Texas Red-labeled Dextran 10000 (Life Technologies) was used to partially aspirate zygotic cytoplasm until the membrane was broken and penetrated. A volume of 6-8pl was injected in each zygote that were then placed into KSOM drops and cultured as described above. A total of 12 manipulations (oocyte batches) were performed.

3.3.4 Quantitative RT PCR

Pools of 4 to 10 blastocysts per group were placed in 20μl of extraction buffer from PicoPure RNA extraction kit (Applied Biosytems, Life Technologies), incubated 30 minutes at 42°C and stored at -80°C until processing in a total of 5 replicates (oocyte batches). Samples were thawed in ice and RNA was extracted according to manufacturer protocol, including a step for incubation with DNase (Qiagen, Hilden,

Germany). Reverse transcription reaction was performed using Superscript II Reverse Transcriptase kit (Invitrogen) with 250ng of random primers (Promega, Madison, WI) following manufacturer instructions. Complementary DNA samples were diluted to 2µg/ml after quantification with Nanodrop spectrophotometer (Thermo Scientific, Rochester, NY). Quantitative PCR reactions were set in duplicates for each sample with SYBR Green 2X PCR Master Mix (Applied Biosystems), 2µl of cDNA and performed using ABI 7000 Detection System. Thermal cycle settings were 40 cycles of 95°C for 15s and 60°C for 60s. The H2A gene was used as housekeeping for normalization of target genes expression. Primers used are described in Table 3.1.

3.3.5 Immunocytochemistry

All solutions were prepared with PBS. Embryos were washed 3 times in PBS and fixed with 4% paraformaldehyde solution for 20 minutes. Then, d7.5 blastocysts were washed 3 times in 0.1% Triton X-100 solution and permeabilized for 15 minutes with 0.5% Triton X-100 solution. Non-specific epitope blocking was performed using 0.1% Triton X-100, 1% BSA and 10% horse serum for 1 hour at room temperature. Embryos were incubated with CDX2 primary antibody (1:50; sc166830, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature. Incubation with SOX2 or OCT4 (1:200, sc17320 and sc8628 respectively, Santa Cruz Biotechnology) primary antibodies were performed overnight at 1:200 at 4°C in 0.1% Triton X-100 and 1% BSA solution. Embryos were then washed 3 times for 15 minutes in 0.1% Triton X-100 solution. Incubation with anti- anti-goat secondary antibody (1:400, Jackson

Immunoresearch, West Grove, PA) followed for 1 hour at room temperature in the dark. Embryos were then washed 3 times for 15 minutes in the dark and mounted on slides with Prolong Gold Antifade Reagent with DAPI (Invitrogen, Life Technologies). Blastocysts were visualized using an inverted spinning-disk confocal microscope and stacks of pictures were obtained using Metamorph software (Molecular Devices, Sunnyvale, CA).

3.3.6 Differential cell staining

Differential staining of blastocysts was performed as described previously (Tang et al. 2009) with minor modifications. Briefly, embryos were incubated with 1% Triton x-100 in PBS containing 100µg/ml propidium iodide for 40s. Blastocysts were fixed overnight in absolute ethanol with 25µg/ml Hoechst 33342 at 4°C and analyzed at an inverted fluorescence microscope (Nikon TE-2000, Tokyo, Japan). Three replicates were performed, with 11 non-injection control embryos, 11 SCR injected and 12 siRNA3 injected blastocysts.

3.3.7 Functional Barrier Assay

Trophectoderm permeability was tested as described before (Moriwaki et al. 2007) with minor modifications. Day 7.5 blastocysts were incubated with 1mg/ml of FITC labeled Dextran 4 kDa in KSOM medium for 10 minutes and then washed in HH medium 9 times. Green fluorescence inside blastocoel was observed at an inverted

fluorescence microscope (Nikon) and those embryos were considered positive. A person blind to the treatments evaluated the images and recorded the number of positive and negative embryos.

3.3.8 Statistical Analysis

Data were analyzed using SAS 9.3 software (SAS Institute, Cary, NC). Treatments, as control, SCR and siRNA, were considered independent variable. Cell number was considered dependent variable and data were analyzed by ANOVA using PROC Mixed, with Tukey's adjustment as post-hoc test for comparison of means. Each batch of oocyte was considered as random effect. Cleavage rates, blastocyst rates and were also considered dependent variables and analyzed by ANOVA using PROC GLM, with Tukey's adjustment as post-hoc test for comparison of means. Quantitative PCR data was analyzed using PROC MIXED of SAS as described previously (Steibel et al. 2009). Data from quantitative PCR is presented in graphs with Log2 distribution and for ease of understanding the fold change is used in the text. Dependent variable for permeability assay had a binary distribution, positive or negative, and was analyzed using PROC GLIMMIX, using treatment as dependent variable in the model and batch as random effect. Statistical significance for all tests was considered if $p \leq 0.05$.

Table 3.1 – Bovine specific primer sequences

Primer Name	Primer Sequences (5' to 3')	Reference
CDX2 siRNA 1	AAGGACGTGAGCATGTATCCCCCTGTCTC AAGGGATACATGCTCACGTCCCCTGTCTC	NM_001206299
CDX2 siRNA 2	AACCAGGACGAAAGACAAATACCTGTCTC AATATTTGTCTTTCGTCCTGGCCTGTCTC	NM_001206299
CDX2 siRNA 3	AAGGATGACATTCTCCTAGATCCTGTCTC AAATCTAGGAGAATGTCATCCCCTGTCTC	NM_001206299
CDX2	TGGGCAGCCAAGTGAAAACCAGG GCGGCCAGTTCGGCTTTCCT	NM_001206299
IFN-T	ACAGTGACTGCGCCTGGGA GGTGATGTGGCATCTTAGTCAGCG	ENSBTAT00000048583
KRT18	AGGTGAGGAGCCTGGAGGCG TCTGCAGAACGATGCGGGCG	NM_001192095
SOX2	AGCGCATGGACAGCTACGCG ATGGGCTGCATCTGAGCGGC	NM_001105463
OCT4	TACTGTGCGCCGCAGGTTGG GCTTTGATGTCCTGGGACTCCTCA	NM_174580
NANOG	TCCAGCAAATGCAAGAACTTTC TTACATTTCATTCTCTGGTTCTGGAA	NM_001025344
EOMES	CGCGCCCACGTCTACCTGTG GGGTCCGCCAGCACCACTTC	NM_001191188
ELF5	TAAATCAGAAGCCCTGGCGAAGA ACATGAGCTGGATGATGGAGCA	Smith et al. 2010, NM_001024569

3.4 Results

3.4.1 Temporal and spatial localization of CDX2 protein in early embryos

In order to verify the dynamics of CDX2 protein in the bovine embryos we collected in vitro fertilized embryos at different developmental stages, from zygote to blastocyst. These embryos were fixed and submitted to immunocytochemistry for detection of CDX2 protein. While embryos from zygote through morula stage did not display CDX2 protein staining, TE cells in blastocysts did (Figure 3.1).

3.4.2 Validation of siRNA injection

Knockdown of *CDX2* during bovine development would allow testing the hypothesis that *CDX2* is not required for TE formation. We designed and produced three siRNA that targeted different exons of the bovine *CDX2* gene. These siRNAs were injected into in vitro fertilized zygotes that were cultured until blastocyst stage, when CDX2 protein was detectable. Blastocysts were then collected for RNA isolation, which was subsequently reverse transcribed into cDNA and used in quantitative PCR. Of the 3 siRNAs used, siRNA1 did not reduce *CDX2* expression, siRNA2 reduced 54% of *CDX2* gene expression and siRNA3 reduced 78% when compared to non-injected controls, while Scramble siRNA (SCR) reduced only 10% of *CDX2* expression (Figure 3.2A). Due to its higher efficiency, we selected siRNA3 to use on the remaining experiments. We further injected zygotes with SCR and siRNA3 and fixed blastocysts for CDX2

immunocytochemistry. Non-injected controls and SCR injected embryos displayed CDX2 protein while siRNA3 injected ones were negative, indicating its successful knockdown (Figure 3.2B).

3.4.3 Development rates and differential cell counting

We assessed cleavage and blastocyst formation rates in non-injected controls, SCR and siRNA3 injected embryos. Cleavage rates were reduced in both injected groups compared to controls (Figure 3.3A). We attributed this to the injection procedure itself since lysed structures were observed after injections with both, SCR and siRNA3. Blastocyst rates and overall morphology were not different among the three groups (Figure 3.3A and 3.3B). We also assessed cell number and allocation in blastocysts by differential staining of ICM and TE cells. No differences were observed among all groups in regards to ICM, TE, total cell number and ratio of TE cells over total cell number (Table 3.2).

Table 3.2 -Cell allocation of day 7.5 IVF blastocysts as determined by propidium iodide and bisbenzimide differential staining. ICM = inner cell mass; TE = trophectoderm

	ICM cells	TE cells	Total cells	TE/Total (%)
CTRL	38.20 ± 2.87	66.64 ± 6.98	98.43 ± 6.82	64.02 ± 2.80
SCR siRNA	33.28 ± 2.92	80.64 ± 6.98	110.82 ± 7.48	70.36 ± 2.78
Cdx2 siRNA	40.58 ± 2.67	72.09 ± 6.69	105.03 ± 5.83	64.45 ± 2.74

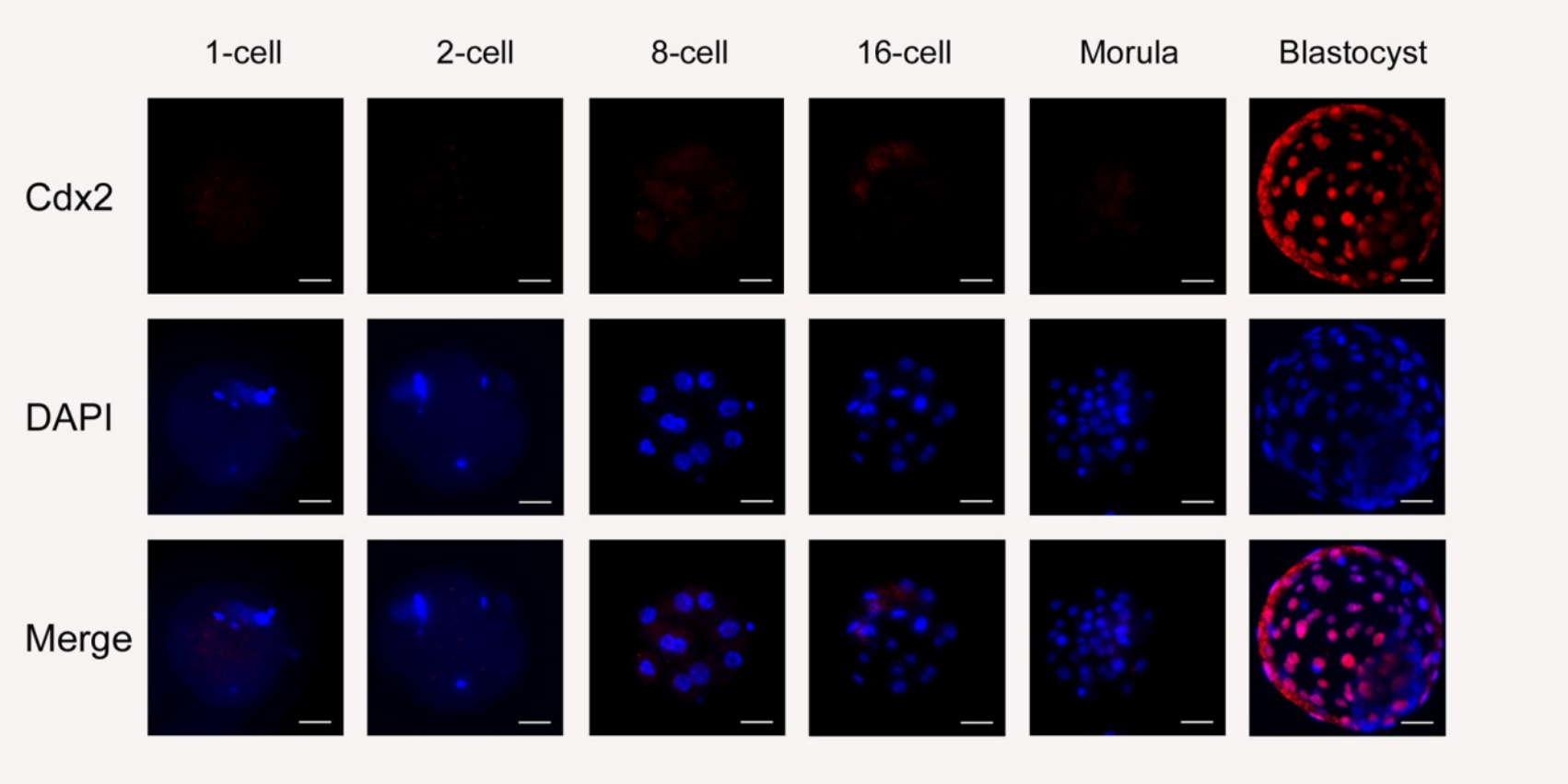

Figure 3.1 – Temporal and spatial characterization of CDX2 during bovine pre-implantation development.

Scale bar is equal to 40μm.

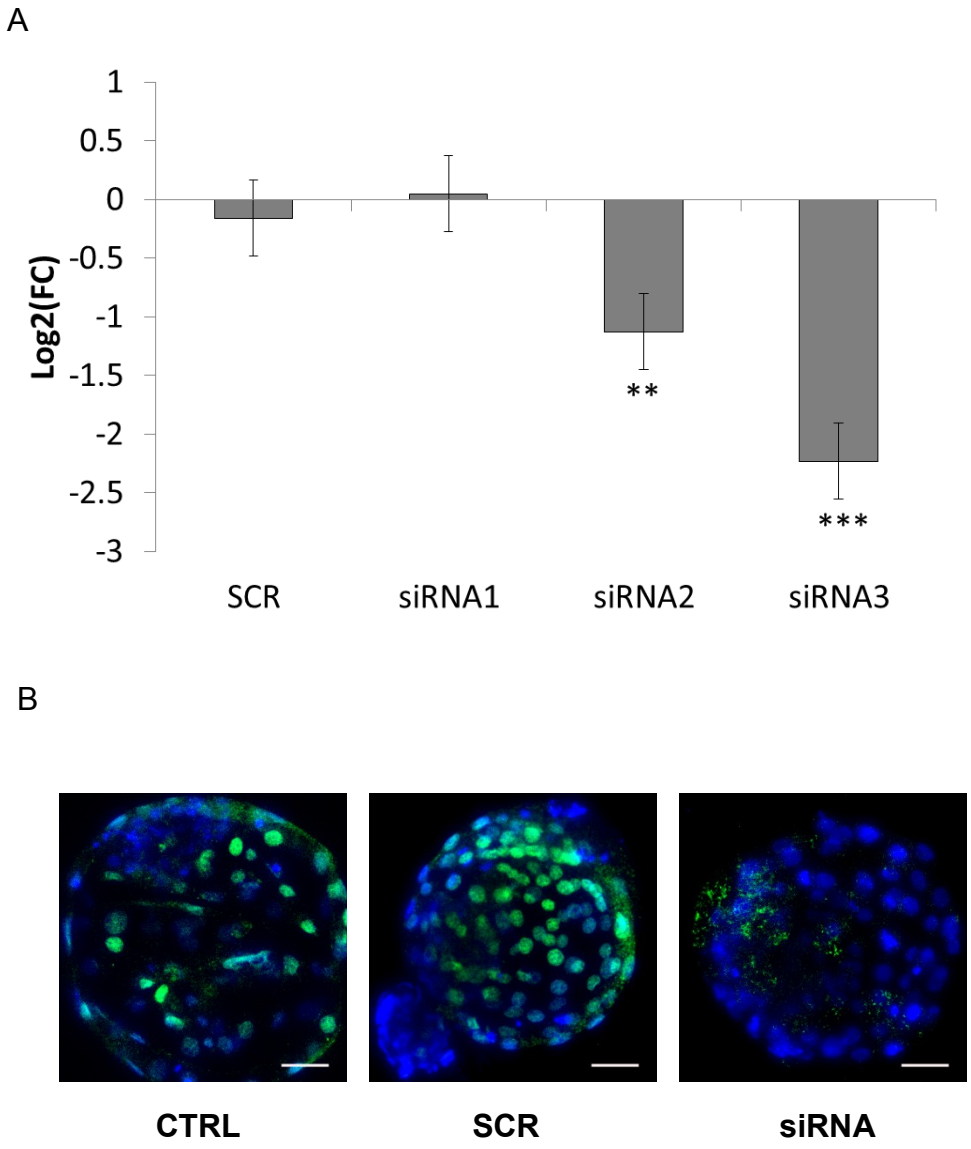

Figure 3.2 – Validation of *CDX2* siRNA. A) Quantification of *CDX2* gene expression in D7.5 after siRNA injection, relative to non-injected embryos. B) CDX2 protein is downregulated in D7.5 blastocysts after siRNA injection. Asterisks indicate significant statistical difference, **$p \leq 0.01$, ***$p \leq 0.001$; n = 5, pools of 4 to 10 embryos. Scale bar is equal to 40µm.

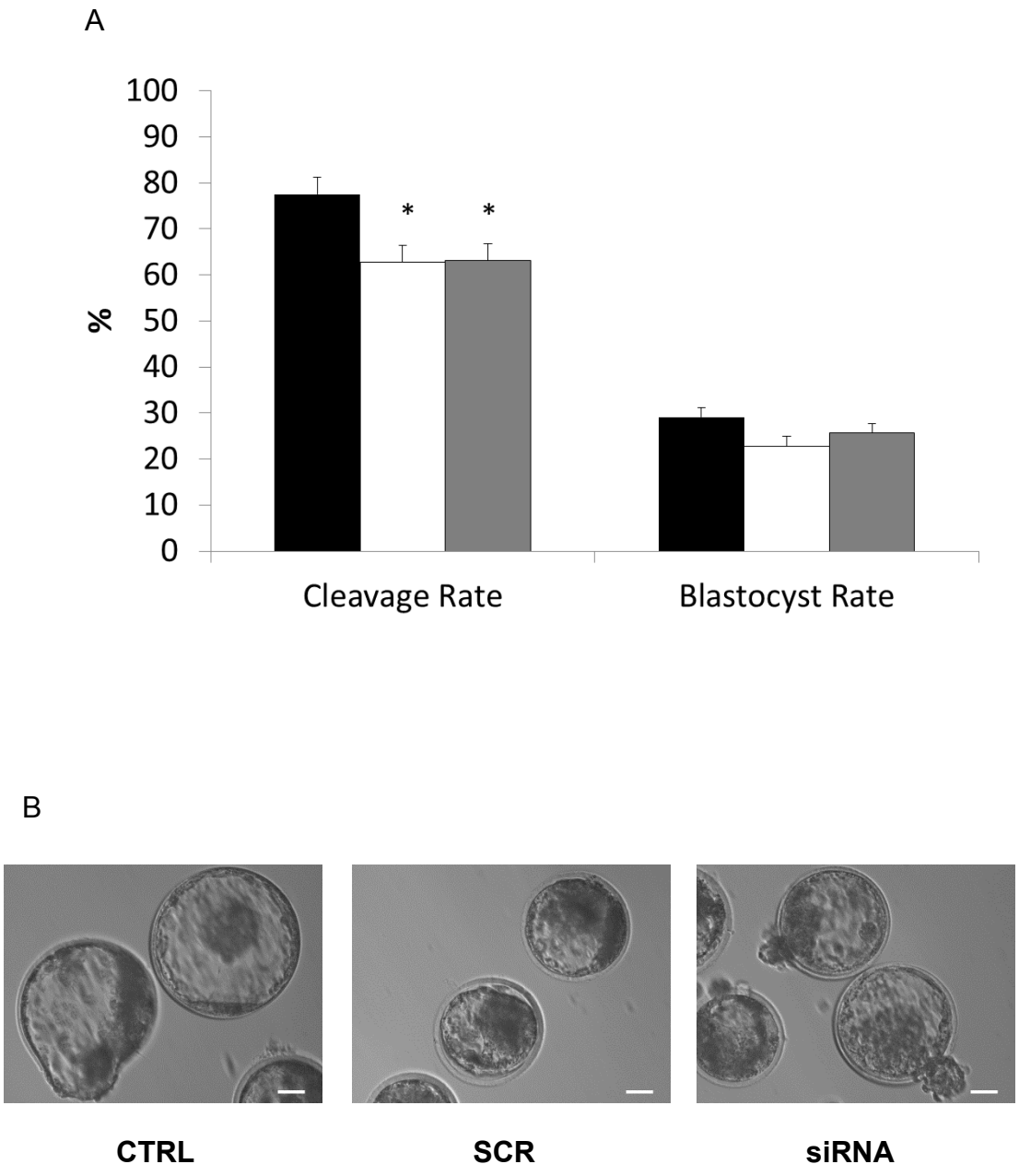

Figure 3.3 – Blastocyst formation after *CDX2* knockdown. A) Cleavage and blastocyst rates of Control (black bars), Scramble (open bars) and siRNA groups (grey bars). B) Phase contrast images of blastocysts from all three groups. Asterisk indicates significant statistical difference ($p \leq 0.05$); n=12. Scale bar is equal to 40μm.

3.4.4 Quantitative PCR gene expression analysis

Five replicates of injected zygotes or non-injected control were cultured until day 7.5 when blastocysts were collected for RNA isolation and subsequent cDNA synthesis. We used qPCR analysis to verify if the knockdown of *CDX2* would cause changes in gene expression of blastocysts. TE-related genes *IFN-T* and *KRT18* were not changed after CDX2 knockdown (Figure 3.4A) and the same was observed for pluripotency related genes *SOX2, OCT4* and *NANOG* (Figure 3.4B). *EOMES* and *ELF5*, other TE specific genes, were tested but amplification signal was too low in all blastocyst samples tested and we considered these genes not expressed at this stage. Data was normalized using housekeeping gene H2A.

3.4.5 Immunofluorescence of OCT4 and SOX2

We performed immunocytochemistry in blastocysts to observe if CDX2 knockdown would interfere with the spatial localization of the pluripotency associated markers OCT4 and SOX2. We observed that OCT4 is expressed in the whole blastocyst regardless of siRNA or SCR injection, as in non-injected control (Figure 3.5A). We had previously observed that SOX2 protein is limited to the ICM of bovine blastocysts (Goissis and Cibelli, 2012 unpublished observations), which coincides with the embryonic stage in which protein expression of CDX2 is exclusively localized in the TE. We speculated that CDX2 could be responsible for the downregulation of SOX2 protein in the TE; however *CDX2* knockdown did not alter the localization of SOX2 protein in bovine embryos (Figure 3.5B).

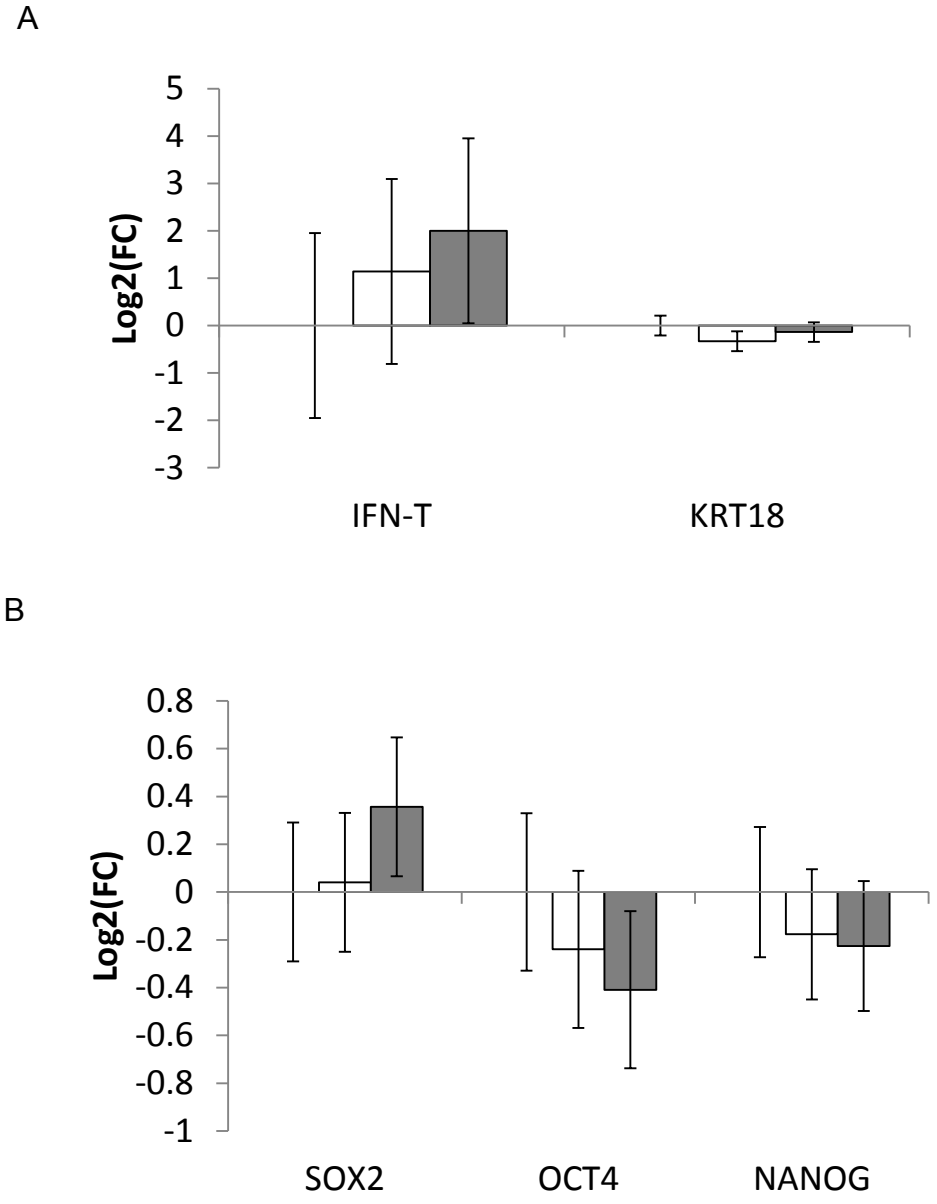

Figure 3.4 – Gene expression analysis in D7.5 blastocysts. A) Quantification of TE-related genes *IFN-T and KRT-18* in Control (set to zero), Scramble (open bars) and siRNA (grey bars) groups. B) Quantification of developmentally important genes *SOX2, OCT4* and *NANOG* in Control (set to zero), Scramble (open bars) and siRNA (grey bars) groups. Asterisk indicates significant statistical difference; n = 5, pools of 4 to 10 embryos.

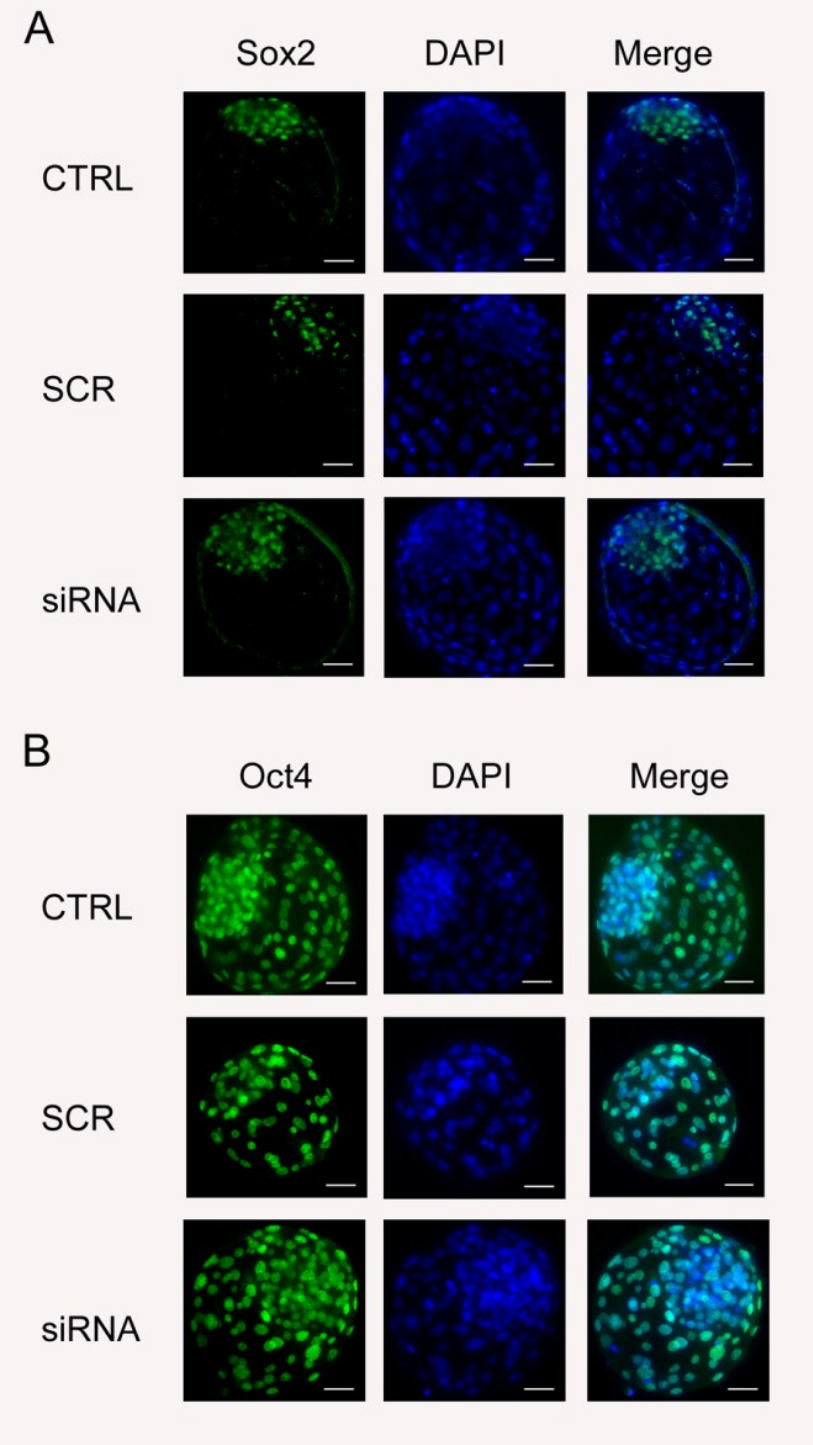

Figure 3.5 - Immunocytochemistry of D7.5 blastocysts. A) SOX2 is restricted to the ICM in all groups; B) OCT4 is present in both ICM and TE in all groups. Scale bar is equal to 40μm.

3.4.6 Permeability assays

Blastocysts from seven replicates were incubated with Dextran-FITC in order to verify integrity of the blastocoels. We performed a statistical analysis on binary data and observed that significantly more embryos injected with *CDX2* siRNA had Dextran-FITC inside of the blastocoels than in non-injected controls and SCR injected groups (Figure 3.6). A total of 53.5% (15/28) of siRNA injected embryos were positive for Dextran-FITC, while 26.4% (9/34) and 26% (6/23) were positive for non-injected controls and SCR groups respectively.

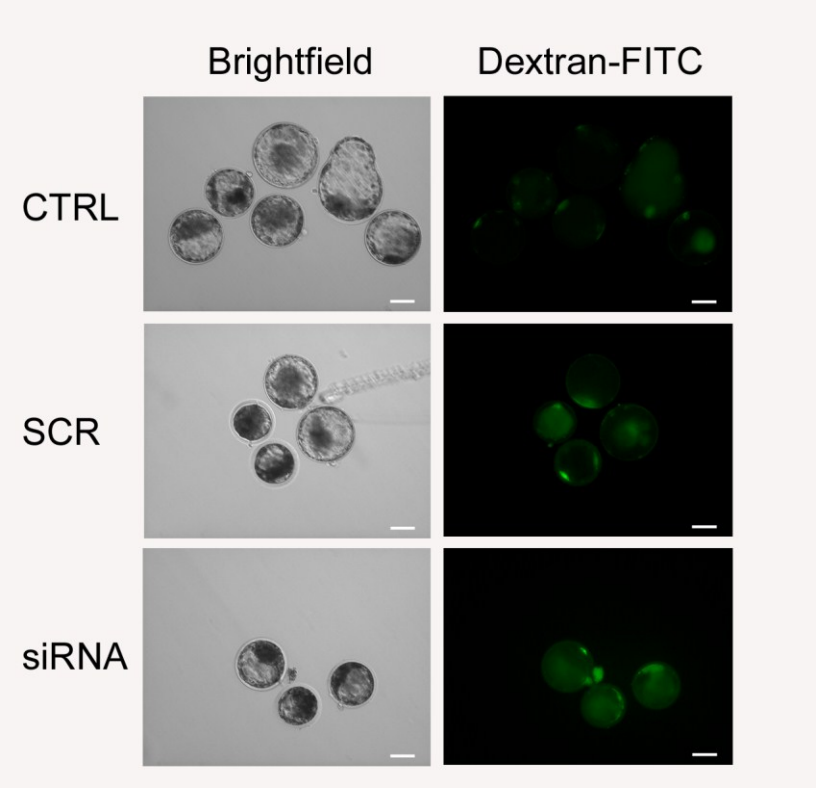

Figure 3.6 – Functional barrier assay. A higher percentage of embryos injected with CDX2 siRNA displayed a defective TE as demonstrated by an increased permeability allowing the extracellular Dextran-FITC to localize inside the blastocoel cavity. Scale bar is equal to 40μm.

3.5 Discussion

Unlike the mouse, studies of the first differentiation events in the early pre-implantation bovine embryos are limited. Knowledge obtained in this area could help improve survival of *in vitro* produced embryos. Mouse embryos have been extensively studied and serve as a model for human early development; however, this is being recently called into question as bovine embryos could be a more suitable model for human embryo development (Berg et al. 2011, Rossant, 2011). CDX2 is a transcription factor that is long been used as a trophectoderm marker and has been shown to be involved in the proper function of the mouse TE (Strumpf et al. 2005, Meissner and Jaenisch, 2006, Wu et al. 2010). In this study, we tested the hypothesis that *CDX2* is dispensable for blastocyst formation as well as TE function in bovine embryos.

Temporal and spatial localization of CDX2 protein during early bovine embryo has only been investigated at the blastocyst stage, where it was shown to be limited to TE cells (Kuijk et al. 2008). We thoroughly analyzed all stages of bovine pre-implantation development for the presence of CDX2 protein and indeed found that from the zygote to morula stage, CDX2 is absent; unlike the mouse embryo in which CDX2 protein can be observed as early as the 8-cell embryo (Dietrich and Hiiragi, 2007, Ralston and Rossant, 2008) and in the outer cells of the 16-cell embryo (Strumpf et al. 2005, Suwinska et al. 2008). In the mouse, CDX2 was shown to act downstream of Tead4, in agreement with the observation that cell polarization occurs before CDX2 expression (Yagi et al. 2007; Nishioka et al. 2007, Ralston and Rossant, 2008). Our

observations together with the knowledge available in the mouse led us to believe that CDX2 would be dispensable for TE establishment in bovine embryos.

To further prove our hypothesis, we injected in vitro fertilized bovine zygotes with siRNA targeting bovine *CDX2* mRNA. The designed siRNAs were tested and one of proved to be 78% effective in the reduction of *CDX2* mRNA expression, leading to the absence of detectable protein by immunocytochemistry. Scramble (SCR) injected zygotes were used as controls as well as non-injected embryos. We observed a decrease in cleavage rates in both injected groups which was probably due to lysis or death caused by the injection itself however CDX2 protein expression in these groups was unaffected. Development to blastocyst was similar among all three groups. Our results are in line with those observed in mouse (Strumpf et al. 2005, Meissner and Jaenisch, 2006, Wu et al 2010), where depletion of *CDX2* does not impair the formation of TE and development to the blastocyst stage.

Knockdown of *CDX2* did not interfere with mRNA expression of TE related genes IFN-T and Krt18 or pluripotency associated genes SOX2 and Nanog. OCT4 mRNA levels were also unchanged as previously reported (Berg et al. 2011). Unlike the mouse, bovine embryos express most pluripotency markers such as OCT4, NANOG, SSEA4, TRA-1-60 and TRA-1-81 in the TE of blastocysts (Kirchhof et al. 2000; Cao et al. 2009; Muñoz et al. 2008). In the mouse TE, CDX2 binds to the *OCT4* promoter and recruits co-repressors that downregulate *OCT4* expression (Niwa et al. 2005, Wang et al. 2009). It was recently shown that the *OCT4* promoter in bovine have a different CR4 promoter region that does not allow CDX2-mediated repression and it is shared with other species as human, horse, dog and rabbit. This same study also showed that

somatic cell nuclear transfer-derived embryos containing siRNA vectors targeting *CDX2* were able to form blastocysts and even develop for 15 days after transferred into recipient (Berg et al. 2011).

We found that OCT4 protein localization was unchanged after knockdown of *CDX2.* SOX2, another pluripotency-related transcription factor, is specifically localized in the ICM of bovine blastocysts (Goissis and Cibelli, unpublished data) allowing us to speculate that CDX2 could be responsible for silencing *SOX2* in the TE and thus embryos injected with CDX2 siRNA would display expression of SOX2 in the TE. Our data revealed that SOX2 protein is confined to the ICM even after CDX2 knockdown. Our data clearly uncouples CDX2 expression from the localization and expression of OCT4 and SOX2.

CDX2 was shown to be responsible for maintaining TE integrity (Strumpf et al. 2005, Meissner and Jaenisch, 2006). We observed that knockdown of *CDX2* caused embryos to be more susceptible to permeabilization with Dextran-FITC solution, indicating a defective epithelial barrier, probably due to defective tight junctions (Wu et al 2010). Noteworthy, not all embryos showed a faulty TE, indicating that at this point of bovine development *CDX2* is not the sole responsible for TE integrity.

In summary, we showed that CDX2 protein temporal and spatial localization in early bovine development is limited to the TE of blastocysts. We also showed that knockdown of *CDX2* in bovine embryos is compatible with blastocyst development; corroborating our initial hypothesis that *CDX2* is not required for TE formation in bovine embryos. Gene expression in these blastocysts was unchanged as well as protein localization of OCT4 and SOX2. Also, *CDX2* knockdown produced more embryos that

had reduced functional epithelial barrier, confirming our hypothesis that *CDX2* plays a role for maintenance of TE integrity.

Our results show that *CDX2* have a similar role in the TE as in the mouse, despite differences in the onset of protein expression. While the precise mechanism for the onset of bovine TE differentiation remains to be elucidated, CDX2 seems to be uncoupled from the expression and localization of key pluripotency transcription factors. Our results give credence to the notion that unlike the mouse, the role of CDX2 in bovine embryos is much later in development. These findings, together with those of previous studies, corroborate the idea that TE lineage specification is not conserved among mammals and that the bovine could also be used as a model for human pre-implantation development. In addition, monitoring CDX2 expression could be used to assess TE integrity of bovine *in vitro* produced embryos.

CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF SOX2 IN BOVINE PRE-IMPLANTATION EMBRYOS

4.1 Abstract

Establishment of pluripotent embryonic stem cells (ESCs) from bovine embryos has been so far, unsuccessful. The lack of reliable pluripotency markers is also an important drawback when attempting to derive these cells. In this study, we aimed to find genes upregulated in the inner cell mass (ICM) of bovine blastocysts, we then selected *SOX2* for further characterization. Spatial and temporal localization of SOX2 protein revealed that its expression starts at the 16-cell stage and is then restricted to the ICM of blastocysts. To study *SOX2* role during bovine early embryo development, we designed and siRNA targeting *SOX2* mRNA. At first, zygotes were injected and blastocyst rate declined when compared to non-injected or Scramble injected controls. When only one blastomere of a two-cell embryo was injected with SOX2 siRNA, we observed similar development rates. Daughter cells of the injected blastomere were tracked by TRITC fluorescence and found to contribute to the ICM along with cells that lacked SOX2. Gene expression analysis revealed a decrease in *SOX2* and *NANOG* gene expression in siRNA injected embryos; however *OCT4* expression was unchanged. We conclude that SOX2 shows an exclusive ICM localization in bovine blastocysts, its downregulation negatively impact pre-implantation development;

however it does not affect ICM formation if injected in one blastomere of a 2-cell embryo.

4.2 Introduction

Pluripotent embryonic stem cells (ESCs) were isolated three decades ago in the mouse (Evans and Kaufman, 1981; Martin, 1981). These cells have been extensively used to produce transgenic mice by injection in the blastocyst (Rossant et al. 1993) and have also shown quite effectively as nuclei donor to improve the outcome of somatic cell nuclear transfer (Rideout et al. 2001). Obtaining ESCs in bovine species would be of great interest to produce transgenic and cloned animals. Several attempts have been made and no bona fide pluripotent ESCs were reported to be derived (Talbot et al. 1995; Cibelli et al. 1998; Saito et al. 2003; Wang et al. 2005; Keefer et al. 2007; Telugu et al. 2010). One obstacle is that the same pluripotency markers established in mouse and human, seem to be ineffective in pointing out bona fide bovine ESCs.

OCT4 (Scholer et al., 1990), *NANOG* (Chambers et al. 2003) and *SOX2* (Avilion et al. 2003) are transcription factors expressed in the inner cell mass (ICM) of mouse blastocysts. These genes are excellent markers for mouse and human ESCs or induced pluripotent cells (Reubinoff et al. 2000; Takahashi et al., 2007). In addition, the cell surface pluripotency marker, SSEA-1, which recognizes mouse ICM (Solter and Knowles, 1978) has shown to be effective on the characterization of mouse ESCs (Martin, 1981). Curiously, SSEA-1 does not mark human ESCs, which they do share with the mouse SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Thomson et al. 1998)

indicating that there are differences across species. These differences point to the fact that while both mouse and human ESCs can display pluripotent characteristics of indefinite self-renewal and differentiation capacities, they differ in their 'pluripotency signature'. Interestingly, in bovine, OCT4, NANOG, SSEA4, TRA-1-60 and TRA-1-81 were shown to be expressed in both ICM and trophectoderm (TE) of bovine blastocysts (Kirchhof et al. 2000, Muñoz et al., 2008, Cao et al. 2009). In the human pre-implantation embryo, OCT4 is not restricted to the ICM (Caufmann et al. 2005, Chen et al. 2009, Roode et al. 2012); however SOX2 and NANOG are expressed in the ICM only (Caufmann et al. 2009, Roode et al. 2012).

Little is known about the expression of SOX2 in the bovine embryo. SOX2 is part of the SOX protein family, which comprises 20 genes containing a DNA-binding HMG domain, both nuclear import and export signals and act as transcription factors (Wegner, 2009; Kormish et al. 2010). In the mouse, *SOX2* mRNA is initially expressed in some cells of the morula and then restricted to the ICM of blastocysts; however, maternal protein is detected in oocytes, and throughout the pre-implantation embryo until the blastocyst stage (Avilion et al. 2003). *SOX2* is required for pluripotency maintenance in mouse ESCs (Masui et al. 2007) and is also one of four factors used to induce pluripotency in differentiated cells (Takahashi and Yamanaka, 2006). SOX2 is also expressed in adult stem cells of several tissues, including testis stem cells that can reconstitute spermatogenesis in infertile mice (Arnold et al. 2011). We hypothesized that SOX2 is a pluripotency marker in bovine embryos and also that it is required for formation of the ICM in blastocysts.

In this study we evaluated the differences in gene expression between ICM and TE by comparing expression of isolated TE with whole blastocysts. We also characterized the temporal and spatial expression of SOX2 protein during bovine embryo development and then we performed knockdown experiments to assess the requirement for SOX2 in bovine pre-implantation development. Our results show that *SOX2* mRNA is expressed in higher levels in the ICM of in vitro derived blastocysts. We also show that SOX2 protein is initially detected at 16-cell stage and continues to be expressed in blastocysts where it is confined to the ICM. Sox2 downregulation will negatively impact embryo development and the expression of other pluripotency genes, highlighting its role in bovine pre-implantation development; however, cells depleted of SOX2 are still able to contribute to the ICM.

4.3 Material and Methods

All reagents were purchased from Sigma Aldrich (St.Louis, MO) unless otherwise stated.

4.3.1 In vitro production of embryos

Bovine oocytes were obtained by aspirating ovaries collected at a commercial slaughterhouse as described (Ross and Cibelli, 2010). Cumulus-oocyte complexes were selected according to their quality and placed into maturation media, which consisted of medium 199 supplemented with 10% FBS, 3 µg/ml LH (Sioux Biochemical,

Sioux Center, IA), 3 µg/ml FSH (Sioux Biochemical), 22 µg/ml sodium pyruvate and 25 µg/ml gentamycin (Life Technologies). After 22h oocytes were submitted to either in vitro fertilization (IVF) of parthenogenetic activation (PA). IVF was carried in TALP-based fertilization media (Parrish et al. 1986) supplemented with 20µg/ml heparin. After thawing live sperm cells were selected by percoll gradient and added to the well containing the oocytes at a concentration of $1x10^6$ sperm cells/ml. Fertilization was carried for 18h at 38.5°C and 5%CO2 in high humidity. After 18h, presumptive zygotes were stripped of cumulus after 3 min of vortexing and washed in HH media before being placed into microdrops of KSOM + AA medium (Millipore, Concord Road, Billerica, MA) under oil and cultured at 38.5°C in high humidity. At day 3 of culture the cleavage rates were assessed and medium was supplemented with 5% FBS. At day 7.5 the blastocyst rates were assessed. For PA, matured oocytes had their cumulus cells removed by 5 min of vortexing with 1mg/ml of hyaluronidase. Oocytes were then washed in HH and activated by 4 min exposure to 5mM Ionomycin (EMD Biosciences, La Jolla, CA) in HH medium, followed by incubation in 200mM DMAP in KSOM at 38.5°C and 5%CO2 in high humidity. After 4h, oocytes were washed in HH and placed into KSOM drops and in vitro cultured as described above.

4.3.2 Gene expression of Trophectoderm and Whole Embryos

Three replicates of in vitro-fertilized blastocysts were collected and divided in 2 groups. A minimum of 7 and a maximum of 11 embryo samples per group were obtained. One of the groups consisted of intact embryos and the other consisted of the

TE separated from the ICM using a sharp splitting blade (Bioniche Animal Health, Belleville, ON, Canada). Intact embryos and TE were placed in Extraction Buffer from PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA), incubated at 42°C for 30 minutes, flash frozen in liquid nitrogen and kept at -80°C until RNA extraction. RNA isolation was carried out using PicoPure following manufacturer instructions, including a DNase (Qiagen, Hilden, Germany) incubation step. Reverse transcription reaction was performed using Superscript II Reverse Transcriptase kit (Invitrogen) with 250ng of random primers (Promega, Madison, WI) following manufacturer instructions. Resulting cDNA was diluted to 2µg/ml after quantification with Nanodrop spectrophotometer (Thermo Scientific, Rochester, NY). Quantitative PCR reactions were set in duplicates for each sample with SYBR Green 2X PCR Master Mix (Applied Biosystems), 2µl of cDNA and performed using ABI 7000 Detection System. Thermal cycle settings were 40 cycles of 95°C for 15s and 60°C for 60s. H2A and GAPDH genes were used as housekeeping for normalization of target genes expression by ΔΔCt method (Pfaffl, 2001). Primers used are described in Table 4.1.

4.3.3 Immunocytochemistry

All solutions were prepared with PBS. In vitro fertilized embryos were collected at the zygote, 2-cell, 4-cell, 8-cell, 16-cell-stage respectively at 16h, 30h, 40h, 52h and 72h post insemination. Morulas were collected at day 5 and blastocysts at day 7.5 post insemination. Embryos were fixed using 4% paraformaldehyde for 20 min at room temperature, followed by 3 washes in PBS with 1mg/ml polyvinylpyrrolidone, in which

they were stored at 4°C until further processing. Embryos were permeabilized using 0.5% Triton X-100 solution for 15 minutes and were then placed 1 hour at room temperature in blocking solution consisting of 0.1% Triton X-100, 1% BSA and 10% horse serum. Incubation with primary antibody against SOX2 (1:200, AB5603, Millipore or SC17320, Santa Cruz Biotechnology, Santa Cruz, CA) was carried overnight at 4°C in 0.1% Triton X-100 and 1% BSA solution. Embryos were then washed 3 times for 15 minutes in 0.1% Triton X-100 solution. Incubation with anti-goat secondary antibody (1:400, Jackson Immunoresearch, West Grove, PA) followed for 1 hour at room temperature in the dark. Embryos were then washed 3 times for 15 minutes in the dark and mounted on slides with Prolong Gold Antifade Reagent with DAPI (Invitrogen, Life Technologies). Embryos were visualized using an inverted spinning-disk confocal microscope and stacks of pictures were obtained using Metamorph software (Molecular Devices, Sunnyvale, CA).

4.3.4 SiRNA synthesis and validation

Target sequences for bovine SOX2 gene (Gene bank accession: NM_001105463) were designed using software TargetFinder from Ambion (no longer available). Oligonucleotides for use with Ambion Silencer siRNA Construction Kit (Life Technologies, Grand Island, NY) were designed using the siRNA Template Design Tool online software also from Ambion (discontinued) and are described in Table 1. We synthesized 3 different siRNAs against 3 different positions of the single-exon coded SOX2 mRNA. We also synthesized a Scramble (SCR) siRNA that would not target any

other bovine gene as verified by NCBI BLAST online resource. In order to validate these siRNAs we collected injected day 5 morulas that were submitted to RNA extraction and subsequent quantitative PCR as described above or fixed for SOX2 immunocytochemistry.

Table 4.1 – Bovine oligonucleotides used for PCR

Primer Name	Sequences (5' to 3')	Reference
CDX2	Fwd -TGGGCAGCCAAGTGAAAACCAGG	NM_001206299
	Rev - GCGGCCAGTTCGGCTTTCCT	
FGFR4	Fwd - CTCAAGCCAGAGTTCAATCCCA	NM_001192584
	Rev - AGGACTTCTACCAAGGCCCAGA	
GDF3	Fwd - ATGCACTCCGTCGACCCGGA	XM_001254180
	Rev - AGCATTTACCCCAAGAGCACCCC	
KLF4	Fwd - AGCAGCAACCCGGCTCTCCT	NM_001105385
	Rev - GCGGCCACGGACTCCTGATG	
LIFR	Fwd - TTTGGAACGGTTGTTTTTGCGGG	NM_001192263
	Rev -GCCCTCCAAGGACGTCGGTC	
LIN28	Fwd - AAACTGCCACCGCAGCCCAA	NM_001193057
	Rev - CCTGGGAGCTGGGAGCTTGC	
NANOG	Fwd - TCCAGCAAATGCAAGAACTTTC	NM_001025344
	Rev -TTACATTTCATTCTCTGGTTCTGGAA	
OCT4	Fwd - TACTGTGCGCCGCAGGTTGG	NM_174580
	Rev - GCTTTGATGTCCTGGGACTCCTCA	
REX1	Fwd - AGCGCATGGACAGCTACGCG	NM_001105463
	Rev - ATGGGCTGCATCTGAGCGGC	
RONIN	Fwd - GCTGCTGGCCATGGCTGTCA	NM_001104994
	Rev - ACGGTGGCCTCAGGTCCCTC	
Scramble siRNA	Fwd - AAGCAGGACAGCAGACTAGATCCTGTCTC	
	Rev - AAATCTAGTCTGCTGTCCTGCCCTGTCTC	
SOX2	Fwd - AGCGCATGGACAGCTACGCG	NM_001105463
	Rev - ATGGGCTGCATCTGAGCGGC	
SOX2 siRNA 1	Fwd - AAGCCTTTCCAAAAAATAATACCTGTCTC	NM_001105463
	Rev –AATATTATTTTTTGGAAAGGCCCTGTCTC	
SOX2 siRNA 2	Fwd - AAGAAGGATAAGTACACACTGCCTGTCTC	NM_001105463
	Rev - AACAGTGTGTACTTATCCTTCCCTGTCTC	
SOX2 siRNA 3	Fwd - AAGAAACAGCATGGAGAAAAACCTGTCTC	NM_001105463
	Rev - AATTTTTCTCCATGCTGTTTCCCTGTCTC	

4.3.5 Microinjection of Zygotes and 2-cell embryos

After 18h of incubation for in vitro fertilization, presumptive zygotes were denuded by vortexing in HH medium. They were washed to remove excessive sperm and cumulus cells and placed into microdrops of HH supplemented with 20% of FBS. Microinjection was performed similarly as described previously (Ross et al. 2008). Briefly, 40μM SOX2 siRNA or SCR siRNA with 2mg/ml TRITC-labeled Dextran 70000 was loaded into a 0.8-1.0 μm diameter glass micropipette. Zygote cytoplasm was aspirated until membrane was broken and then a volume of 6-8pl was injected in each zygote. Injected zygotes were placed into KSOM microdrops and cultured as described above. Day 5 morulas were collected for quantitative PCR to validate siRNAs. Blastocyst rate was assessed at day 7.5.

In order to inject one blastomere of a 2-cell embryo, PA-derived embryos were cultured in KSOM until 30h after activation. Injections were carried as above, however a volume of 3-4pl was injected in only one of the cells. Day 5 morulas were collected for immunocytochemistry to verify knockdown of SOX2. Day 7.5 blastocysts were collected for gene expression analysis and immunocytochemistry as described above and also, for differential cell counting.

4.3.6 Differential cell counting

Differential staining of blastocysts was performed as described previously (Tang et al. 2009) with minor modifications. Briefly, embryos were incubated with 1% Triton x-

100 in PBS containing 100µg/ml propidium iodide for 40s. Blastocysts were fixed overnight in absolute ethanol with 25 µg/ml Hoechst 33342 at 4°C and analyzed at an inverted fluorescence microscope (Nikon TE-2000, Tokyo, Japan). Four replicates were performed, with 27 non-injection control embryos, 34 SCR injected and 23 siRNA3 injected embryos.

4.3.7 Statistical Analysis

Data were analyzed using SAS 9.3 software (SAS Institute, Cary, NC). Independent variables were group (whole Blastocyst or TE) and treatments (control, SCR and siRNA). Cell number was considered dependent variable and data were analyzed by ANOVA using PROC Mixed, with Tukey's adjustment as post-hoc test for comparison of means. Each replicate consisted of a different oocyte batch and was included in the model as a random variable. Cleavage rates and blastocyst rates were also considered dependent variables and analyzed by ANOVA using PROC GLM, with Tukey's adjustment as post-hoc test for comparison of means. Quantitative PCR data was analyzed using PROC MIXED as described previously (Steibel et al. 2009). Results in graphs are presented in Log2 distribution and throughout the text the relative fold change is used to facilitate data interpretation.

4.4 Results

4.4.1 Differences of gene expression between trophectoderm and whole embryos

In vitro-derived blastocysts were divided in two groups for RNA isolation, reverse transcription and relative PCR quantification. One of the groups consisted of intact blastocysts (BL) and the other had the TE mechanically isolated by splitting with a sharp blade. We expected with this design to identify genes that were either exclusively or highly expressed in the ICM of bovine blastocysts, as those would be expressed in lower levels or not present in the TE. In this experiment we focused on genes known to be related with pluripotency in mouse and human. Results are summarized in Figure 4.1. We observed different expression of *LIFR*, *KLF4* and *OCT4*, which displayed a trend ($P<0.10$) to be increased in BL samples compared to TE. Genes that showed significant upregulation in the ICM were *LIN28,* 2-fold; *FGFR4*, 4-fold; *NANOG*, 11-fold and *SOX2*, 7-fold. *NANOG* protein was already characterized in the bovine embryo (Muñoz et al., 2008; Cao et al. 2009), thus we decided to explore the roles of *SOX2*. It is important to mention that all genes tested were detected in TE samples albeit at relatively lower levels.

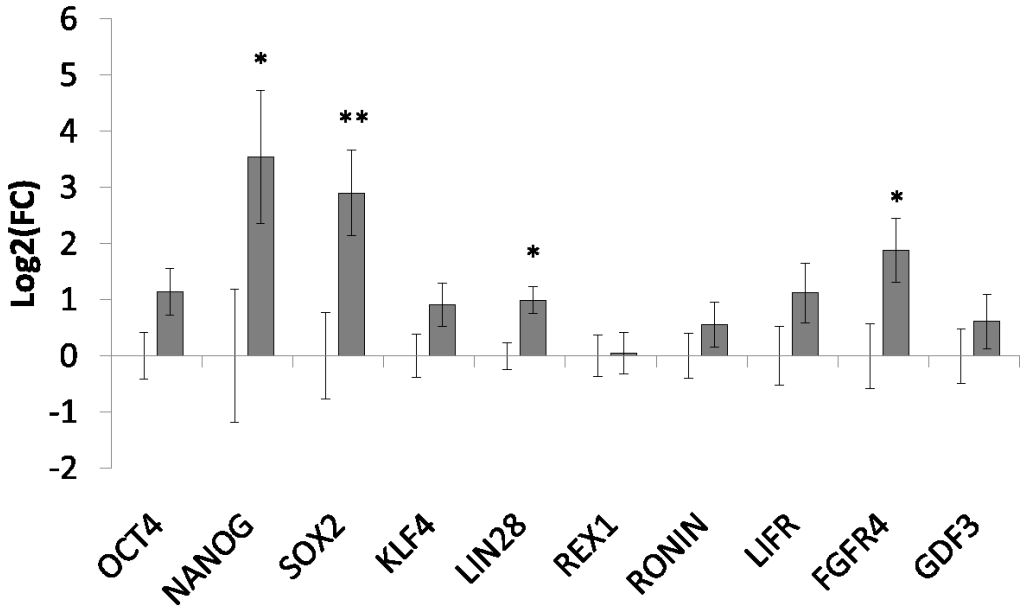

Figure 4.1 - Quantification of pluripotency candidate genes of IVF-derived blastocysts relative to mechanically isolated TE samples. Asterisks indicate significant statistical difference (*p≤ 0.05; **p≤ 0.01); n = 4, pools of 4 to 12 embryos.

4.4.2 Temporal and spatial localization of SOX2 protein,

In order to establish the dynamics of SOX2 protein expression we collected pre-implantation embryos at all stages of development, from zygote to blastocyst, and fixed them for immunocytochemistry. From the zygote to 8-cell stage embryo, we observed no noticeable SOX2 protein expression, however, at 16-cell-stage, SOX2 protein was detected in all cells of most embryos a pattern also observed at the morula stage (Figure 4.2). Curiously, at the blastocyst stage SOX2 positive cells were restricted to the ICM (Figure 4.2), which would characterize SOX2 as the first specific protein marker of bovine ICM. We used two different antibodies at the blastocyst stage to ensure that the staining was indeed of SOX2 protein (Figure 2), which is mostly nuclear within the cells.

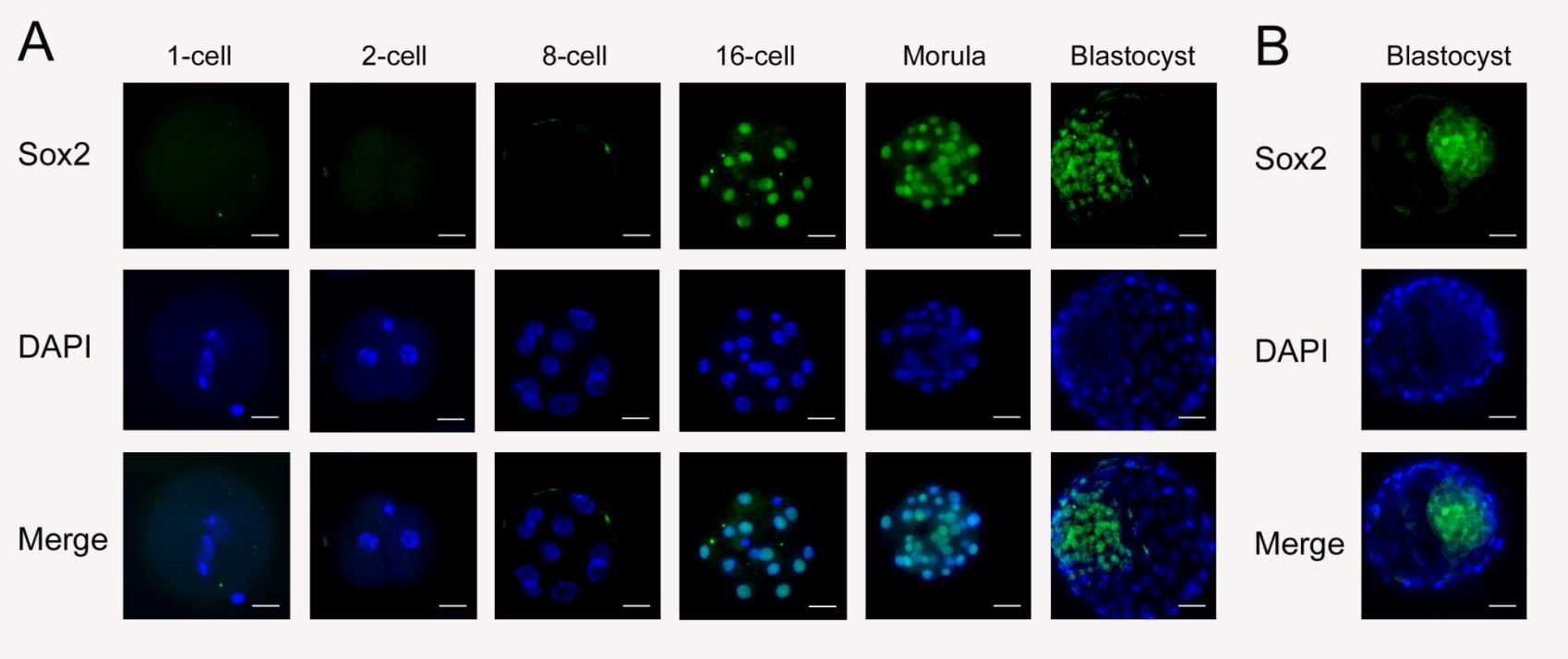

Figure 4.2 –Representative images of SOX2 immunocytochemistry throughout bovine embryo development: A) using a SOX2 antibody from SantaCruz (sc17320). B) using a SOX2 antibody from Millipore (AB6503). Scale bar is equal to 40μm.

4.4.3 SOX2 siRNA injection

We designed three different siRNAs against bovine *SOX2* mRNA sequence. In vitro fertilized zygotes were injected with these siRNAs and SCR siRNA. Non-injected zygotes served as control. At day 5 we collected morulas for RNA isolation, knowing that at this time these embryos were already expressing SOX2. Using quantitative PCR we observed that siRNA1 reduced about 11% of SOX2 mRNA levels while siRNA2 and siRNA3 reduced *SOX2* levels to about 45% (Figure 4.3A). We decided to combine siRNA2 and siRNA3 and together they showed a 74% knockdown of SOX2 (Figure 4.3A). Embryos injected with siRNA2 + 3 were cultured until blastocyst stage, however, development to blastocyst was significantly reduced (Figure 4.3B).

This reduction in blastocyst development could have been caused by impairment of ICM formation or by blocking development of all cells in the embryo. To answer this question we decided to inject one blastomere of a 2-cell embryo and to trace such cell and its daughter cells with a co-injection of TRITC-Dextran 70000 (Figure 4.4A) that is incapable of escaping the cells once delivered intracellularly. In order to maximize the number of 2-cell embryos at the time of injection we opted to produce embryos by parthenogenesis, artificially activating them with ionomycin. We observed that some cells of the embryo, but not all, were stained with TRITC at 8-cell stage (Figure 4.4B) and that in about half of the cells of morulas, SOX2 protein was not detected by immunocytochemistry (Figure 4.4C).

Cleavage beyond the 2-cell stage was assessed and its rates tended to be lower in SCR ($p=0.07$) and were significantly lower in siRNA injected groups when compared

to non-injected controls (Figure 4.5A). However, blastocyst formation rate was unchanged among the three groups (Figure 4.5A). Blastocysts derived from siRNA injections displayed TRITC staining at the ICM (Figure 4.5B) and had cells negative for SOX2 protein in the ICM (Figure 4.6A) and even one blastocyst that had no positive SOX2 cells (Figure 4.6B), suggesting that *SOX2* is not essential for ICM formation.

Even though blastomeres injected with *SOX2* siRNA could contribute to the ICM, we speculated that the number of ICM cells in such embryos could be reduced. We performed differential staining analysis in day 7.5 blastocysts and no difference in ICM cell number, TE cell number, total cell number or ICM:TE ratio were observed among all treatment groups (Table 4.2).

Table 4.2 - Cell allocation of day 7.5 IVF blastocysts as determined by propidium iodide and bisbenzimide differential staining. ICM = inner cell mass; TE = trophectoderm

	ICM cells	TE cells	Total Cells	ICM/TE Ratio
Control	31.19 ± 1.75	53.11 ± 2.51	83.66 ± 2.60	0.6023 ± 0.036
SCR	30.54 ± 1.56	58.67 ± 2.68	89.06 ± 3.35	0.5440 ± 0.032
siRNA	29.74 ± 1.99	59.41 ± 3.73	91.64 ±5.26	0.5126 ± 0.039

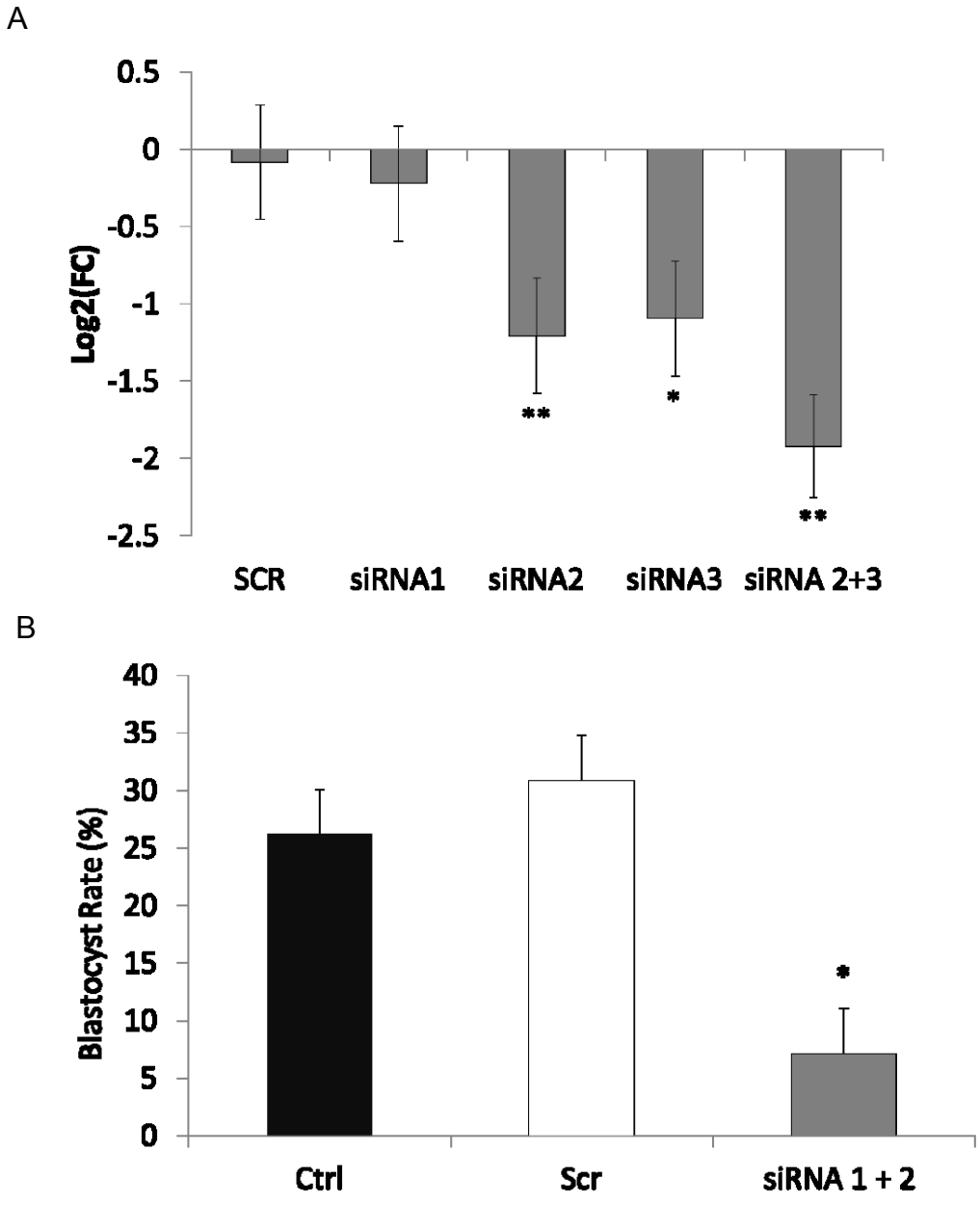

Figure 4.3 - Verification of siRNA efficiency. A) Quantification of SOX2 knockdown efficiency in day 4.5 morulas relative to non-injected controls. Asterisks indicate significant statistical difference from Control group (*p≤ 0.05; ** p≤ 0.01); n = 3, pools of 7 to 10 embryos. B) Blastocyst rates from non-injected, SCR injected or siRNA injected zygotes. Asterisk indicate significant statistical difference; n=5.

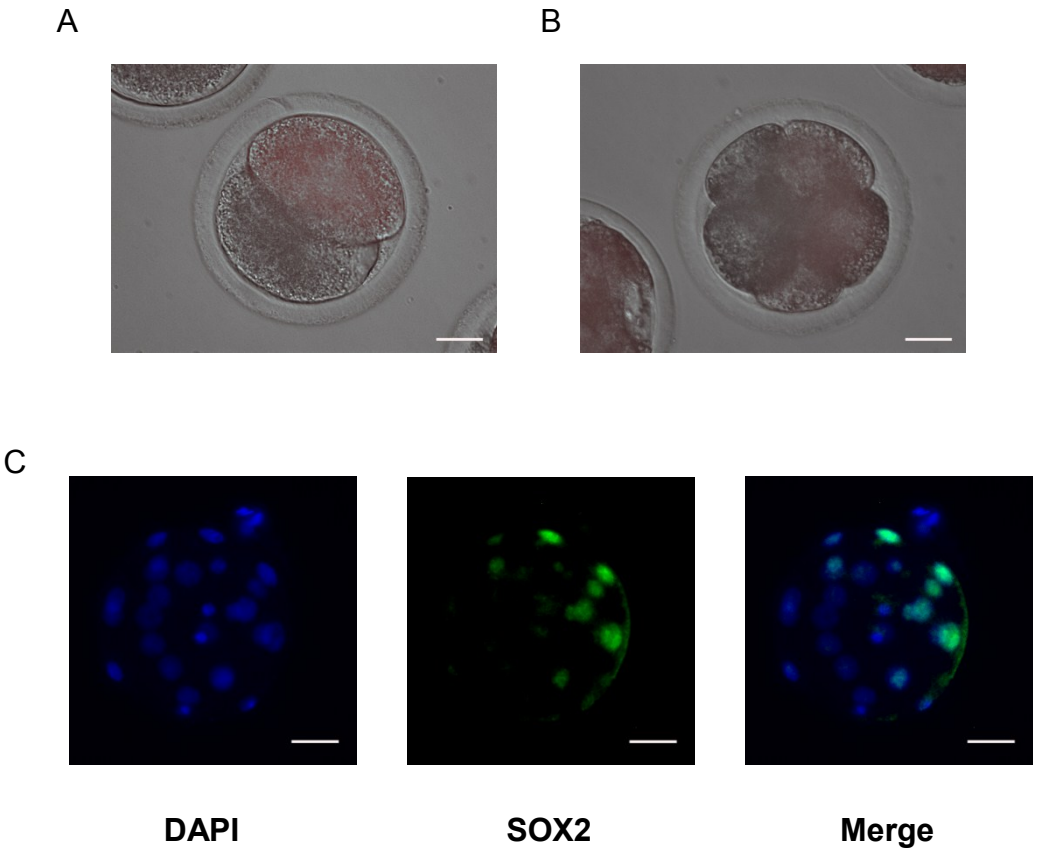

Figure 4.4 – Validation of 2-cell embryo injections. A) Representative image of a 2-cell embryo with one blastomere injected with SOX2 siRNA and Dextran-TRITC. B) Representative image of an 8-cell embryo that had one blastomere injected at 2-cell stage with SOX2 siRNA and Dextran-TRITC. C) Representative images of SOX2 immunocytochemistry of a morula that had one blastomere injected at 2-cell stage with SOX2 siRNA and Dextran-TRITC. Scale bar is equal to 40μm

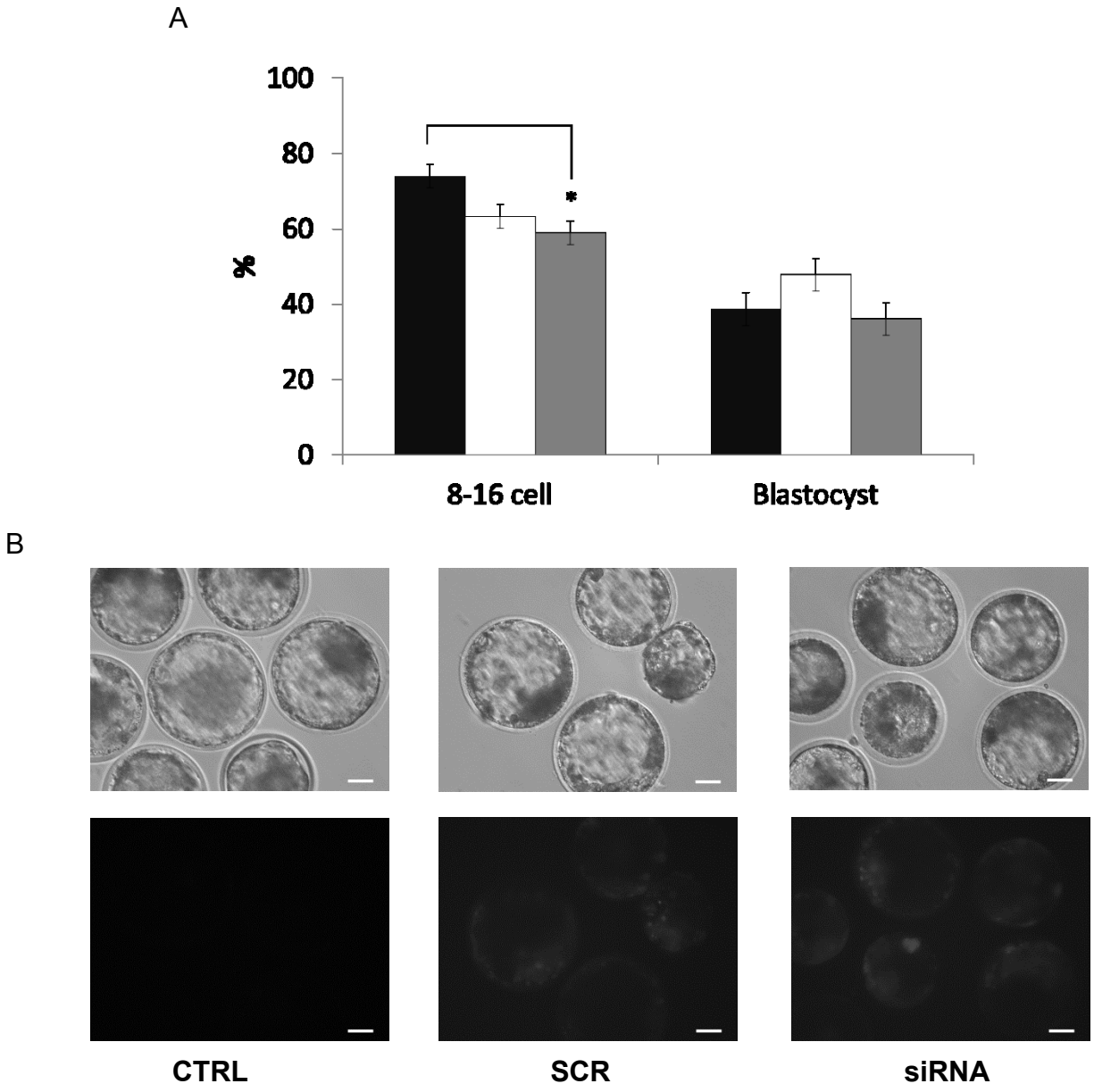

Figure 4.5 –Development to blastocyst after 2-cell injection. A) Developmental rates to 8-16-cell stage or blastocyst, of CTRL (black bars), SCR (open bars) or siRNA (grey bars) groups. B) Representative brightfield (top) and TRITC fluorescent (bottom) images of D7.5 blastocysts that were non-injected, injected with SCR or injected with SOX2. siRNA at 2-cell stage. Asterisk indicate significant statistical difference ($p \leq 0.05$), n = 9. Scale bar is equal to 40μm.

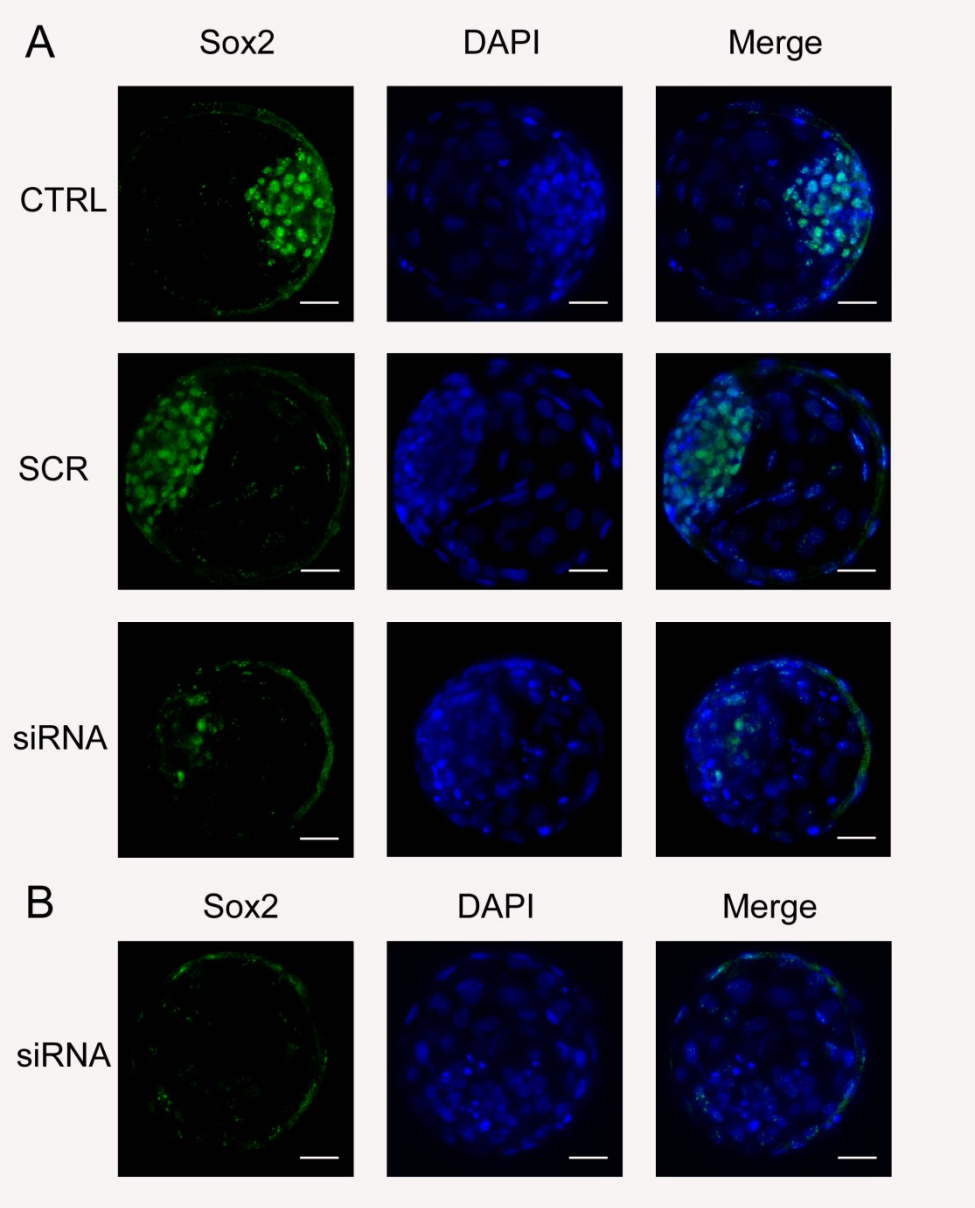

Figure 4.6 – SOX2 immunocytochemistry of D7.5 blastocysts. A) Representative images of non-injected, SCR injected or siRNA injected 2-cell embryos. B) Image of one embryo injected with SOX2 siRNA that was fully negative for SOX2 staining. Scale bar is equal to 40μm

4.4.4 Quantitative PCR gene expression analysis

Embryos that had one blastomere injected at the 2-cell stage were obtained after 7.5 days in culture and submitted to RNA isolation and quantitative RT-PCR. *SOX2* levels were reduced 43% in siRNA group compared to non-injected controls while SCR injected embryos had only 1% reduction in SOX2 expression (Figure 4.7). Embryos injected with siRNA had 55% reduction in *NANOG* expression while SCR injected ones had only 9% reduction compared to controls (Figure 4.7). We tested expression of *OCT4*, which was not statistically different among the three groups (Figure 4.7). Based on our initial data, *FGFR4* was upregulated in the ICM; however knockdown of *SOX2* did not alter the expression levels of this gene (Figure 4.7). We also had previously observed that *CDX2* protein is expressed only in the blastocyst stage (Goissis and Cibelli, unpublished observations), concurrent with the restriction of SOX2 to the ICM; however, knockdown of *SOX2* does not interfere with *CDX2* gene expression levels (Figure 4.7).

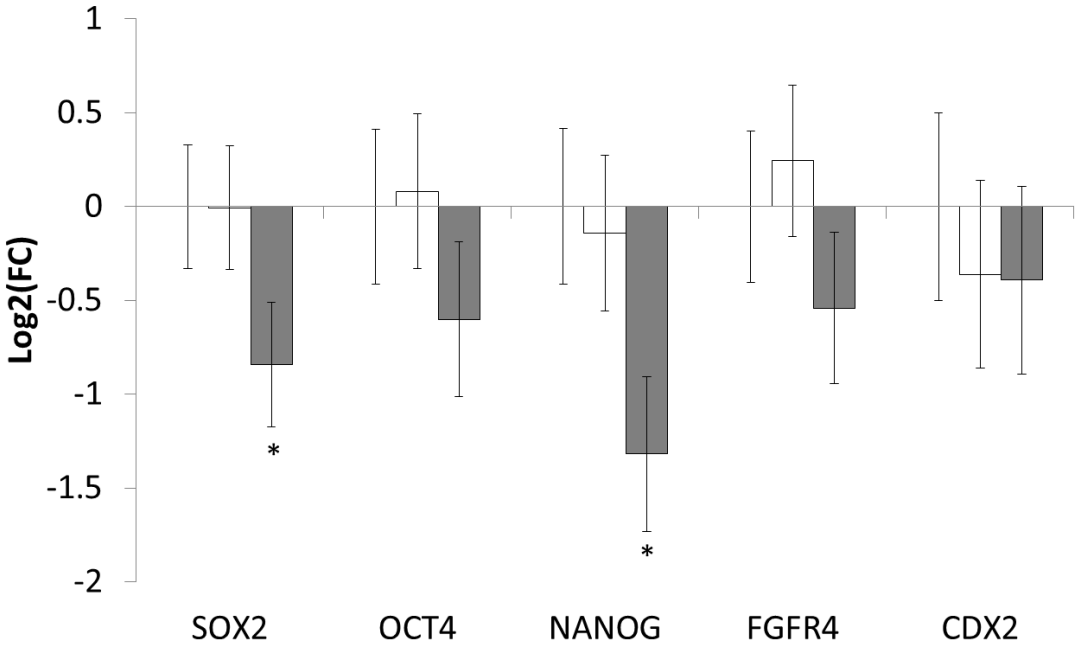

Figure 4.7 - Quantification of developmentally important genes expression in day 7.5 blastocysts SCR (open bars) and siRNA (grey bars) groups relative to IVF group (set to zero). Asterisk indicates significant statistical difference ($p \leq 0.05$); n = 5, pools of 5 to 10 embryos.

4.5 Discussion

Establishment of pluripotent ESCs from bovine embryos has been so far, unsuccessful. Interestingly, bovine blastocysts display a different pattern of pluripotency markers when compared to mouse embryos. Some of the most common markers, such as OCT4 and NANOG, are expressed in both ICM and TE (Kirchhof et al. 2000, Muñoz et al., 2008, Cao et al. 2009). SOX2, a transcription factor widely used as a pluripotency marker and required for pluripotency maintenance in mouse (Masui et al. 2007), has not been properly studied in the bovine embryo. In this study we assessed the difference of gene expression between mechanically isolated TE and whole blastocysts; we verified SOX2 protein localization in the blastocyst and tested the hypothesis that SOX2 is required for bovine ICM formation and therefore a potentially important marker for pluripotent cells.

In the search for ICM markers in the bovine blastocyst, we obtained RNA from mechanically isolated TE or whole blastocysts. We reasoned that any gene significantly upregulated in the 'whole blastocyst' group would indicate genes enriched in the ICM. *FGFR4* was one of the genes significantly upregulated samples containing the ICM. In mouse, *FGFR4* is expressed both in ICM and TE but restricted to the epiblast and primitive endoderm later in development (Rappolee et al. 1998), this may indicating temporal differences between bovine and mouse embryos. *SOX2*, *NANOG* and *LIN28* were also significantly higher in samples containing the ICM when compared to TE. Since *NANOG* protein localization was already shown in bovine (Muñoz et al. 2008,

Cao et al. 2009) and *LIN28* fold-change was not as pronounced as *SOX2*, we concentrated our efforts in the later one.

By immunocytochemistry, we showed that SOX2 expression is first observed at the 16-cell stage, which differs from the 2-cell stage in the mouse (Avilion et al. 2003) and coincides with the bovine embryonic genomic activation, occurring at the 8-16 cell stage (Telford et al. 1990, Meirelles et al. 2004). SOX2 protein expression at the blastocyst stage is restricted to the ICM, different than other pluripotency markers, such as OCT4, NANOG, SSEA-4 and TRA-1-60 (Muñoz et al. 2008, Cao et al. 2009). Interestingly, SOX2 mRNA was also detected in the TE samples of our preliminary gene expression experiment although we have been unable to detect protein expression in the TE using two different antibodies. However we cannot rule out the possibility that SOX2 is expressed albeit at such low levels that are below the sensitivity levels of our technique. Another possibility would be that in the TE cells possess *SOX2* mRNA is postranscriptionally regulated by microRNAs. Our data indicates that SOX2 is shown as the first transcription factor described in the bovine embryo that could be used as a specific marker for bovine ICM. During the preparation of this manuscript, Khan and colleagues confirmed our findings that SOX2 protein is restricted to the ICM in D7 bovine embryos, even though *SOX2* mRNA is present in the TE (Khan et al. 2012).

In order to test the hypothesis that *SOX2* is required for ICM formation in bovine blastocysts, we designed and injected siRNA into zygotes. In the mouse, functional knockdown of *SOX2* using siRNA was not possible due to the presence of maternal protein (Pan and Schultz, 2011); however we were able to knockdown *SOX2* in bovine due to the absence of maternal SOX2 protein. A lower number of siRNA injected

embryos reached blastocyst stage, thus we performed siRNA injections in one blastomere of a 2-cell embryo. Embryos that had only one cell injected with siRNA were able to reach blastocyst stage at the same rate as non-injected and SCR controls. Unexpectedly, injected cells contributed to the ICM, confirmed by tracking the cells with co-injection of Dextran-TRITC and the lack of SOX2 positive cells within the ICM itself. More interestingly, we observed one embryo that completely lacked SOX2 protein, providing more evidence that SOX2 does not seem to be required for ICM formation. Injection of *SOX2* siRNA also did not influence cell number and allocation. The role of SOX2 in the mouse is still subject to debate. Homozygous deletion of SOX2 yielded normal blastocysts (Avilion et al. 2003) that failed to survive right after implantation. Knockdown of SOX2 arrested embryos at the morula stage, reducing expression of TE-related proteins at that stage (Keramari et al. 2011). However, Pan and Schultz (2011) point that maternal SOX2 protein would still be present in these aforementioned studies.

The decline of bovine blastocyst rate after SOX2 siRNA injection in zygotes could be caused by a failure to activate the embryonic genome (EGA) cause by a reduction of SOX2, as observed after overexpression or dominant negative injection of SOX2 in mouse (Pan and Schultz, 2011) or, by failure to regulate trophoblast genes at the morula stage (Keramari et al. 2011). Based on our data, the second scenario seems unlikely due to the contribution of daughter cells of an injected blastomere also to the TE. Considering the first scenario, we believe the siRNA injected cells were able to overcome developmental arrest due to the presence of other cells undergoing EGA. It was shown that phosphatidylinositol 3-kinase (PI3K) signaling through AKT pathway is required for mouse EGA (Zheng et al. 2010). PI3K-AKT signaling can be activated by

different embryotropins that act, in relation to the embryo itself, on an autocrine manner (reviewed by O'Neill et al. 2012). Based on this information, we speculate that non-injected cells would secrete specific embryotropins that would act through PI3K-AKT signaling in injected cells, allowing them to undergo proper EGA. Further studies will be required to discover the mechanisms of the developmental arrest after SOX2 knockdown in zygotes.

SOX2 is part of the so called pluripotency network, in which plays a central role along with *OCT4* and *NANOG* (Loh et al. 2006). These genes self-regulate each other and often bind to same target genes, maintaining the pluripotency program (Boyer et al. 2005, Chew et al. 2005, Rodda et al. 2005, Chen et al. 2008). Knockdown of *SOX2* led to a reduction in *NANOG* expression in bovine blastocysts, probably due to *SOX2* regulation of *NANOG* as mentioned above. *NANOG* null mouse embryos are able to form ICM, however these embryos are not viable and cannot be used for ES cell derivation, as cells cannot retain pluripotency and differentiate into endoderm-like cells (Mitsui et al. 2003), trophoblast cells or undergo cell death (Silva et al. 2009). Curiously, *OCT4* expression was not reduced as it would be expected in terms of the pluripotency network. However, it was suggested that during bovine development *OCT4* and *SOX2* would not interact as it occurs in mouse or human (Xie et al. 2010), which could explain the observed result.

Further studies will be required to determine the long term effect of embryos having a *SOX2*-deficient ICM. In vitro study of these embryos would benefit from the derivation of bona fide bovine ESCs, unfortunately such cells have not been reported yet. Transfer of these embryos into recipient cows would offer limited information due to

short knockdown effect of SOX2 siRNA injections. Considering the role of SOX2 in the adult animal, conditional knockdown using shRNA coded in the genome could be the best option.

Our study revealed that SOX2 protein is limited to the ICM of bovine blastocysts. Knockdown of *SOX2* since zygote stage had a negative impact on development to blastocyst; however, injection of *SOX2* siRNA in a single blastomere of a 2-cell embryo did not. Moreover, these injected cells were able to contribute to the ICM, even though NANOG expression was also reduced in blastocysts. Based on the data presented, we conclude that SOX2 is a protein marker for ICM although it is not required for ICM formation in bovine embryos.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Reproductive biotechnologies are important to increase efficiency in livestock production and in breeding programs, especially cattle. Among them, somatic cell nuclear transfer (SCNT) could be used to generate copies of high genetic merit individuals. However, its efficiency has remained low for 15 years since it was first achieved, hampering its broader application. Pluripotent embryonic stem cells (ESC) as shown in the mouse, were thought be a valid alternative to improve SCNT efficiency, unfortunately, bona fide ESCs have not been isolated from bovine embryos so far. The understanding of molecular mechanisms governing the first lineage specification in the bovine pre-implantation embryo could aid to resolve problems associated with SCNT and in the derivation of true bovine ESC. In this dissertation we used different approaches to study roles of developmentally important genes, *OCT4, CDX2* and *SOX2*, in bovine embryo development.

In Chapter 2, we described an attempt to improve SCNT by overexpressing the pluripotency factor *OCT4* in donor cells prior to fusing it with an oocyte. We observed an increase in the number of trophectoderm (TE) cells, however no other significant change was observed and alterations related to the SCNT process, such as increased *XIST* expression, were still present. Based on our results, we believe that *OCT4* over expression could be used to improve SCNT if combined with other strategies, such as chromatin modifiers, which have been reported to increase histone acetylation in SCNT embryos (Su et al. 2011b, Wang et al. 2011b, Xu et al. 2012). Knockdown of *XIST* until blastocyst stage is another strategy, since knockout of one *XIST* allele in the mouse

significantly increased the number of live offspring (Inoue et al. 2010). Reduction of histone variant MacroH2A could also increase efficiency as it is increases resistance of X chromosome reprogramming (Pasque et al. 2011).

In order to gain knowledge on the process of lineage specification in bovine embryos, in Chapter 3 we characterized the expression of CDX2 protein and also reduced its expression during development by injection of siRNA. The collected data revealed that *CDX2* is not required for blastocyst formation; however it is important in maintaining TE integrity. Ruling out CDX2 as the gatekeeper of TE formation will allow us to focus on other likely candidates as master regulators such as *TEAD4,* which is regulated by Hippo signaling pathway in the mouse (Nishioka et al. 2009). Understanding these processes will, in turn, help us asses proper reprogramming in SCNT embryos, as most problems during gestation involve the placenta.

In Chapter 4, we characterized spatial and temporal localization of SOX2 protein, which starts at the 16-cell embryo and is restricted to the ICM in blastocysts. This information reveals SOX2 as a potentially unique ICM marker that could be used to aid true ESC derivation in bovine. In addition, brings us closer to finding a mechanism for ICM specification eventually improving the chances of ESC derivation. One interesting fact observed is that while there was an absence of SOX2 protein, *SOX2* mRNA was detected in TE, albeit in lower levels. This fact is in agreement with *in situ* hybridization data already published (Khan et al. 2012). It is possible to speculate that post-transcriptional regulatory mechanisms are precluding SOX2 protein to be expressed in the TE. MicroRNA-145 was shown to downregulate *SOX2* in mouse ESC (Xu et al. 2009) thus a new line of inquiry with regards to microRNAs and their role in ICM

specification should be further explored. Experiments on overexpression and knockdown microRNAs that target *SOX2* during embryo development could indeed lead to unveiling a powerful role in ICM and TE specification.

Knockdown of *SOX2* by injecting zygotes reduced blastocyst formation. Recently, *SOX2* was implicated as a critical transcriptional regulator during embryonic genome activation (EGA - Pan and Schultz, 2011). It is possible that in bovine it has a similar role, which explains why its knockdown would reduce blastocyst formation. Curiously, injection of one blastomere of a 2-cell embryo did not interfere with development rates. We speculate that non-injected cells were able to rescue the injected ones by secreting factors that would activate specific pathways, leading to EGA. In mouse it was shown that phosphatidylinositol 3-kinase (PI3K) signaling through AKT pathway is required for mouse EGA (Zheng et al. 2010).

A first experiment would be to determine if *SOX2* knockdown in zygotes interfere with EGA. That could be tested by measuring brUTP incorporation and compare to non-injected controls at the 8-cell stage. Also, expression of genes associated with EGA (Vigneault et al. 2009) could be analyzed. Another experiment would be to verify if PI3K signaling is involved with EGA in bovine. This could be tested by adding selective inhibitors such as Wortmannin or LY294002 in culture media followed by verification of BrUTP incorporation and expression of EGA related genes.

Surprisingly, we show that cells lacking SOX2 protein can contribute to the ICM of bovine embryos, despite the fact that SOX2 is apparently the first exclusive marker of ICM cells. This is intriguing and raises the possibility that genes different than those already described for mouse and human pluripotent cells are involved in ICM

specification or, that ICM specification is the default outcome of totipotent blastomeres and only TE specification requires expression of different genes, silencing the gene signature program of pluripotency.

With the recent debate about the use of mouse or bovine embryos as a model for human embryo development (Berg et al. 2011, Rossant, 2011), our studies provide information that could also be used to further understand the mechanisms of lineage segregation in human embryos. Overall, the studies presented here advanced the knowledge of genes responsible for bovine pre-implantation development and generated a large number of questions about lineage specification in bovine, creating novel opportunities for further hypothesis driven experiments.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ahmed K, Dehghani H, Rugg-Gunn P, Fussner E, Rossant J, Bazett-Jones DP. Global chromatin architecture reflects pluripotency and lineage commitment in the early mouse embryo. PLoS One. 2010 5(5):e10531.

Avilion AA, Nicolis SK, Pevny LH; Perez, L; Vivian, N; Lovell-Badge, R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 17: 126–140.

Beck F, Erler T, Russell A, James R. Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. Dev Dyn. 1995 204(3):219-27.

Berg DK, Smith CS, Pearton DJ, Wells DN, Broadhurst R, Donnison M, Pfeffer PL. Trophectoderm lineage determination in cattle. Dev Cell. 2011 20(2):244-55.

Beyhan Z, Ross PJ, Iager AE, Kocabas AM, Cunniff K, Rosa GJ, Cibelli JB. Transcriptional reprogramming of somatic cell nuclei during pre-implantation development of cloned bovine embryos. Dev Biol. 2007 305(2):637-49.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005 Sep 23;122(6):947-56.

Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature. 2007 448(7150):191-5.

Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A. Capture of authentic embryonic stem cells from rat blastocysts. Cell. 2008 135:1287-98.

Campbell KH, Fisher P, Chen WC, Choi I, Kelly RD, Lee JH, Xhu J. Somatic cell nuclear transfer: Past, present and future perspectives. Theriogenology. 2007 68 Suppl 1:S214-31.

Canovas S, Cibelli JB, Ross PJ. Jumonji domain-containing protein 3 regulates histone 3 lysine 27 methylation during bovine pre-implantation development. Proc Natl Acad Sci U S A. 2012 109:2400-5.

Cao S, Wang F, Chen Z, Liu Z, Mei C, Wu H, Huang J, Li C, Zhou L, Liu L.Isolation and culture of primary bovine embryonic stem cell colonies by a novel method. J Exp Zool Part A Ecol Genet Physiol. 2009 311:368-76

Cauffman G, Van de Velde H, Liebaers I, Van Steirteghem A. Oct-4 mRNA and protein expression during human pre-implantation development. Mol Hum Reprod. 2005 11(3):173-81.

Cauffman G, De Rycke M, Sermon K, Liebaers I, Van de Velde H. Markers that define stemness in ESC are unable to identify the totipotent cells in human pre-implantation embryos. Hum Reprod. 2009 24(1):63-70.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell. 2003 113:643-55.

Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J, Loh YH, Yeo HC, Yeo ZX, Narang V, Govindarajan KR, Leong B, Shahab A,Ruan Y, Bourque G, Sung WK, Clarke ND, Wei CL, Ng HH. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell. 2008 133(6):1106-17.

Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, Cowan C, Fitz-Gerald C, Zhang K, Melton DA, Eggan K. Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines.Cell Stem Cell. 2009 4(2):103-6

Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P, Ng HH. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol. 2005 25(14):6031-46.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de León FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science. 1998a 280(5367):1256-8.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de León FA, Robl JM. Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat Biotechnol. 1998b 16:642-6.

Cibelli J. Developmental biology. A decade of cloning mystique. Science. 2007; 316:990-2.

Chavatte-Palmer P, Camous S, Jammes H, Le Cleac'h N, Guillomot M, Lee RS. Review: Placental perturbations induce the developmental abnormalities often observed in bovine somatic cell nuclear transfer. Placenta. 2012 33 Suppl:S99-S104.

Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL, Renard JP, Chavatte-Palmer P. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. Biol Reprod. 2006 75(1):122-30.

Dietrich JE, Hiiragi T. Stochastic patterning in the mouse pre-implantation embryo. Development. 2007 134(23):4219-31.

Ding X, Wang Y, Zhang D, Wang Y, Guo Z, Zhang Y. Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. Theriogenology. 2008 70:622-30.

Enright BP, Kubota C, Yang X, Tian XC. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine. Biol Reprod. 2003 69(3):896-901.

Enright BP, Sung LY, Chang CC, Yang X, Tian XC Methylation and acetylation characteristics of cloned bovine embryos from donor cells treated with 5-aza-2'-deoxycytidine. Biol Reprod. 2005 72:944-8.

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981 292(5819):154-6.

Fujii T, Moriyasu S, Hirayama H, Hashizume T, Sawai K. Aberrant expression patterns of genes involved in segregation of inner cell mass and trophectoderm lineages in bovine embryos derived from somatic cell nuclear transfer. Cell Reprogram. 2010 12:617-25.

Gordon JW, Ruddle FH. Integration and stable germ line transmission of genes injected into mouse pronuclei. Science. 1981 214(4526):1244-6.

Han X, Han J, Ding F, Cao S, Lim SS, Dai Y, Zhang R, Zhang Y, Lim B, Li N. Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. Cell Res. 2011 21(10):1509-12

Hanna J, Markoulaki S, Mitalipova M, Cheng AW, Cassady JP, Staerk J, Carey BW, Lengner CJ, Foreman R, Love J, Gao Q, Kim J, Jaenisch R. Metastable pluripotent states in NOD-mouse-derived ESCs. Cell Stem Cell. 2009 4(6):513-24.

Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME. Evidence for placental abnormality as the major cause

of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod. 2000 63:1787-94.

Hill JR, Chavatte-Palmer P. Pregnancy and neonatal care of cloned animals. In: Cibelli J, Lanza RP, Campbell KHS, West MD (Ed.), Principles of Cloning, Academic Press, 2002 pp, 247-266

Hublitz P, Albert M, Peters AH. Mechanisms of transcriptional repression by histone lysine methylation. Int J Dev Biol. 2009 53:335-54.

Iager AE, Ragina NP, Ross PJ, Beyhan Z, Cunniff K, Rodriguez RM, Cibelli JB. Trichostatin A improves histone acetylation in bovine somatic cell nuclear transfer early embryos. Cloning Stem Cells. 2008 10:371-9.

Inoue K, Kohda T, Sugimoto M, Sado T, Ogonuki N, Matoba S, Shiura H, Ikeda R, Mochida K, Fujii T, Sawai K, Otte AP, Tian XC, Yang X, Ishino F, Abe K, Ogura A. Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. Science. 2010 330:496-9.

Jähner D, Stuhlmann H, Stewart CL, Harbers K, Löhler J, Simon I, Jaenisch R. De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature. 1982 298:623-8.

Keefer CL, Pant D, Blomberg L, Talbot NC. Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. Anim Reprod Sci. 2007 98(1-2):147-68.

Keramari M, Razavi J, Ingman KA, Patsch C, Edenhofer F, Ward CM, Kimber SJ. Sox2 is essential for formation of trophectoderm in the pre-implantation embryo. PLoS One. 2010 5(11):e13952.

Khan DR, Dubé D, Gall L, Peynot N, Ruffini S, Laffont L, Le Bourhis D, Degrelle S, Jouneau A, Duranthon V. Expression of Pluripotency Master Regulators during Two Key Developmental Transitions: EGA and Early Lineage Specification in the Bovine Embryo. PLoS One. 2012 7(3):e34110.

Kim JB, Sebastiano V, Wu G, Araúzo-Bravo MJ, Sasse P, Gentile L, Ko K, Ruau D, Ehrich M, van den Boom D, Meyer J, Hübner K, Bernemann C, Ortmeier C, Zenke M, Fleischmann BK, Zaehres H, Schöler HR. OCT4-induced pluripotency in adult neural stem cells. Cell. 2009 136:411-9

Kirchhof N, Carnwath JW, Lemme E, Anastassiadis K, Schöler H, Niemann H. Expression pattern of Oct-4 in pre-implantation embryos of different species. Biol Reprod. 2000 63(6):1698-705.

Kocabas AM, Crosby J, Ross PJ, Otu HH, Beyhan Z, Can H, Tam WL, Rosa GJ, Halgren RG, Lim B, Fernandez E, Cibelli JB. The transcriptome of human oocytes. Proc Natl Acad Sci U S A. 2006 103:14027-32

Koo DB, Kang YK, Choi YH, Park JS, Kim HN, Oh KB, Son DS, Park H, Lee KK, Han YM. Aberrant allocations of inner cell mass and trophectoderm cells in bovine nuclear transfer blastocysts. Biol Reprod. 2002 67:487-92.

Kormish JD, Sinner D, Zorn AM. Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. Dev Dyn. 2010 239(1):56-68.

Kuijk EW, Du Puy L, Van Tol HT, Oei CH, Haagsman HP, Colenbrander B, Roelen BA. Differences in early lineage segregation between mammals. Dev Dyn. 2008 237:918-27.

Kurosaka S, Eckardt S, McLaughlin KJ. Pluripotent lineage definition in bovine embryos by OCT4 transcript localization. Biol Reprod. 2004 71:1578-82.

Latham KE. Early and delayed aspects of nuclear reprogramming during cloning. Biol Cell. 2005 97(2):119-32.

Li W, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S. Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell. 2009 4(1):16-9.

Li G, Margueron R, Ku M, Chambon P, Bernstein BE, Reinberg D. Jarid2 and PRC2, partners in regulating gene expression. Genes Dev. 2010 24:368-80.

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH.The OCT4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet. 2006; 38:431-40.

Lucifero D, Suzuki J, Bordignon V, Martel J, Vigneault C, Therrien J, Filion F, Smith LC, Trasler JM. Bovine SNRPN methylation imprint in oocytes and day 17 in vitro-produced and somatic cell nuclear transfer embryos. Biol Reprod. 2006 75(4):531-8.

Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981 78(12):7634-8.

Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS, Niwa H. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol. 2007 9(6):625-35.

Meirelles FV, Caetano AR, Watanabe YF, Ripamonte P, Carambula SF, Merighe GK, Garcia SM. Genome activation and developmental block in bovine embryos. Anim Reprod Sci. 2004 82-83:13-20

Meissner A, Jaenisch R. Generation of nuclear transfer-derived pluripotent ES cells from cloned Cdx2-deficient blastocysts. Nature. 2006 439(7073):212-5.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003 113(5):631-42.

Muñoz M, Rodríguez A, De Frutos C, Caamaño JN, Díez C, Facal N, Gómez E. Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines. Theriogenology. 2008 69(9):1159-64.

Navarro P, Avner P. When X-inactivation meets pluripotency: an intimate rendezvous. FEBS Lett. 2009 583(11):1721-7.

Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. Mech Dev. 2008 125(3-4):270-83.

Nishioka N, Inoue K, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N, Makita R, Kurihara H, Morin-Kensicki EM, Nojima H, Rossant J, Nakao K, Niwa H, Sasaki H. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell. 2009 16(3):398-410.

Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. 1998 12(13):2048-60.

Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell. 2005 123(5):917-29.

Nolen LD, Gao S, Han Z, Mann MR, Gie Chung Y, Otte AP, Bartolomei MS, Latham KE. X chromosome reactivation and regulation in cloned embryos. Dev Biol. 2005; 279:525-40.

O'Neill C, Li Y, Jin XL. Survival signaling in the pre-implantation embryo. Theriogenology. 2012 77(4):773-84.
Pan H, Schultz RM. Sox2 modulates reprogramming of gene expression in two-cell mouse embryos. Biol Reprod. 2011 85(2):409-16.

Panarace M, Agüero JI, Garrote M, Jauregui G, Segovia A, Cané L, Gutiérrez J, Marfil M, Rigali F, Pugliese M, Young S, Lagioia J, Garnil C, Forte Pontes JE, Ereno Junio JC, Mower S, Medina M. How healthy are clones and their progeny: 5 years of field experience. Theriogenology. 2007 67(1):142-51

Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. 1986 25(4):591-600.

Pasque V, Gillich A, Garrett N, Gurdon JB. Histone variant macroH2A confers resistance to nuclear reprogramming. EMBO J. 2011 May 30(12):2373-87.

Peippo J, Machaty Z, Peter A. Terminologies for the pre-attachment bovine embryo. Theriogenology. 2011 76(8):1373-9.

Pesce M, Schöler HR. Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells. 2001 19:271-8.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001 29(9):e45.
Plachta N, Bollenbach T, Pease S, Fraser SE, Pantazis P. Oct4 kinetics predict cell lineage patterning in the early mammalian embryo. Nat Cell Biol. 2011 13(2):117-23.

Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007 448(7151):313-7

Ralston A, Rossant J. Genetic regulation of stem cell origins in the mouse embryo. Clin Genet. 2005 68(2):106-12.

Ralston A, Rossant J. Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. Dev Biol. 2008 313(2):614-29.

Rappolee DA, Patel Y, Jacobson K. Expression of fibroblast growth factor receptors in peri-implantation mouse embryos. Mol Reprod Dev. 1998 51(3):254-64.

Rathbone AJ, Fisher PA, Lee JH, Craigon J, Campbell KH. Reprogramming of ovine somatic cells with Xenopus laevis oocyte extract prior to SCNT improves live birth rate. Cell Reprogram. 2010 12:609-16.

Reik W, Walter J. Evolution of imprinting mechanisms: the battle of the sexes begins in the zygote. Nat Genet. 2001 27(3):255-6.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000 18(4):399-404.

Rideout WM 3rd, Eggan K, Jaenisch R. Nuclear cloning and epigenetic reprogramming of the genome. Science. 2001 293(5532):1093-8.

Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, Robson P. Transcriptional regulation of nanog by OCT4 and SOX2. J Biol Chem. 2005 280(26):24731-7.
Roode M, Blair K, Snell P, Elder K, Marchant S, Smith A, Nichols J. Human hypoblast formation is not dependent on FGF signalling. Dev Biol. 2012 361(2):358-63.

Ross PJ, Ragina NP, Rodriguez RM, Iager AE, Siripattarapravat K, Lopez-Corrales N, Cibelli JB. Polycomb gene expression and histone H3 lysine 27 trimethylation changes during bovine pre-implantation development. Reproduction. 2008a 136:777-85.

Ross PJ, Beyhan Z, Iager AE, Yoon SY, Malcuit C, Schellander K, Fissore RA, Cibelli JB. Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. BMC Dev Biol. 2008b 19;8:16.

Ross PJ, Rodriguez RM, Iager AE, Beyhan Z, Wang K, Ragina NP, Yoon SY, Fissore RA, Cibelli JB. Activation of bovine somatic cell nuclear transfer embryos by PLCZ cRNA injection. Reproduction. 2009 137:427-37.

Ross PJ, Cibelli JB. Bovine somatic cell nuclear transfer. Methods Mol Biol. 2010; 636:155-77.

Rossant J, Bernelot-Moens C, Nagy A. Genome manipulation in embryonic stem cells. Philos Trans R Soc Lond B Biol Sci. 1993 339(1288):207-15.

Rossant J. Developmental biology: A mouse is not a cow. Nature. 2011 471(7339):457-8.

Saito S, Sawai K, Ugai H, Moriyasu S, Minamihashi A, Yamamoto Y, Hirayama H, Kageyama S, Pan J, Murata T, Kobayashi Y, Obata Y, Yokoyama KK. Generation of cloned calve and transgenic chimeric embryos from bovine embryonic stem-like cells. Biochem Biophys Res Commun. 2003 309(1):104-13.

Sangalli JR, De Bem TH, Perecin F, Chiaratti MR, Oliveira LD, de Araújo RR, Valim Pimentel JR, Smith LC, Meirelles FV. Treatment of Nuclear-Donor Cells or Cloned Zygotes with Chromatin-Modifying Agents Increases Histone Acetylation But Does Not Improve Full-Term Development of Cloned Cattle. Cell Reprogram. 2012 [Epub ahead of print]

Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W, Dean W. Epigenetic marking correlates with developmental potential in cloned bovine pre-implantation embryos. Curr Biol. 2003 13:1116-21

Santos F, Dean W. Epigenetic reprogramming during early development in mammals. Reproduction. 2004; 127:643-51

Sasaki H. Mechanisms of trophectoderm fate specification in pre-implantation mouse development. Dev Growth Differ. 2010 52(3):263-73.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science. 1997 278(5346):2130-3

Schöler HR, Dressler GR, Balling R, Rohdewohld H, Gruss P.Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J. 1990 9(7):2185-95.

Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem. 2007 76:75-100.

Sharov AA, Masui S, Sharova LV, Piao Y, Aiba K, Matoba R, Xin L, Niwa H, Ko MS. Identification of Pou5f1, Sox2, and Nanog downstream target genes with statistical confidence by applying a novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. BMC Genomics. 2008 9:269.

Silva J, Nichols J, Theunissen TW, Guo G, van Oosten AL, Barrandon O, Wray J, Yamanaka S, Chambers I, Smith A. Nanog is the gateway to the pluripotent ground state. Cell. 2009 138(4):722-37.

Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides Nature. 1988 336(6200):688-90

Smith C, Cloning and genetic improvement of beef cattle. Anim Prod. 1989 49:49-62.

Smith CS, Berg DK, Berg M, Pfeffer PL. Nuclear transfer-specific defects are not apparent during the second week of embryogenesis in cattle. Cell Reprogram. 2010 12:699-707.

Smith SL, Everts RE, Tian XC, Du F, Sung LY, Rodriguez-Zas SL, Jeong BS, Renard JP, Lewin HA, Yang X. Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. Proc Natl Acad Sci U S A. 2005 102(49):17582-7.

Solter D, Knowles BB. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci U S A.1978 75(11):5565-9.

Somers J, Smith C, Donnison M, Wells DN, Henderson H, McLeay L, Pfeffer PL. Gene expression profiling of individual bovine nuclear transfer blastocysts. Reproduction. 2006 131:1073-84.

Steibel JP, Poletto R, Coussens PM, Rosa GJ. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data.Genomics. 2009 94(2):146-52.

Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development. 2005 132(9):2093-102.

Su JM, Yang B, Wang YS, Li YY, Xiong XR, Wang LJ, Guo ZK, Zhang Y. Expression and methylation status of imprinted genes in placentas of deceased and live cloned transgenic calves. Theriogenology. 2011a 75(7):1346-59.

Su J, Wang Y, Li Y, Li R, Li Q, Wu Y, Quan F, Liu J, Guo Z, Zhang Y. Oxamflatin significantly improves nuclear reprogramming, blastocyst quality, and in vitro development of bovine SCNT embryos. PLoS One. 2011b 6(8):e23805.

Sumer H, Liu J, Malaver-Ortega LF, Lim ML, Khodadadi K, Verma PJ. NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. J Anim Sci. 2011 89(9):2708-16.

Suwińska A, Czołowska R, Ozdzeński W, Tarkowski AK. Blastomeres of the mouse embryo lose totipotency after the fifth cleavage division: expression of Cdx2 and Oct4 and developmental potential of inner and outer blastomeres of 16- and 32-cell embryos. Dev Biol. 2008 322(1):133-44.

Suzuki J Jr, Therrien J, Filion F, Lefebvre R, Goff AK, Smith LC. In vitro culture and somatic cell nuclear transfer affect imprinting of SNRPN gene in pre- and post-implantation stages of development in cattle. BMC Dev Biol. 2009 9:9.

Suzuki J Jr, Therrien J, Filion F, Lefebvre R, Goff AK, Perecin F, Meirelles FV, Smith LC. Loss of methylation at H19 DMD is associated with biallelic expression and reduced development in cattle derived by somatic cell nuclear transfer. Biol Reprod. 2011 84(5):947-56.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 126(4):663-76.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 131(5):861-72

Talbot NC, Powell AM, Rexroad CE Jr. In vitro pluripotency of epiblasts derived from bovine blastocysts. Mol Reprod Dev. 1995 42(1):35-52.

Tang S, Wang Y, Zhang D, Gao Y, Ma Y, Yin B, Sun J, Liu J, Zhang Y. Reprogramming donor cells with oocyte extracts improves in vitro development of nuclear transfer embryos. Anim Reprod Sci. 2009 115:1-9.

Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: A comparison of several species Mol Reprod Dev. 1990 26: 90-100

Telugu BP, Ezashi T, Roberts RM. The promise of stem cell research in pigs and other ungulate species. Stem Cell Rev. 2010 6(1):31-41

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RD. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature. 2007 448(7150):196-9.

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A. 1995 92(17):7844-8.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998 282(5391):1145-7

Vassena R, Han Z, Gao S, Latham KE. Deficiency in recapitulation of stage-specific embryonic gene transcription in two-cell stage cloned mouse embryos. Mol Reprod Dev. 2007 74(12):1548-56.

Vigneault C, Gravel C, Vallée M, McGraw S, Sirard MA. Unveiling the bovine embryo transcriptome during the maternal-to-embryonic transition. Reproduction. 2009 137(2):245-57.

Wang L, Duan E, Sung LY, Jeong BS, Yang X, Tian XC. Generation and characterization of pluripotent stem cells from cloned bovine embryos. Biol Reprod. 2005 73(1):149-55.

Wang K, Sengupta S, Magnani L, Wilson CA, Henry RW, Knott JG. Brg1 is required for Cdx2-mediated repression of Oct4 expression in mouse blastocysts. PLoS One. 2010 5(5):e10622.

Wang YS, Xiong XR, An ZX, Wang LJ, Liu J, Quan FS, Hua S, Zhang Y. Production of cloned calves by combination treatment of both donor cells and early cloned embryos with 5-aza-2/-deoxycytidine and trichostatin A.Theriogenology. 2011a 75(5):819-25.

Wang LJ, Zhang H, Wang YS, Xu WB, Xiong XR, Li YY, Su JM, Hua S, Zhang Y. Scriptaid improves in vitro development and nuclear reprogramming of somatic cell nuclear transfer bovine embryos. Cell Reprogram. 2011b 13:431-9.

Wee G, Shim JJ, Koo DB, Chae JI, Lee KK, Han YM. Epigenetic alteration of the donor cells does not recapitulate the reprogramming of DNA methylation in cloned embryos. Reproduction. 2007 134(6):781-7.

Wegner M. All purpose Sox: The many roles of Sox proteins in gene expression. Int J Biochem Cell Biol. 2010 42(3):381-90.

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature. 2007 448(7151):318-24.

Whetstine JR, Nottke A, Lan F, Huarte M, Smolikov S, Chen Z, Spooner E, Li E, Zhang G, Colaiacovo M, Shi Y. Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. Cell. 2006 125:467-81.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature. 1997 385(6619):810-3.

Wu G, Gentile L, Fuchikami T, Sutter J, Psathaki K, Esteves TC, Araúzo-Bravo MJ, Ortmeier C, Verberk G, Abe K, Schöler HR. Initiation of trophectoderm lineage specification in mouse embryos is independent of Cdx2. Development. 2010 137(24):4159-69.

Wu X, Li Y, Xue L, Wang L, Yue Y, Li K, Bou S, Li GP, Yu H. Multiple histone site epigenetic modifications in nuclear transfer and in vitro fertilized bovine embryos. Zygote. 2011 19:31-45.

Xie D, Chen CC, Ptaszek LM, Xiao S, Cao X, Fang F, Ng HH, Lewin HA, Cowan C, Zhong S. Rewirable gene regulatory networks in the pre-implantation embryonic development of three mammalian species. Genome Res. 2010 20(6):804-15.

Xu C, Rosler E, Jiang J, Lebkowski JS, Gold JD, O'Sullivan C, Delavan-Boorsma K, Mok M, Bronstein A, Carpenter MK. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells. 2005 23(3):315-23.

Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell. 2009 137(4):647-58.

Xu W, Wang Y, Li Y, Wang L, Xiong X, Su J, Zhang Y. Valproic Acid improves the in vitro development competence of bovine somatic cell nuclear transfer embryos. Cell Reprogram. 2012 14(2):138-45.

Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. Development. 2007 134(21):3827-36

Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat Genet. 2007 39(3):295-302.

Yao N, Wan PC, Hao ZD, Gao FF, Yang L, Cui MS, Wu Y, Liu JH, Liu S, Chen H, Zeng SM. Expression of interferon-tau mRNA in bovine embryos derived from different procedures. Reprod Domest Anim. 2009; 44:132-9.

Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. Nature. 2008 453(7194):519-23.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson J. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007 318(5858):1917-20

Zhang M, Wang F, Kou Z, Zhang Y, Gao S. Defective chromatin structure in somatic cell cloned mouse embryos. J Biol Chem. 2009 284:24981-7.

Zheng W, Gorre N, Shen Y, Noda T, Ogawa W, Lundin E, Liu K. Maternal phosphatidylinositol 3-kinase signalling is crucial for embryonic genome activation and pre-implantation embryogenesis. EMBO Rep. 2010 11(11):890-5.

Zhou H, Li W, Zhu S, Joo JY, Do JT, Xiong W, Kim JB, Zhang K, Schöler HR, Ding S. Conversion of mouse epiblast stem cells to an earlier pluripotency state by small molecules. J Biol Chem. 2010 285(39):29676-80.