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Effect of tissue source on adult equine multipotent stromal cell pluripotency induction treatment with synthetic mRNA

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EFFECT OF TISSUE SOURCE ON ADULT EQUINE MULTIPOTENT STROMAL CELL
PLURIPOTENCY INDUCTION TREATMENT WITH SYNTHETIC MRNA

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program of
Animal and Dairy Sciences

by
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	x
ABSTRACT	xii
CHAPTER I: INTRODUCTION	1
CHAPTER II: LITERATURE REVIEW	4
Stem Cell Biology	4
Embryonic Stem Cells	7
Adult Multipotent Stromal Cells	9
Induced Pluripotent Stem Cells	12
Pluripotency Genes	13
Reprogramming Techniques: Methods to Produce iPSCs	17
Synthetic mRNA Transfection	19
Potential Future for iPSCs	21
CHAPTER III: EFFECT OF TRANSFECTION WITH SYNTHETIC MRNA IN DIFFERENT CELLS TYPES	24
Introduction	24
Materials and Methods	26
Experimental Design	30
Results	34
Discussion	47
CHAPTER IV: SUMMARY AND CONCLUSIONS	51
LITERATURE CITED	53
APPENDIX A	66
Plasmid Preparation	66
Mini Prep Isolation	66
Clone Selection	67
mRNA in vitro Synthesis	67
Cell Collection	70
Lipofectamine Transfections	73
Evaluation of Induction of Pluripotency Treatment	74
RNA Isolation	75

Conversion of mRNA into cDNA.....	76
Calibrator Development	77
Retro Transcriptase Polymerase Chain Reaction	80
Purification of PCR Products	81
qRT-PCR.....	82
Immunocytochemistry.....	83
Flow Cytometry	86
Differentiation into Specific Lineages	87
Evaluation of Trilineage Differentiation.....	89
Cell Proliferation Assay	93
APPENDIX B	94
VITA	107

LIST OF TABLES

B.1 PCR Primers	94
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LIST OF FIGURES

2.1	Reported sites for SCs collection in the horse	6
3.1	Cell morphology under conventional microscope (200x) A: ASCs B: BMSCs C: Fibroblasts	35
3.2	Cell morphology under confocal microscope stained with 488 phalloidin F-actin (green) and Hoechst dye (blue) (200x) A: ASCs B: BMSCs C: Fibroblasts.....	35
3.3	Immunophenotyping with CD44, CD90, CD105 and CD29 of ASCs (A, B, C and D) and Fibroblasts (E, F, G and H) (black histogram) compared to the auto fluorescence of the cell (green histogram)	36
3.4	Multipotent stromal cells after differentiation and stainedwith Oil red O (A), Alizarin Red (B) and Alcian Blue (C)	36
3.5	ASCs (A), BMSCs (B) and fibroblast (C) colonies obtained after 14 days of pluripotency induction treatment compared to their respective controls (D, E and F) under light microscopy (40x)	37
3.6	Close caption of representative colonies at different magnification: 200x (A, B and C) and 400x (D, E and F)	38
3.7	Immunocytochemistry of pluripotency factors after 7 days of treatment with synthetic mRNA to induce pluripotency	39
3.8	Immunocytochemistry of pluripotency factors after 14 days of treatment with synthetic mRNA to induce pluripotency	39
3.9	Expression ratios of pluripotency genes in ASCs determined by qRT-PCR	40
3.10	Expression ratios of pluripotency genes in BMSCs determined by qRT-PCR.....	40
3.11	Expression ratios of pluripotency genes in fibroblast determined by qRT-PCR	40
3.12	(A) Standard curve between arbitrary fluorescence units and cell number (B) Linear regression between arbitrary fluorescence units and cell number	41
3.13	Arbitrary Fluorescence units after incubation of alamarBlue® dye for 2h with induced and not induced ASCs, BMSCs and fibroblast over a time period of 21 days	41
3.14	Adipogenic differentiation of induced and not induced ASCs after cell expansion	42
3.15	Adipogenic differentiation of induced and not induced BMSCs after cell expansion.....	43

3.16	Adipogenic differentiation of induced and not induced fibroblast after cell expansion ...	43
3.17	Osteogenic differentiation of induced and not induced ASCs after cell expansion.....	44
3.18	Osteogenic differentiation of induced and not induced BMSCs after cell expansion	44
3.19	Osteogenic differentiation of induced and not induced fibroblast after cell expansion....	45
3.20	Chondrogenic differentiation of induced and not induced cells after cell expansion	46
B.1	Amplicon size validation for OCT4, KLF4 and 18SrRNA (not used in further experiments).....	94
B.2	Amplicon size validation for KLF4 and SOX2.....	95
B.3	Amplicon size validation for TNC, PAP and HPRT-1	95
B.4	Temperature gradient for the KLF4 primer tested against equine sequence.....	96
B.5	Temperature gradient for the KLF4 primer tested against human sequence	96
B.6	Temperature gradient for the OCT4 primer tested against equine sequence	97
B.7	Temperature gradient for the OCT4 primer tested against human sequence.....	97
B.8	OCT4 qRT-PCR Amplification/Cycle graph.....	98
B.9	OCT4 Standard curve.....	98
B.10	OCT4 Melting curve	99
B.11	KLF4 qRT-PCR Amplification/Cycle graph	99
B.12	KLF4 Standard curve	100
B.13	KLF4 Melting curve.....	100
B.14	SOX2 qRT-PCR Amplification/Cycle graph.....	101
B.15	SOX2 Standard curve.....	101
B.16	SOX2 Melting curve	102
B.17	PAP qRT-PCR Amplification/Cycle graph	102
B.18	PAP Standard curve	103

B.19	PAP Melting curve	103
B.20	HPRT-1 qRT-PCR Amplification/Cycle graph	104
B.21	HPRT-1 Standard curve	104
B.22	HPRT-1 Melting curve.....	105
B.23	TNC qRT-PCR Amplification/Cycle graph.....	105
B.24	TNC Standard curve.....	106
B.25	TNC Melting curve	106

LIST OF ABBREVIATIONS

ASCs	Adipose Derived Multipotent Stromal Cells
ATP	Adenosine 5'-triphosphate
BMSCs	Bone Marrow Derived Multipotent Stromal Cells
CD	Cluster of differentiation
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
ESC	Embryonic Stem Cells
ES-like cells	Embryonic Stem Like Cells
FBS	Fetal Bovine Serum
GTP	Guanosine 5'-triphosphate
HPRT-1	Hypoxanthine phosphoribosyltransferase-1
ICM	Inner Cell Mass
IFN	Interferon
iPSCs	Induced Pluripotent Stem Cells
KLF4	Kruppel-like factor 4
LIF	Leukemia Inhibitor Factor
miRNA	micro RNA
MSCs	Adult Multipotent Stromal Cells
OCT4	Octamer-binding transcription factor 4
PAP	Polyadenylate Polymerase
PI	Propidium Iodide
PFA	Paraformaldehyde

P/S	Penicillin/Streptomycin
qRT-PCR.....	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RT	Reverse Transcription
RT-PCR.....	Reverse Transcriptase Polymerase Chain Reaction
SCs	Stem cells
SCNT	Somatic Cell Nuclear Transfer
SDFP	Superficial Digital Flexor Tendon
SOX2.....	Sex determining region Y-box 2
ssRNA.....	single stranded RNA
STAP.....	Stimulus-Triggered Acquisition of Pluripotency
TNC.....	Tenascin

ABSTRACT

Autogenous and autologous adult multipotent stromal cells are applied to treat equine musculoskeletal injuries in clinical practice. However, options for autologous therapy in the equine specie are restricted due to the limited biological material obtained. Induced pluripotent stem cells represent an alternative to overcome these limitations.

Traditionally, iPSCs are produced by introducing DNA for early embryonic genes into cells with viruses. The method evaluated, avoids the use of viral vectors and potential for exogenous DNA integration into the cell's genome by utilizing a lipid delivery vehicle carrying synthetic mRNA coding for the human pluripotency factors OCT4, KLF4 and SOX2. Cells from different tissue have specific epigenetic profiles that determine their phenotype and functionality; hence they might have a distinct susceptibility to induction of pluripotency. We tested the expansion capacity and potency of equine adult MSCs from adipose tissue, bone marrow, and fibroblast following chemical transfection with synthetic mRNA of human sequences for embryonic genes, OCT4, KLF4 and SOX2. Target gene mRNA and cell protein expression was compared among passage (P) 3 cells from the different tissue sources before and after 7 and 14 days of transduction. Additionally, multilineage capacity and expansion rate were compared in P10 cells. Target protein expression was higher in transduced cells at both time points compared to untreated cells based on immunocytochemistry. Based on qRT-PCR, OCT4 and SOX2 expression was significantly higher in transfected cells after 14 days of transduction compared untreated cells (Oct4: ASCs, $P=0.030$; BMSCs, $P=0.005$ and fibroblast, $P<0.001$; Sox-2: ASCs, $P=0.012$; BMSCs, $P=0.001$ and fibroblast, $P=0.005$). Multilineage differentiation was detected in transfected versus untreated cells both at passage 10 by histological staining after differentiation.

Expansion rates of transduced and untreated cells were not different with the exception of BMSCs ($P < 0.05$). Based on these results, dedifferentiation of equine MSCs with synthetic mRNA increases in vitro potency and expansion capacity.

The ability to induce pluripotency from MSCs obtained from multiple equine tissue sources will significantly increase accessibility to highly characterized stem cells. This will in turn improve the ability to predict cell behavior for customized therapies, becoming an important model for future human applications.

CHAPTER I: INTRODUCTION

Musculoskeletal injuries result in a major economical loss for the equine industry not only due to time away from competition but also because of the high rate of re-injury (Williams et al., 2001; Clegg, 2012). Though the initial tendon injury often heals to some degree, the new tissue has less functionality and greater potential to re-injury than the original (Paris and Stout, 2010). Multipotent stromal cells are an attractive alternative for clinicians to treat tendon injuries, whether obtained from bone marrow or adipose tissue, are reported to improve tendon repair tissue in other species (Behfar et al., 2013). However, questions about practical aspects related to the use of this therapy are still unanswered. For instance, autologous therapy is restricted due to limited biological material that can be harvested and there is a general lack of characterization by immunophenotyping and trilineage differentiation before implantation. Another concern is that cells from different tissues have distinct epigenetic profiles that can result in phenotypically and functionally distinct cells from genetically identical precursors (Aranda et al., 2009) and reducing the predictability of cell behavior in the patient. Differential gene expression is not only seen in stable and discrete cells (differentiated cells) but also in undifferentiated cells. Plus, in vitro culture expansion reduces multipotentiality and regenerative capacity (Zhironkina et al., 2012).

Induced pluripotent stem cells represent an alternative to overcome these limitations. Subjecting MSCs to a pluripotent inducing treatment would permit in vitro culture expansion (Gourronc and Klingelutz, 2012) to obtain material to treat a musculoskeletal injury. In addition, it would permit autologous treatment with a fairly homogenous population of cells in an injured horse. The concept of iPSc introduced by Yamanaka consists of subjecting a differentiated cell to a gene reactivation treatment, generally by introducing genes that are

normally expressed in early embryos (Takahashi and Yamanaka, 2006). Traditionally, iPSCs are produced by introducing early embryonic genes into cells with recombinant DNA carried by viruses. A limitation with use of recombinant DNA is that, unless is directed by another technique, integration into the cell genome is non-specific and can potentially activate endogenous oncogenic sequences. Hence, the use of recombinant DNA in these techniques has significant limitations for clinical translation (Bayart and Cohen-Haguenauer, 2013; Okano et al., 2013). Recently, mRNA transfection with lipofectamine was introduced as an alternative to induce pluripotency (Warren et al., 2010). This method avoids the utilization of viral vectors and introduction of recombinant DNA to obtain induced pluripotent stem cells. One of the goals of this project was to determine if the tissue source of multipotent stromal cells subjected to pluripotency induction treatment would respond differently as determined by expression of introduced genes and allowing cell proliferation without losing multipotent characteristics. In series of investigations, in vitro behavior including expansion capacity and multilineage differentiation was determined in equine adult adipose, and bone marrow multipotent stromal cells as well as adult fibroblasts before and after induction of pluripotency by introduction of embryonic gene mRNA. There is limited information surrounding the response of adult equine ASCs, BMSCs and fibroblasts to transfection for purposes of dedifferentiation. It is especially important for the problem of tenogenesis to confirm improved expansion capabilities and multilineage differentiation of treated cells from all three tissues. To date, there has been no comparison of the induction of pluripotency of different sources of cells before utilization for regenerative therapy by transfection of synthetic mRNA. Also, there is limited information about the potential for induced pluripotency in equine BMSCs and ASCs for the purpose of tenogenesis. The other goal of this study is to provide a side by side comparison of the tenogenic

capacity of adult equine ASCs and BMSCs before and after induction of pluripotency with synthetic mRNA. It is expected that in vitro tenogenesis will be different due to suspected high level of mRNA of tenogenic anabolic gene (tenascin) in ASCs following tenogenic induction in vitro compared to the other cell types prior to induction of pluripotency owing to the fact that ASCs are present in a stromal environment similar to the one in tendons. Further, it is assumed that cells will retain comparable expansion capacities following induction of pluripotency and those differences in tenogenesis will be resolved by the same.

CHAPTER II: LITERATURE REVIEW

Stem Cell Biology

Stem cells are in the focus of the biomedical research field due to their diverse use in therapeutic applications for various disorders and trauma (Terskikh et al., 2006) specially in the area of regenerative medicine (Brehm et al., 2012). These types of cells are appealing since they have the ability to differentiate into diverse cell types, as well as the capacity of self-renewal, while maintaining their characteristics. However, the number of distinct tissues they can differentiate into and the ability to keep their properties in prolonged culture are subjected to their previous potency state and their plasticity (Zaidi and Nixon, 2007). Hence, we have different types of SCs that can be mainly classified according to their potency they could be classified in totipotent, pluripotent, multipotent and unipotent cells (Spencer et al., 2011). This potency state determines the multiplicity of tissue types a certain cell type can derive. The higher the potency, the grater the types of tissue they can form. Plasticity is defined as the ability to give rise to cell types different to the ones is determined by their lineage commitment (Lakshmipathy and Verfaillie, 2005). A totipotent cell has the ultimate plasticity, since it is not committed to any lineage and can form all the tissues in an organism; this includes the embryonic and trophoblastic cells, which would form the embryo and part of the placenta respectively. Pluripotent cells are characterized for being able to form all embryonic tissue types (endoderm, mesoderm and ectoderm lineage) but lack the capacity to differentiate into trophoblast. Multipotent cells are more committed than the latter, since they naturally can only give rise to a specific spectrum of cell types determined by the embryological lineage they followed. Normally they are in charge of replacing damaged cells due to physiological turnover or injury of the tissue they reside (Bjornson et al., 1999). Thus, they are the precursors of specialized cells (Zaidi and Nixon, 2007). While the commitment into a specific lineage increases the plasticity is reduced, that is

why unipotent cells, also known as progenitor cells, are the least plastic and cannot self-renew. Hence we could state that the types and number of cells into which SCs can develop are determined by their potency state. However, there have been reports of cells being able to transdifferentiate across lineage once they have been committed into one (Bjornson et al., 1999; Polak and Bishop, 2006), and of cells that de-differentiate gaining a higher potency state (Molchadsky et al., 2010; Tapia and Schöler, 2010). These phenomena are leading to ongoing reconsiderations not only of the definitions of potency and plasticity, but also of the whole concept of stem cell (Lakshmipathy and Verfaillie, 2005; Polak and Bishop, 2006).

SCs could also be classified based on the source as they can be isolated from embryos, fetuses or from adult tissue (Polak and Bishop, 2006). In adult horses several reports stated that adipose tissue (de Mattos Carvalho et al., 2009; Braun et al., 2010; Pascucci et al., 2011; Carrade et al., 2012; Ranera et al., 2012; Burk et al., 2013; Radtke et al., 2013; Reed and Johnson, 2014), bone marrow (Frisbie et al., 2009; Crovace et al., 2010; Lee et al., 2010; Godwin et al., 2012; McCarthy et al., 2012; Ranera et al., 2013; Watts et al., 2013; Lange-Consiglio et al., 2013b), peripheral blood (Marfe et al., 2012; Spaas et al., 2013a; Spaas et al., 2013b) amnion (Lange-Consiglio et al., 2013a; Lange-Consiglio et al., 2013b), amniotic fluid (Sang-Bum et al., 2011), umbilical cord tissue (Hoynowski et al., 2007; Corradetti et al., 2011), umbilical cord blood (Koch et al., 2007; Reed and Johnson, 2008; Toupadakis et al., 2010; De Schauwer et al., 2013a; Reed and Johnson, 2014), tendon (Stewart et al., 2009), muscle (Stewart et al., 2009; Radtke et al., 2013), periosteum (Radtke et al., 2013), and cornea (Moriyama et al., 2013) could be used as sources of SCs (Fig 2.1). In this case, since cells are isolated from a fully differentiated tissue and they have the property of cells renewal plus differentiation into a specific lineage are

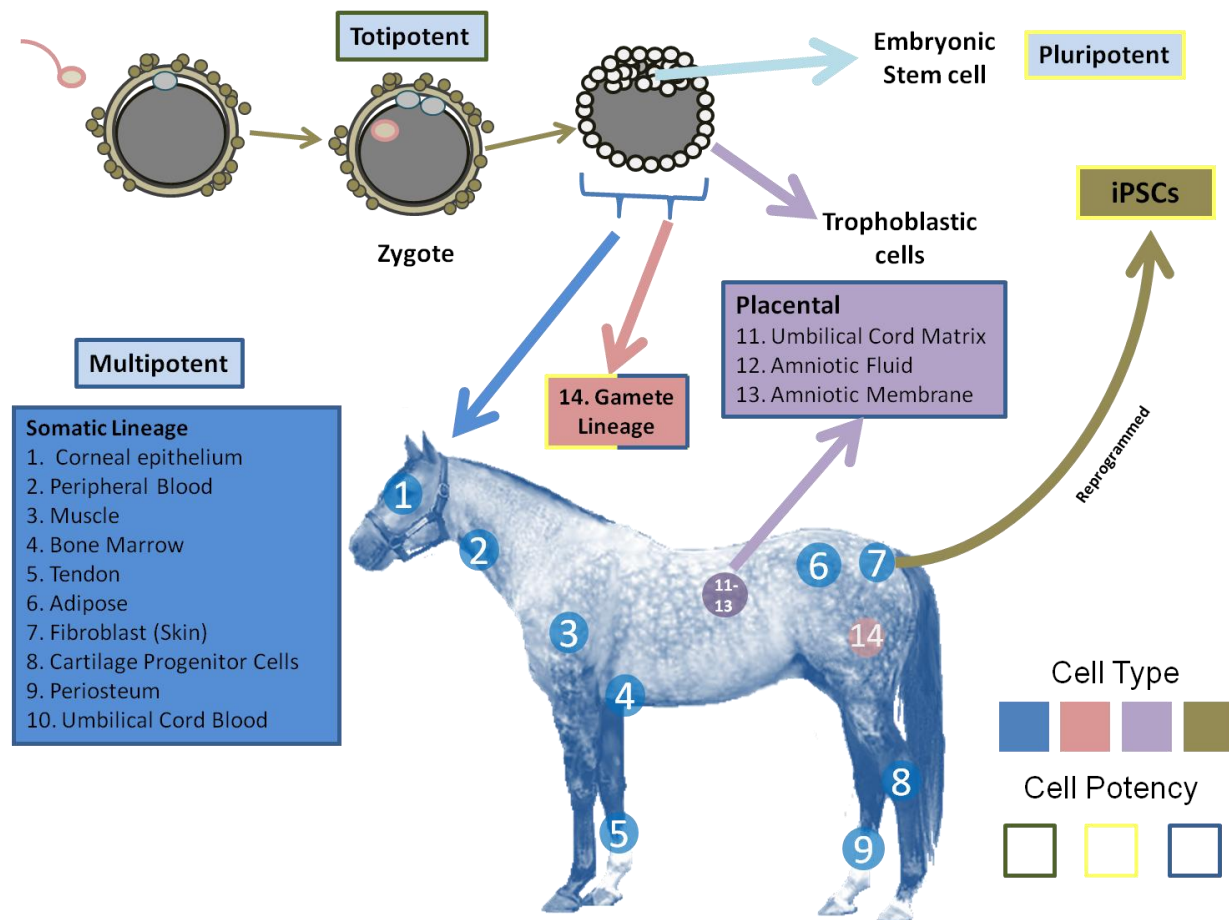


Fig 2.1 Reported sites for SCs collection in the horse

classified as MSCs. Stem cells have also been isolated from equine embryos in the blastocyst stage (Saito et al., 2002; Li et al., 2006; Guest and Allen, 2007; Guest et al., 2010) by physical or immune dissection of the inner cell mass (Keefer et al., 2007). SCs derived from embryos are classified as ESCs and present characteristics of SCs in a pluripotent state. Stem cells with similar properties have been isolated from embryos in a further developmental stage when obtained from primordial germ cells and named embryonic germ cells (Lakshmipathy and Verfaillie, 2005). Totipotent cells are only present in the period of time from fertilization to the initiation of embryonic divisions.

Thus, it can be summarized that totipotent cells occur only in the first stages of embryo development, pluripotent in later embryonic development, multipotent in late fetal and fully developed (adult) tissue stages, and unipotent, cells in adult stages.

These classifications SCs can be separated based on whether they occur naturally or induced. In 2006, Dr. Yamanaka was the pioneer of the technique induction of pluripotency when he introduced exogenous genes coding for transcription factors normally expressed in embryos, obtaining stable colonies of cells resembling those from ESCs that were called induced pluripotent stem cells (Takahashi and Yamanaka, 2006).

A recent publication showed that an appropriate non-specific exogenous stimulus, such as a reduction in the pH, is enough to induce changes in a fully committed cell converting them into a pluripotent state. After this phenomenon was reported, a new term was coined: stimulus-triggered acquisition of pluripotency (Obokata et al., 2014). Strong similarities were observed in this work when the properties of the STAP stem cells were compared with ESCs. Further studies are needed to determine if any other exogenous stimulus that are known to increase expression of pluripotent genes, Trituration and cell membrane damage with streptomycin O are able to induce the formation of stable STAP stem cells colonies (Obokata et al., 2014).

Embryonic Stem Cells

The first reports of derivation of ESCs in mammals were in 1981 in mice (Evans and Kaufman, 1981; Martin, 1981). Since then, different research groups tried to replicate the isolation in domestic species and in humans. Similar characteristics were observed between mouse ESCs and primate ESCs (Thomson et al., 1995; Thomson et al., 1998) namely: infinite self-renewal, differentiation into the three germ layers, teratoma formation in vivo and embryo body formation.

However, not all the characteristics met with mouse ESCs were demonstrated by primate ESCs, such as incorporation into the blastocyst ICM by chimera formation or the incorporation into the germ line was proved. These criteria were also not met with ESCs isolates in domestic species (Keefer et al., 2007). This led to the finding that there are two types of ESCs derived from the ICM: those derived from the early formed epiblast, derived from the ICM cells that are not exposed to the blastocyst cavity, called naïve ESCs (also known as “truly” ESCs) and those derived from the post-implantation epiblast, known as primed ESCs (Nichols and Smith, 2009; De Los Angeles et al., 2012). Thus, this poses the question of whether the isolated cells in non rodent species (Keefer et al., 2007), especially in the equine, are truly ESCs (Paris and Stout, 2010). As such, the cells are often designated as embryonic stem-like cells.

In the case of the horse, isolation of ES-like cells were performed from in vivo produced embryos (Saito et al., 2002; Li et al., 2006; Guest and Allen, 2007; Guest et al., 2010). However, the separation between epiblast and hypoblast (cells derived from the ICM cells that are exposed to the blastocyst cavity) occurs around day 7 in the horse (Enders et al., 1993) when embryos are typically recovered from the mare, showing the difficulties that are imposed in equine ESCs research. This could explain why several groups tried to isolate and culture equine ESCs with no published reports of successful isolation of ESCs (Paris and Stout, 2010). The limitations with trying to use ESCs in regenerative medicine do not seem to end there.

One of the characteristics of truly ESCs is the formation of teratomas when transferred to immunocompromised mice. However, no teratoma formation was reported when these cells that met all the others requirements for considered them truly ESCs were transplanted in immunocompromised mice (Li et al., 2006). Although, it has been stated that tumor formation by truly ESCs is more common when the host is of the same species as the derived ESCs (Erdö

et al., 2003), no teratoma formation was observed after equine ES-like cells were transferred for 90 days into mice (Guest et al., 2010). The lack of teratoma formation observed using equine embryonic derived stem cells might be due to a lack of potentiality properties from these ES-like cells or maybe is a special feature of equine ESCs compared with properties reported in human or mouse ESCs.

Immune rejection is a concern that scientist are working to remedy and this response is hampering the advances of therapies with ESCs is a possible immune rejection from the host after transplantation of these cells (Takahashi and Yamanaka, 2006). Only one group has tested the immune response using equine ESCs in an homologous host (Guest et al., 2010). Although their findings suggest that no immune reaction was induced by the transplantation of stem cells derived from embryos, it is important to keep in mind that these cells were not probably truly ESCs. Migration of ESCs after transplantation has been reported in other species (Srivastava et al., 2006). Thus, this is another factor to consider when evaluating potential ESCs therapies, if migration is unwanted in the host.

Last but not least, ethical concerns of using ESCs for regenerative therapies are always present, because the isolation of these cells implies the destruction of a viable embryo. The removal of the ICM from a blastocyst interrupts the formation of a new organism, depicting a controversial fine line of when life begins (Bahadur et al., 2010). Even though ESCs present characteristics that would make them an interesting tissue source for regenerative therapy, certain aspects are a clear barrier for the application in the regular clinic.

Adult Multipotent Stromal Cells

The use of MSCs in regenerative therapy not only appears to be an appealing alternative to ESCs but also it is the type of SCs that has been studied the longest. First reports of isolation

of MSCs date to 19th century studies however the majority of the work was performed within the last few decades (Friedenstein et al., 1968; Dominici et al., 2006; Spencer et al., 2011). However, some aspects of MSCs are still undefined introducing a high variability between studies setting hurdles for clinical applications and a need for uniformity (De Schauwer et al., 2011).

As mentioned before, there are different sources where MSCs can be isolated. Likewise, they have the advantage that the isolation techniques, in most cases, are fairly simple and tested in several species including the human (Vidal et al., 2006) and the ethical issue of destroying embryos to obtain SCs would be avoided.

Nevertheless, the fact that diverse types of tissue are accessible for isolation of MSCs it has been reported that cell variations between and within isolates are detectable (Giovannini et al., 2008; Reed and Johnson, 2008).

There are different protocols of isolation (Siddappa et al., 2007) and differences in collection are believed to be of the factors adding to the variability observed between cell isolates (Vidal et al., 2007; Bourzac et al., 2010) turning it into an important factor to consider when designing an applied therapy with these cells. Even SCs isolated from the blastocyst inner cell mass may vary in developmental potential (Thomson et al., 1998), but this seems to be worse in the case of MSCs since heterogeneity among cells within isolates increases with donor age due to commitment toward specialization (Siddappa et al., 2007).

Another negative aspect reported, was that undifferentiated cells isolated from a given tissue retained characteristics of the tissue of origin due to epigenetic marks, restraining cell differentiation capability (Eilertsen et al., 2008). This is also supported by studies performed in the horse that reported that MSCs have a tendency to differentiate into the tissue that was harvested (Toupadakis et al., 2010; McCarthy et al., 2012). This factor known as cell

commitment implies that in order to have a cell product with the highest potential available one would have to isolate SCs from the same type of tissue as that tissue needing treatment, situation that is not always feasible.

Another issue of feasibility when trying to apply MSCs in a normal clinical situation is the difference in time between the occurrence of the lesion and the initiation of treatment (Guest et al., 2010). However, this could be solved by cryopreservation of cells of horses that are in sport activities and are more prone to injuries. In fact it was demonstrated that the characteristics and functions of cryopreserved MSCs are not different from fresh (Haack-Sorensen et al., 2007). Recent publications state that transplantation of undifferentiated cells produce trophic factors that reduce the immune response of the host, turning the application of allogeneic cells an alternative to autologous therapeutic treatments (Toupadakis et al., 2010; De Schauwer et al., 2014).

Since most musculoskeletal injuries in the horse require large amounts of biological material to repair, the expansion capacity of the MSCs is vital (Vidal et al., 2007). Not only in vitro expansion of MSCs needs to be analyzed but also the evaluation of in vivo migration and proliferation should be considered. Reports in the equine pointed out a different cell propensity to division when comparing ES-like cells and MSCs (Guest et al., 2010). In an in vivo model of an injury in the superficial digital flexor tendon side by side comparison of undifferentiated ESCs and MSCs transplantation showed that ESCs had better survival than MSCs at all time points examined with <5% of the MSCs injected cells surviving after 10 days (Guest et al., 2010). They hypothesized that the lack of proliferation might be due to the inability to divide indefinitely caused by cell senescence. These findings suggest that MSCs are not differentiating into tenocytes and becoming part of the regenerated tissue, supporting the idea that the

improvement in scar formation when MSCs treatment are compared with controls is due to the recruiting of native cells by the secretion of growth and immunomodulatory factors (Burk et al., 2013; De Schauwer et al., 2013b). It is due to determine if turning MSCs into cells that not only modulate but that also become part of the final tissue using genetic engineering techniques, would improve the healing process.

It appears that MSCs could be a great alternative to the ESCs for regenerative therapies when considering immunogenicity, collection and ethical issues. However, certain features of adult stem cells such as their limited potency, the inability to successfully expand in vitro without reducing their multipotency characteristics, their cell origin retention and variation between isolates make them an inconsistency and unpredictable source of cells for tissue regeneration. In search of a treatment that might start to solve these conundrums, induction of pluripotency qualifies as candidate since gaining cell potency and self-renewal could be achieved with this technique.

Induced Pluripotent Stem Cells

The concept of iPSCs introduced by Yamanaka in 2006 consists of subjecting a differentiated cell to a gene reactivation treatment, generally by introducing genes that are normally expressed in early embryos (Takahashi and Yamanaka, 2006). This treatment changes the potency state of the cell to one of pluripotency, closely resembling the state of ESCs. This undifferentiated state allows for cell expansion a characteristic lost during cell differentiation. The major goal of this technique is to establish an environment where the exogenous pluripotent genes are expressed and lead to the activation of the endogenous counterpart. A subsequent inactivation of the introduced genes is needed to guarantee that the cells were truly induced to pluripotency. Of the genes evaluated that are normally expressed in early development

Yamanaka in 2006 narrowed down the list to only four factors needed to induce pluripotency: OCT4, SOX2, KLF4 and c-MYC. When one of these genes from the cocktail is removed no colonies of iPSCs are formed (removal of OCT4, SOX2 or KLF4) or their characteristics do not resemble those observed in ESCs (removal of c-MYC) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The genes used for induction of pluripotency code for transcription factors that work together to maintain ESCs in a pluripotent state. After the first reports of iPSCs derived from mouse and humans cells, this technique was repeated only in domestic species (including the equine specie) (Nagy et al., 2011; Khodadadi et al., 2012; Breton et al., 2013) and those that are endangered (Ezashi et al., 2009; Shimada et al., 2010; Ben-Nun et al., 2011; Han et al., 2011; Koh and Piedrahita, 2014) showing that these factors are part of conserved mechanisms for pluripotency maintenance.

Pluripotency Genes

The mechanism involved in the regulation of pluripotency has not been completely elucidated and most of the studies were performed using ESCs. It has been stated that OCT4, SOX2 and NANOG are the core regulatory factors in ESCs (Boyer et al., 2005), however, the addition of NANOG to the reprogramming cocktail appears to be dispensable (Takahashi et al., 2007). The OCT4 factor also known as POU5F1(POU class 5 homeobox 1) appears to be the more important of them since it is necessary for the activation of the other transcription factors (Pesce and Schöler, 2001). It binds not only to 623 promoters in genes coding for diverse proteins but also to its own regulatory region creating a positive feedback for its expression (Boyer et al., 2005). This particular feature is needed for the reactivation of the endogenous OCT4 gene by exogenous translated sequence, one of the objectives of the induction of pluripotency technique. This positive feedback has been reported for SOX2 and NANOG, which

regulate 1271 and 1687 promoters, respectively. It has been determined that these three transcription factors interact in the regulation of 353 genes (Boyer et al., 2005). It was reported that as embryo development progresses expression of OCT4 is restricted to the ICM without expression in the trophoblastic cells. This regulation of the expression of OCT4 appears to be closely orchestrated, because differential expression of this factor directs the further tissue differentiation (Niwa et al., 2000). Expression of OCT4 has been detected in equine MSCs derived from different tissues namely bone marrow, adipose tissue and chord tissue and blood (Reed and Johnson, 2008; Ranera et al., 2012; Seo et al., 2013) adding more evidence to the statement that MSCs have an intermediate state of potency between multipotency and pluripotency gaining the ability of transdifferentiation as we discussed earlier.

One of the other agents regulating cell pluripotency and differentiation is the sex determining region Y-box 2 gene (Avilion et al., 2003). It is detected during early embryo development through the morula stage of mouse embryos (Niwa et al., 2000) and has a high relevance directing tissue differentiation into the neuronal and extra-embryonic ectoderm and gut endoderm (Avilion et al., 2003; Adachi et al., 2010). As seen with OCT4 expression, deregulation of SOX2 leads to predominant formation of trophoblastic cells or altered lineage differentiation (Zhao et al., 2004; Masui et al., 2007; Adachi et al., 2010).

However, one of the most important characteristics of SOX2 is the interaction with OCT4 in maintaining the pluripotency state in ESCs, because they work synergistically to activate Oct–Sox enhancers. These enhancers increase the expression of pluripotent stem cell- specific genes, such as NANOG, OCT4 and SOX2 itself. Using SOX2 knock out mouse models it was determined that SOX2 is necessary for maintaining sufficient OCT4 levels in ESCs (Masui et al.,

2007). This close interaction is such that OCT4 levels also influence the expression of SOX2 (Hattori et al., 2004).

The last member of the triad is NANOG, a homeodomain protein that directs the propagation of undifferentiated cells (Chambers et al., 2003). The transcription factor coded by this gene has an importance implication in the endoderm differentiation of ESCs. When the expression of NANOG is down-regulated the pluripotent cells in the epiblast differentiate to endodermic cells (Mitsui et al., 2003). Thus, this factor is another key component for maintaining the pluripotent state in ESCs.

The interaction OCT4-SOX2-NANOG is a complex set of connected pathways that control pluripotency, self-renewal, and cell fate determination (Kuroda et al., 2005; Rodda et al., 2005; Loh et al., 2006).

Krüppel-like factor 4 and v-myc avian myelocytomatosis viral oncogene homolog are the others factors reported by Yamanaka to be necessary for inducing pluripotency of adult somatic cells (Takahashi and Yamanaka, 2006). These two factors are normally related with cell proliferation and renewal and due to this properties they are normally identify in tumors, thus frequently know as oncogenes (Huangfu et al., 2008). Genomic changes involving c-MYC such as mutations, overexpression, rearrangement and translocation are present in different hematopoietic tumors, including Burkitt lymphoma (Mangani et al., 2013). It is reported that c-MYC not only has a great influence in gene control due to the large number of receptor sites in the genome (Cawley et al., 2004) but also in epigenetic control. The interaction of c-MYC with the histone acetyltransferase complex p300/CBP is suspected to be one of the reasons for the presence of this gene in the reprogramming cocktail because it increases the ability of other transcription factors (such as OCT4 and SOX2) to bind to their respective sites in the genome

(Faiola et al., 2005; Takahashi and Yamanaka, 2006). The interaction between transcription factors such as OCT4 and the recruitment of this complex for the regulation of gene expression by epigenetic modifications has been demonstrated showing a synergic relation between c-MYC and OCT4 (Hochedlinger and Plath, 2009). KLF-4 has also been implicated in cell cycle control leading to potential tumor formation when this gene is altered (Ray et al., 2013). Its normal function is apoptosis induction via the bcl-2/bax pathway, thus controlling possible tumorigenic cells (Li et al., 2010). However, it also stimulates cell proliferation as it represses the protein p53 which is considered to be a tumor suppressor gene (Rowland et al., 2005). Apart from regulating cell cycle, KLF4 controls the expression of SOX2 and OCT4, which also regulates NANOG (Wei et al., 2009).

Due to the described propensity of tumorigenic formation of KLF4 and c-MYC ongoing studies are attempting to identify chemicals to produce the epigenetic changes initiated by these transcription factors (Huangfu et al., 2008; Lyssiotis et al., 2009; Li and Rana, 2012; Hou et al., 2013). The production of equine iPSCs cells without the presence of c-MYC in the reprogramming cocktail has been reported (Khodadadi et al., 2012); therefore we used this approach when defining our treatments in these experiments.

These findings are leading to a different approach for cellular reprogramming based entirely on small chemical molecules. However, is due to determine if these chemical compounds produce a broad and unspecific epigenetic editing that might lead to deleterious effects when compared with the specific protein function carried by the introduction of sequences coding for specific transcription factors.

Reprogramming Techniques: Methods to Produce iPSCs

The addition of exogenous genes is traditionally performed by transduction, a viral mediated transfection, as it relies in the intrinsic capacity of viruses to introduce genes into the host cells. Different types of viruses have alternative strategies to induce the transcription and/or translation of their sequences by the cell's machinery. The most frequent type of virus used for the induction of pluripotency technique is a lentivirus (a subtype of retrovirus), since they have the ability to affect different types of cells regardless the stage of their cell cycle. Normally these viruses are engineered such that they lack the information that causes pathological changes in a cell and lead to the formation of new viral particles, and they are labeled as replication-defective virus. Hence, these infections are considered terminal. This approach for gene delivery allowed the development of different protocols used for induction of pluripotency as well as sufficient iPSCs to test culture media preparation (Yamanaka, 2012). Another characteristic of this type of virus is its ability to incorporate these sequences into the host genome, a phenomenon known as DNA recombination. This allows the sequence not to be diluted during subsequent cell division. However, this property that might seem, at a first glance, as an advantage posed a question in whether this technology was going to be applied in regenerative therapy (Bayart and Cohen-Haguenauer, 2013; Okano et al., 2013), since most of the protocols involve a non-specific DNA integration that could potentially activate endogenous oncogenic sequences or induced insertional mutagenesis resulting in an altered cell cycle (Kim et al., 2008). One of the genes normally used in this process is oncogenic, c-MYC, leading to reported tumors in derived mouse during evaluation of chimera formation and germline transmission (Okita et al., 2007). Later reports stated that mice derived from retroviral obtained iPSCs present this problem when the exogenous sequence of c-MYC is not silenced (Aoi et al., 2008; Nakagawa et al., 2008;

Yamanaka, 2012). Since the goal is to develop a source of SCs to use in regenerative therapy, results and conclusions obtained from mouse studies should not be freely extrapolated to expected outcomes in other species, thus alternatives to circumvent the DNA recombination protocols must be identified. Hence, different approaches were developed to avoid the use of recombinant DNA such as plasmids (Okita et al., 2008; Okita et al., 2011), Sendai virus (Fusaki et al., 2009), adenovirus (Stadtfield et al., 2008), synthesized RNAs (Warren et al., 2010), proteins (Kim et al., 2009) and small molecules (Hou et al., 2013).

It has been reported that independent of the type of virus used for generating iPSCs, whether if it uses a recombinant or non-recombinant strategies. Immunological response from the host was detected when cells obtained from the same animal were transferred after the induction of pluripotency treatment using viral particles (Zhao et al., 2011). Apparently the inability of a complete removal of all the viral factors after obtaining iPSCs (after several passages) with virus types that replicate constitutively like the Sendai virus are another reason for lowering the rating of this techniques when considering cell therapy (González et al., 2011).

Previous reports of induction of pluripotency using viral vectors showed that using specific exogenous sequences of pluripotency related genes to activate the endogenous genes can be applied throughout a variety of species, indicating the robustness of this technique (Yamanaka, 2012). As well as providing aid to developing a protocol without the use of feeder cells for synthetic mRNA utilization (Warren et al., 2012).

Reports establishing a virus-free technique for obtaining iPSCs based on episomal systems still have the problematic use of DNA that could potentially integrate into the host genome (Miyoshi et al., 2011). Those approaches involving removable transposon like PiggyBac

were reported to produce insertional mutations due to an integration bias for specific parts of the genome (Davis et al., 2013).

Although the use of proteins and small molecules to induce reprogramming to pluripotency looks promising from a future therapy perspective, the low efficiency of induction and the difficulties of reproducible purifications and with sufficient amount for a treatment, makes them an expensive and poorly practical technique (González et al., 2011).

Recently a ground breaking report stated that iPSCs could be obtained by subjecting cells to stressing exogenous stimulus (Obokata et al., 2014). As we mentioned earlier this technique, stimulus-triggered acquisition of pluripotency, still needs to be validated to determine if unspecific gene reprogramming has the same robustness that the other pluripotency induction techniques have.

The use of synthetic mRNA to obtain iPSCs appears to circumvent all these limitations and may become an important tool for tissue engineering.

Synthetic mRNA Transfection

The RNA transfection system was developed to induce pluripotency without the need of plasmids or viral vectors (González et al., 2011). It is based in the incorporation of a foreign molecule of RNA coding for the pluripotency related factors. This molecule will then be translated by the cell into functional proteins. In order to synthesize this molecule in vitro, plasmids carrying a DNA copy of the gene of interest are subjected to a transcription step performed by an RNA polymerase. The synthetic mRNA molecules obtained have to resemble those that are normally transcribed by an eukaryotic cell in order to be processed and to avoid the endogenous antiviral cell response to single stranded RNA, a cell mechanism conserved to eliminate RNA virus (Warren et al., 2010; González et al., 2011). The addition of a 5' guanine

cap and a poly (A) tail to the transcript are required for initiation of translation. Likewise, the use of modified bases is mandatory to escape from the intracellular immune response and the production of type 1-interferon genes (Angel and Yanik, 2010; Warren et al., 2010; McLenachan et al., 2013). The stimulation of the IFN signaling primes the cells for apoptosis (McLenachan et al., 2013) reducing cell number specially in protocols requiring multiple transfections. The uncapped 5' end of mRNA molecule displays free triphosphates reported to be one of the agents stimulating the immune response (Warren et al., 2010). Incorporation of modified nucleotides bases during the RNA in vitro step synthesis substituting 5-methylcytidine for cytidine and pseudouridine for uridine (González et al., 2011), resembling the vast posttranscriptional modifications that endogenous mRNAs experience eukaryotic cells (Warren et al., 2010).

The delivery of mRNA into mammalian cells can be performed via electroporation or complexing the RNA with a cationic vehicle to facilitate uptake by endocytosis (Warren et al., 2010). In order to maintain protein expression levels of these pluripotency related factors that could switch-on the expression of endogenous genes (Yakubov et al., 2010) a repeated treatment needs to be performed due to the short life of the transcriptions factors and the mRNA (Warren et al., 2010). Since a repetitive delivery of mRNA is needed, the use of a cationic vehicle such as lipofectamine is the best approach of the two available mechanisms (Warren et al., 2010; González et al., 2011; McLenachan et al., 2013).

The treatment protocol employed for obtaining iPSCS with synthetic mRNA was thoroughly evaluated regarding timing, ratio and concentration of the transcripts (Warren et al., 2010; McLenachan et al., 2013). No toxicity effect in cells treated with lipofectamine alone was reported (Warren et al., 2010; McLenachan et al., 2013). Although most of the limitations involved in reprogramming of adult cells are addressed by transfection with mRNA, the high

gene dosages of the reprogramming factors resulting from direct mRNA delivery may represent an oncogeneic risk (González et al., 2011) specially in those systems that use c-MYC, an oncogenic factor per se. It is to determine if this protocol could be utilized for different species and cell types.

Furthermore, the stepwise analysis of the inclusion of different factors at different time points to further understand their relevance as well as their sequential activity is manageable with this approach, because different mRNA cocktails could be prepared and delivered using the same method (Warren et al., 2010). Not only for reprogramming to pluripotency but also for directing cell differentiation in an specific manner different than one commonly used with media supplementation, because it has been reported that differentiation of pluripotent stem cells towards muscle cells can be induced with mRNA coding for specific myogenic factors (Warren et al., 2010).

Reports utilizing transfection of microRNAs to induce pluripotency show the strength of this technique (Miyoshi et al., 2011). These ribonucleotides are known regulators of gene expression and are highly expressed in ESCs controlling pluripotency related genes (Houbaviy et al., 2003; Miyoshi et al., 2011). Using specific miRNA alone without the transcription factors can reprogram somatic cells to iPSC, but with a great degree of mosaicism between cells (Miyoshi et al., 2011; Heng et al., 2013).

Potential Future for iPSCs

One of the most important reasons for investing research time and resources in streamlining the techniques for induction of pluripotency is that they do not have the ethical concerns of ESC. Furthermore, the possibility of developing autologous therapies and of costumed made cells for studying a specific pathology it is appealing for tissue regeneration and

disease modeling research areas (Kriks et al., 2011; Yamanaka, 2012). The latter seems to be a promising field not only for the increasing number of publications in the last years but also for the importance of developing cures for devastating maladies (Yamanaka, 2012). Patient specific iPSCs were used to recreate the behavior of cells caused by different diseases such as Parkinson's, Alzheimer's and schizophrenia (Brennand et al., 2011; Devine et al., 2011; Israel et al., 2012; Yamanaka, 2012). Cells induced to pluripotency could also become a step in gene editing procedures for restoring faulty genes of specific diseases (Zhu et al., 2013), setting an important background for genetic disease in the horse like hyperkalemic periodic paralysis, glycogen branching enzyme deficiency and type 1 polysaccharide storage myopathy (Tryon et al., 2009). Moreover, wild approaches like microinjecting rat iPSCs into mouse blastocysts presenting genetic modifications for the development of such organ were also demonstrated for developing entire organs in a heterospecific host (Kobayashi et al., 2010).

Induced pluripotent stem cells have a promising future not only in the field of regenerative medicine. Endangered species best represents the importance of maintaining the health and well being of any valuable specimen that should be addressed with all possible resources and state-of-the-art treatments. Somatic cell nuclear transfer is an important reproductive assisted technique for the preservation of endangered animals. Since the availability of oocytes from endangered species is scarce the need for using related species (in this case *Equus caballus*) as egg donor for heterospecific cloning is necessary. Using enucleated oocytes of a different species reduces the efficiency of SCNT due to cytoplasmic incompatibility. It has been reported that the success rate of cloning could be improved depending on the type of cell used as nuclear donor (Wakayama T., 2007). A pluripotent cell would reprogram to totipotentiality with a greater efficiency than a differentiated cell during the cloning procedure.

Moreover, cloning procedures utilizing intracytoplasmic injection of a plasmid carrying the pluripotency genes into the oocyte prior to enucleation were reported in the horse with statistically significant increase in blastocyst rate and higher pregnancy rates (Olivera et al., 2013). Thus, the use of a safe technique to induce pluripotency like synthetic mRNA, would aid in developing protocols to improve the efficiency of equine SCNT while beneficial for endangered species such as the Przewalski's Horse (*Equus ferus ssp. Przewalskii*) or the Grevy's Zebra (*Equus grevyi*) in an heterospecific cloning program.

CHAPTER III: EFFECT OF TRANSFECTION WITH SYNTHETIC MRNA IN DIFFERENT CELLS TYPES

Introduction

Equine multipotent stromal cells are routinely applied to treat tendon lesions in clinical practice. However, autologous therapy is unpredictable due to individual differences in cell multipotentiality (Aranda et al., 2009). Furthermore, time is required for cell expansion prior to clinical application. A readily available and fully characterized cell product will obviate these limitations to cell therapy. In order to solve these issues, a pluripotency induction treatment would be an interesting approach. It would allow the possibility of expanding cells without losing their multipotentiality characteristics in order to have enough biological material not only to treat a musculoskeletal injury but also to fully characterize the cells used in therapy before applying them.

Different techniques to induce pluripotency have been reported (González et al., 2011). However, the use of synthetic mRNA appears to be the alternative more suitable if these cells are intended to be used in a clinical application. This is due to the fact that non recombinant DNA is involved in this technique, avoiding the threats implied with traditional viral reprogramming such as activation of endogenous oncogenic sequences or induction insertional mutagenesis (Kim et al., 2009).

Delivery of mRNA into the cell can be performed via electroporation or complexing the RNA with a cationic vehicle to facilitate uptake by endocytosis (Warren et al., 2010). The proteins coded by the genes used to induce pluripotency have a short half life in the cells and because no stable DNA sequence is used in this technique, a repeated transfection is needed to supply enough transcripts until reactivation of the endogenous pluripotency sequences occurs by self-regulation (Yakubov et al., 2010). The process required to electroporate cells implies to have

them in suspension, adding an extra stress to the electrical stimulus that the cells are suffering: an enzymatic treatment to detach the cells from the culture surface every time they have to be transfected. Thus, the use of a cationic vehicle such as lipofectamine, appears to be the alternative.

Exogenous mRNA sequences are not innocuous. Specific viruses use the strategy of delivering their genomic material as ribonucleotides to be used by the host cell's translational machinery. Hence, mammalian cells have active mechanisms to face the presence of an exogenous mRNA molecule. To avoid the immunological cell response, the synthetic mRNA used in the pluripotency treatment needs to be modified. For that reason, modified bases were used during the *in vitro* transcription process. The cytosine and uracil bases are replaced by 5-methylcytidine-5'-triphosphate and pseudouridine-5'-triphosphate bases respectively, to reduce the immune response from the treated cell.

The pluripotency genes normally used for inducing differentiated cells into a higher potency state are OCT4, SOX2, KLF4 and c-MYC (Takahashi and Yamanaka, 2006). However, reported changes in the expression of c-MYC are involved in tumor formation and specific diseases (Mangani et al., 2013). Likewise, successful induction of pluripotency utilizing equine cells was achieved without the inclusion of c-MYC in the reprogramming cocktail (Khodadadi et al., 2012). Hence, the lipofectamine treatment was carried out only with the first three mentioned factors.

This study was designed to provide an analysis of the response of different tissue sources such as adult equine ASCs, BMSCs and fibroblast to an induction of treatment with synthetic mRNA. Their ability of translating an exogenous mRNA sequence as well as the correct localization of these proteins was assessed. The gain of a higher potency state was evaluated by

gene expression and immunocytochemistry. The ability of maintaining their multipotency state after a simulation of cell expansion until passage 10 was determined. Plus, their proliferation capacity was evaluated by a cell viability assay after 21 days of culture.

Furthermore, the capability of differentiating into a clinically important tissue in the horse such as tenogenic tissue was compared between cells types before and after treatment with the pluripotency genes.

The information from this study will be vital for controlled clinical trials of stem cell therapies in the horse as well as for creating a future treatment model for musculoskeletal injuries in humans.

Materials and Methods

Cell isolation

BMSCs, ASCs and FSCs were harvested from adult horses (n=4), isolated and culture expanded using standard procedures (see Appendix A, “Cell Collection”). Original cell isolates were cultured to P2 in DMEM-F12 supplemented with 5% fetal bovine serum and 1% antibiotic-antimycotic medium (Stromal medium) with a 5000 cell/cm² seeding density and passage at 70% confluence under standard culture conditions (5% CO₂ in air, 37°C) and cryopreserved in 90% FBS with 10% DMSO and stored in cryogenic vials at -80°C.

Immunophenotype

Immunophenotyping of the isolated cells was performed before the induction to pluripotency treatment using flow cytometry. (see Appendix A, “Flow Cytometry”). Cells were incubated with antibodies against PE-CD105, CD29, PE-CD90, and CD44 for 30 minutes at room temperature. A secondary antibody was used for unconjugated antibodies,

FITC-conjugated goat anti-mouse IgG for 30 minutes. For autofluorescence, control cells will not be incubated with antibodies.

Cell morphology

Morphology of the primary cultures was assessed by immunocytochemistry using the 488 phalloidin F-Actin staining for filamentous actin to evaluate the cytoskeleton and counterstain with Hoechst 33258 to determine nucleus location (see Appendix A, “Immunocytochemistry”).

Differentiation into specific lineages

Cell multipotentiality was determined before the induction treatment by differentiation into the chondrogenic, osteogenic and adipogenic lineage using specific media. For adipogenic differentiation the induction media consisting of: DMEM-Ham’s F12, 10% FBS, 33 μ M biotin (Sigma, B4639), 17 μ M pantothenate (Sigma, P5155), 2 μ M insulin (Sigma, I5500), 1 μ M dexamethasone (Sigma, D4902), 500 μ M isobutylmethylxanthine (Sigma, I5789), and 5 μ M rosiglitazone (AK Scientific, 70325). For osteogenic, cells were cultured in monolayer for 21 days in this differentiation medium: DMEM-Ham’s F12, 10%FBS, 10mM β -glycerophosphate (USB Corporation, 21655), 100nM dexamethasone and 0.25mM ascorbic acid 2-phosphate. The chondrogenic differentiation media used to maintain the pellets for 21 days was DMEM high glucose supplemented with 100nM dexamethasone, 0.25mM ascorbic acid 2-phosphate (Sigma, A8960), 40 μ g/ml proline (Sigma, P0380) and 10ng/ml TGF- β (R&D System, 243-B3). Evaluation of the differentiation was performed with histological staining using Oil Red O, Alcian blue and von Kossa to assess the differentiation into adipogenic, chondrogenic and osteogenic lineages respectively. For tenogenic lineage cells in monolayer were differentiated with a two step protocol. First, cells were incubated for 24h with stromal media supplemented with 10ng/ml of BMP-12 (BioVision, 4572) reported to augment the formation of tendon-like

tissue in adult multipotent stromal cells in human. Then, cells were cultured in DMEM, 15% FBS, 0.34mM proline and 0.17mM ascorbic acid (tenogenic differentiation media) for 21 days. Control cells were cultured in stromal media for the same period and stained as described previously. For further details see Appendix A “Differentiation into Specific Lineages” and “Evaluation of Trilineage Differentiation”.

Induction of pluripotency treatment

Synthetic mRNA was produced with the Ambion® mMessage mMachine® T7 kit (Applied Biosystems, Foster City, California) using amplified samples of human DNA sequences for OCT4, KLF4 and SOX2 using a 3:1:1 stoichiometry. Poly (A) tails were added using the Poly(A)Tailing kit (Ambion®). The products were purified using Ambion® MEGAClear™ kit (For further details see Appendix A, “Plasmid Preparation”, “Mini Prep Isolation”, “Clone Selection” and “mRNA in vitro Synthesis”). The purified synthetic mRNA for each of the three genes was bound with Lipofectamine® reagent (Invitrogen™, Grand Island, NY) with a total RNA of 2pmol per well of 24-well plate and was applied to P3 cell cultures in stromal medium every day for 14 days incubated in a modular incubator containing 5% CO₂, 5% O₂ and 90%N₂ at 38°C (see Appendix A, “Lipofectamine Transfections”).

Immunocytochemistry for pluripotency factors

Specific localization of the pluripotency related proteins was assessed with Immunocytochemistry using the Fluorescent Human ES/iPS cell characterization kit (Milipore, scr078) for detection of OCT4, SOX2 and NANOG expression using fluorescent microscopy after 0, 7 and 14 days of induction (see Appendix A, “Immunocytochemistry”)

Gene expression

Cells were trypsinized from each well and two replicates of each group were combined (approximately 1×10^6 cells) into one 1.5ml tube each for each group. The cells were pelleted and subjected to the mRNA isolation protocol using Dynabeads[®] mRNA DIRECT[™] Kit (Ambion, 61011) following the “standard” procedure. The mRNA was eluted in 30 μ l of DNase free water. To perform quantitative PCR all of the samples were converted to cDNA with the two step reverse transcription kit of QIAGEN (Quantitect[®] Reverse transcription Kit, 205310) that eliminates genomic DNA. Quantitative PCR was used to evaluate gene expression in the target cells. The quantitative PCR reactions were performed with Bio-Rad SsoFast[™] EvaGreen[®] Supermix was used with primers from Table B.1. qRT-PCR was performed with an enzyme activation at 95°C (1 minute) followed by 40 repeats of denature at 95°C (5 seconds) and annealing at 60°C (30 seconds). A final denature and extension step was performed at 95°C (1 minute) and 55°C (1 minute) respectively. A melt-curve analysis was carried out by repeating 80 times and increasing 5°C every 10 seconds beginning at 55°C. Gene expression efficiency was normalized against housekeeping genes GAP and HPRT-1 as internal controls. Quantitative data was analyzed using the REST 2009© program. For further details see Appendix A: “RNA Isolation”, “Conversion of mRNA into cDNA”, “Calibrator Development”, “Retro Transcriptase Polymerase Chain Reaction”, “Purification of PCR Products” and “qRT-PCR”.

Cell proliferation assay

The capability of the cells to remain mitotically active after induction of pluripotency and expansion until P10 was assessed using the alamarBlue[®] dye. The alamarBlue[®] assay is quantitative with respect to time and dose as seen by the ability of metabolically active cells to convert the reagent into a fluorescent and colorimetric indicator. First, a standard curve was

developed to determine the time of incubation of the dye with the cells (see Appendix A, “Cell Proliferation Assay”). The measurements for each time point were taken in three independent replicates on days 0, 7, 14 and 21 of culture.

Statistical Analysis

Gene expression data was analyzed using the REST 2009[®] statistical program. The program uses the geometric mean of multiple reference genes to normalize the results against genes of interest since ratios are used for data evaluation; this is called the Normalization Factor. Individual expressions are calculated relative to each reference gene and averaged using the geometric mean. Cell proliferation assay was analyzed using the Prism 5 software using a two-way ANOVA.

Experimental Design

Experiment 1: Validation of the cells before induction of pluripotency

In order to evaluate the effect of the pluripotency treatment in different cell types, we first determined if the cells were actual ASCs and BMSCs to compare them with fibroblast. Four horses ranging from 2 to 10 years old that were euthanized for clinical reasons were used as source of the tissues for the study. Multipotent stromal cells from adipose and bone marrow targeted were isolated and characterized based on their morphological aspects, surface markers and trilineage differentiation.

Morphology assessment

Staining with F-actin (cytoskeleton) Hoechst 33258 (nucleus) viewed with fluorescent microscopy to determine morphological aspects in culture. Cells were seeded in a Chamber slide at a concentration of 5000/cm², fixed and stained with these labels.

Immunophenotyping

The cell morphology facilitated their isolation and enrichment in the first passages but they are not sufficient to determine their truly potency state. Expression of specific cell markers was added to the characterization procedure of these cultured cells. The CD evaluated were: CD105 (endoglin, type I membrane glycoprotein), CD29 (Integrin beta 1, receptor involved in cell adhesion), CD90 (Thy-1, marker for a variety of stem cells) and CD44 (surface glycoprotein for cell to cell interactions, cell adhesion and migration) using flow cytometry. For each sample/antibody at least 2.0×10^5 cells at P2 were used. For further details see Appendix A, “Flow Cytometry”.

Osteogenic, Adipogenic and Chondrogenic differentiation

Multipotent stromal cells have the ability to differentiate into specific cell types when subjected to specific differentiation media. Cells used in this experiment were differentiated into the adipogenic, chondrogenic and osteogenic lineage (see Appendix A “Differentiation into Specific Lineages” and “Evaluation of Trilineage Differentiation”) as another assessment of their potency state before starting the induction of pluripotency treatment.

Experiment 2: Effect of the pluripotency treatment with synthetic mRNA

The different types of cells were treated with the induction of pluripotency treatment as described previously. Throughout the 14 days of treatment wells were evaluated each day for colony formation. Furthermore, a sample of 4.0×10^4 cells treated at day 7 and 14 cells was taken to seed the Chamber slide for immunocytochemistry. The remaining cells were used for gene expression analysis.

Morphology assessment: Colony formation/loss of contact inhibition

Evaluation of the treatment was determined at first under bright field microscopy throughout the entire induction of pluripotency treatment to confirm the formation of tight clumps of cells growing on top of each indicating loss of contact inhibition, a feature that is presented by pluripotent cells.

Immunocytochemistry: pluripotency induction factors

To determine if the treatment with synthetic mRNA was successful qualitative assessments of gene expression and protein localization were performed.

After 0, 7 and 14 days of induction, expression of OCT4, SOX-2 and NANOG were qualitatively evaluated with immunocytochemistry (Fluorescent Human ES/iPS cell Characterization Kit, Millipore, MA) using fluorescent microscopy. For further information refer to Appendix A “Immunocytochemistry”.

Gene expression

In order to quantitatively evaluate the response of the MSCs to the pluripotency induction treatment, expression levels of the pluripotency genes were evaluated by qRT-PCR. Because exogenous mRNA was utilized in the treatment, an extra step during primer design was needed to determine the specificity of the oligonucleotides used on equine sequences. Temperature gradients were performed to set the annealing temperature when the oligonucleotides amplified the equine sequence for the transcription factors but not the human sequence used to synthesize the exogenous mRNA (See Appendix A, “RNA Isolation”, “Conversion of mRNA into cDNA”, “Calibrator Development”, “Retro Transcriptase Polymerase Chain Reaction”, “Purification of PCR Products” and “qRT-PCR”)

Experiment 3: Evaluation of the in vitro expansion of the pluripotency induced cells

One of the goals of the induction of pluripotency treatment was to gain certain state potency that would permit a sufficient cell expansion to obtain enough biological material to treat an injury. Following the treatment with the pluripotency factors, cells were passaged until they reached P10. Once proliferated, cell growth and the ability to differentiate into the adipogenic, chondrogenic and osteogenic lineage were evaluated.

Cell growth

The capability of the cells to remain mitotically active after being proliferated was assessed using the alamarBlue® dye. A standard curve between arbitrary fluorescence units and a known cell number was utilized to determine the time of incubation with the dye when the fluorescence is linear to the number of cells. The number of cells evaluated for linearity was determined by the number of cells expected to be in a 96-well seeded at a concentration of 1000cells/cm² after 21 days of culture. (see Appendix A, “Cell Proliferation Assay”)

Differentiation into osteogenic, chondrogenic and adipogenic lineage

Trilineage differentiation was evaluated in the same way as in experiment 1 with cells that were induced or not induced and then expanded to P10 to simulate the cell passaging that would had been needed to perform a clinical treatment if these cells were used. Non differentiated controls were analyzed in the same way as the treated cells to discard artifacts due to the technique employed

Experiment 4: Effect of pluripotency induction to the tenogenic differentiation potential of MSC

The first step of inducing the cells into a less differentiated state with the pluripotency treatment and subsequently expanded to increase the amount of biological material produced were evaluated. The next step is differentiating those cells into the tenogenic lineage, thus in

future studies we can utilize these cells to compare them with undifferentiated cell transplantation.

Evaluation of the differentiation media: Gene expression

To test the differentiation media MSCs that were collected as described in previous experiments and differentiated into the tenogenic lineage. Expression of the tenogenesis anabolic marker tenascin was evaluated with qRT-PCR (using the procedure as described previously) using the three different cell types ASCs, BMSCs and fibroblast subjected to the differentiation media (treated cells, T) and compared with cells cultured in stromal media (non-treated cells, NT).

Effect of the pluripotency treated cells in tenogenic differentiation: Gene expression

The influence of the induction to a pluripotent state in the differentiation towards tenogenic tissue was evaluated. The three different sources of cells were treated with the synthetic mRNA as described previously and then subjected to the tenogenic differentiation media (double induced cells: induced to pluripotency and later to tenogenic lineage, 2I). Controls for each cell type were induced to pluripotency but cultured in regular media (only one induction: induction to pluripotency, I).

Results

Experiment 1: Validation of the cells before induction of pluripotency

Morphology assessment

There was no difference observed between cell types. All cell types presented a fusiform morphology and plastic adherence property, both typical features of isolated MSCs after culture of the stromal vascular fraction and subjected to subsequent cell passaging (Fig 3.1 and 3.2)

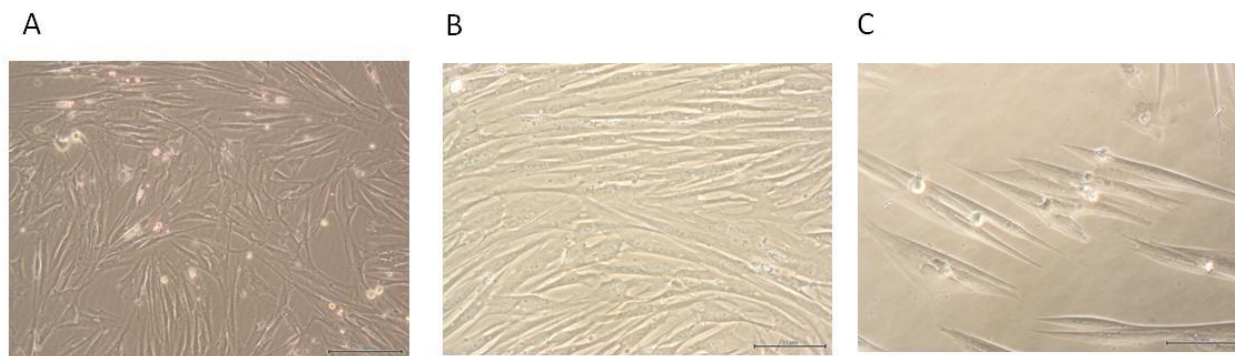


Figure 3.1 Cell morphology under conventional microscope (200x) A: ASCs B: BMSCs C: Fibroblasts.

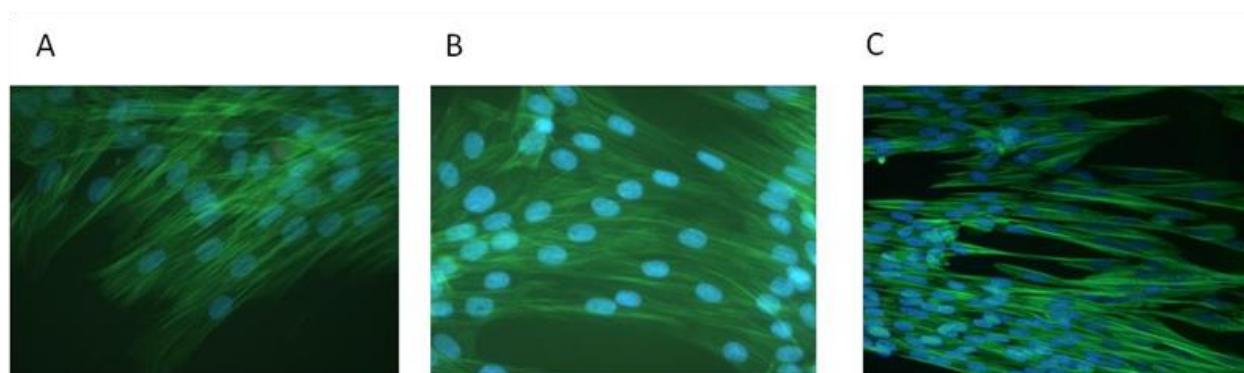


Figure 3.2 Cell morphology under fluorescent microscope stained with 488 phalloidin F-actin (green) and Hoechst dye (blue) (200x) A: ASCs B: BMSCs C: Fibroblasts

Immunophenotyping

The expression of CD105, CD29, CD90 and CD44 was between the ranges normally accepted for the characterization of human MSCs (Dominici et al., 2006). (Fig 3.3). Multipotent stromal cells expression of CD44 was 98.6%, CD29 98%, CD105 98.7% and CD90 91.7%. Fibroblast expression of CD44 was 91.24%, CD29 93.9%, CD105 54.6% and CD90 10.17%.

Osteogenic, Adipogenic and Chondrogenic differentiation

After differentiation cells were stained with Oil Red O, Alizarin Red and Alcian blue to show adipogenic, chondrogenic and osteogenic characteristics respectively (Fig 3.4). All cell isolates were able to generate the intra- (adipogenesis) or extra- (chondrogenesis and

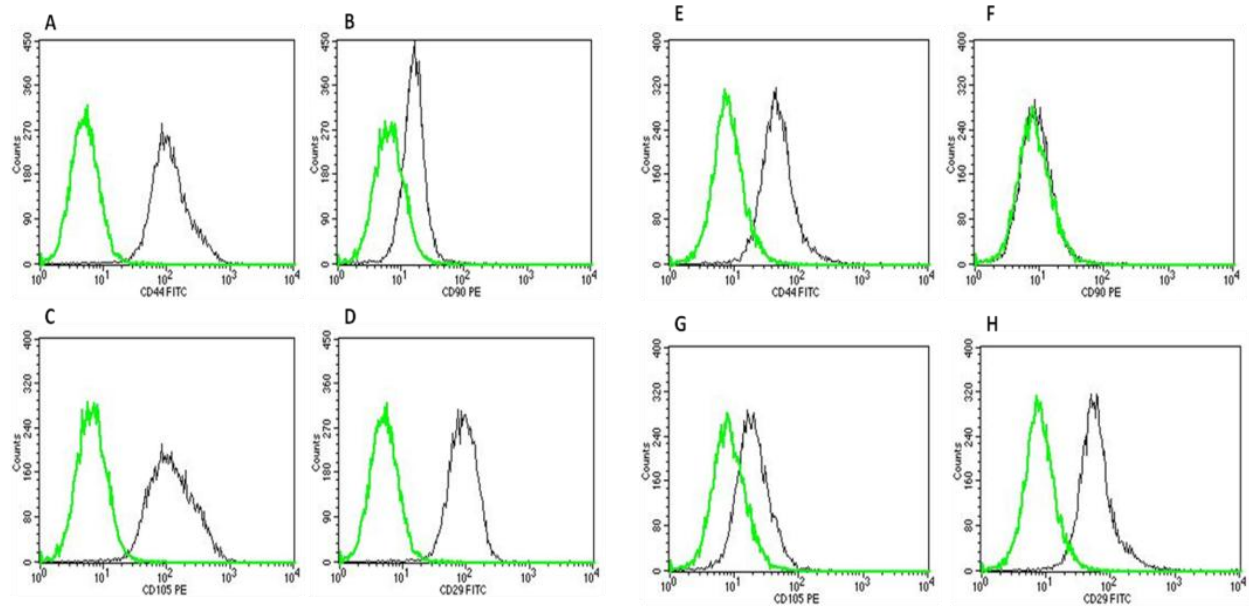


Figure 3.3 Immunophenotyping with CD44, CD90, CD105 and CD29 of ASCs (A, B, C and D) and Fibroblasts (E, F, G and H) (black histogram) compared to the auto fluorescence of the cell (green histogram)

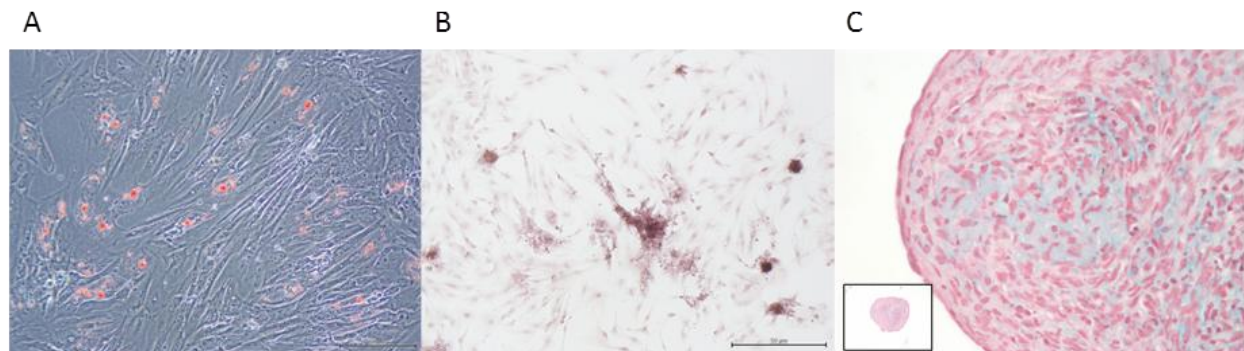


Figure 3.4 Multipotent stromal cells after differentiation and stained with Oil red O (A), Alizarin Red (B) and Alcian Blue (C)

osteogenesis) cellular products that show their capability of trilineage differentiation. Lipid droplet formation within the cytoplasm (indicated by the red staining with the Oil Red O), calcium deposition (indicated by the stained aggregates) and the presence of proteoglycans in the extracellular matrix (light blue staining depicting the form of the lacunae where the nuclei are observed due to the counterstaining with Nuclear Fast Red) indicating the adipogenic, osteogenic and chondrogenic differentiation potential respectively.

Experiment 2: Effect of the pluripotency treatment with synthetic mRNA

Morphology assessment: Colony formation/loss of contact inhibition

The colonies (Fig 3.5) presented characteristics similar to those found in mouse embryonic (Keefer et al., 2007) and induced pluripotent colonies (Takahashi and Yamanaka, 2006) presenting a compact group of cells with a spherical 3D shape and a clear edge that differentiated them from the non-clustered cells (Fig 3.6). No cell aggregates were observed in control wells cultured over the same period of time.

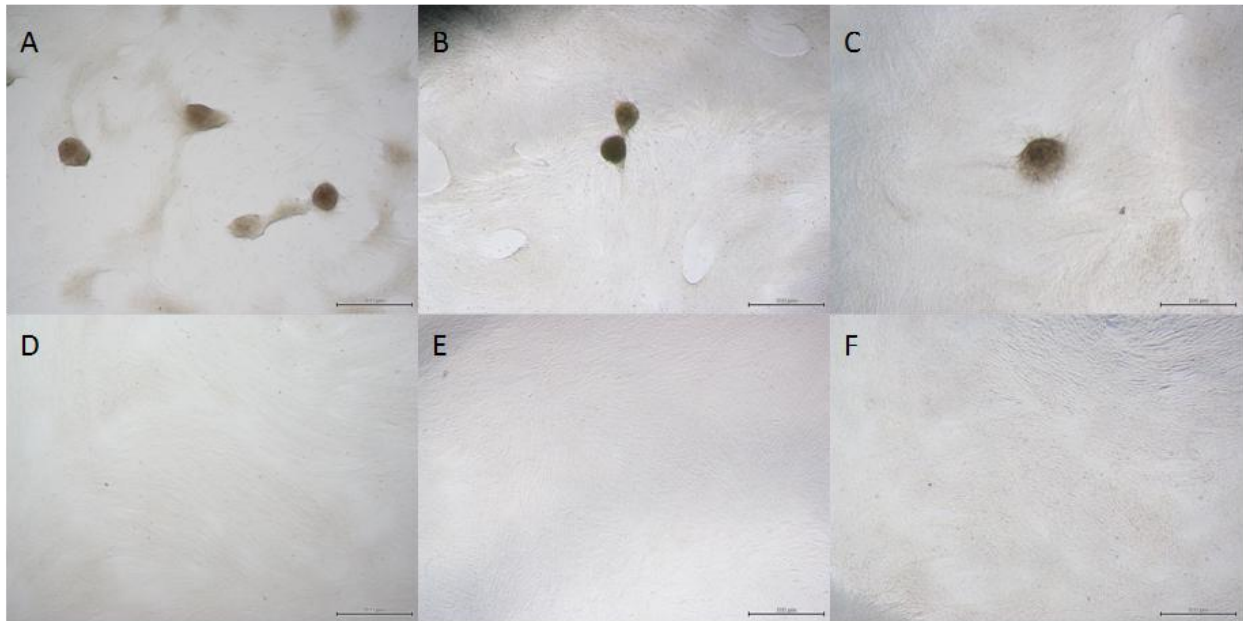


Figure 3.5 ASCs (A), BMSCs (B) and fibroblast (C) colonies obtained after 14 days of pluripotency induction treatment compared to their respective controls (D, E and F) under light microscopy (40x)

Immunocytochemistry: pluripotency induction factors

Higher signal intensity was observed in each cell type pre- and post-induction under the microscope with an appropriate localization of the signal, consistent with increased protein levels of the embryonic-related proteins compared with the controls (“pre-induction”) (Fig 3.7). Presence of these proteins was also detected on day 14 in induced cells forming colonies (Fig 3.8).

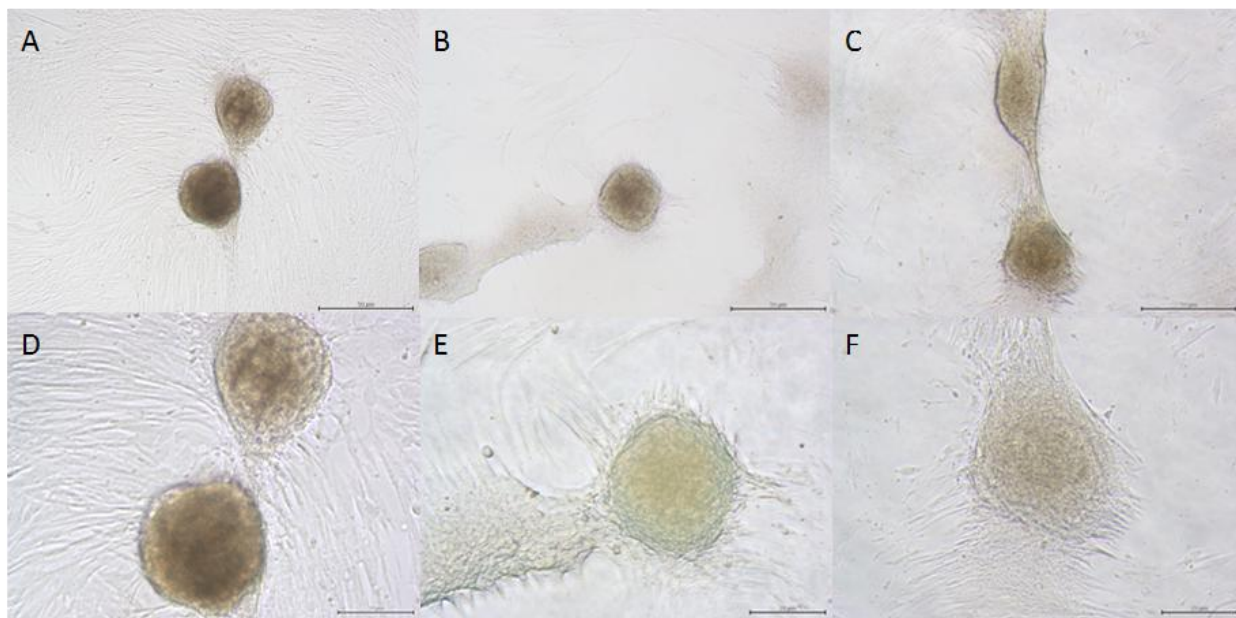


Figure 3.6 Close caption of representative colonies at different magnification: 200x (A, B and C) and 400x (D, E and F)

Gene expression

Significant up-regulation ($P < 0.05$) of OCT4 and SOX2 was detected in all the cell types compared to control cells (Fig 3.9, 3.10 and 3.11). The mean fold changes observed for OCT4 compared to the reference genes were: ASCs: 23.074, $P = 0.030$; BMSCs: 35.448, $P = 0.005$ and Fibroblast: 30.95, $P < 0.001$. While for Sox-2 the results were: ASCs: 230.006, $P = 0.012$; BMSCs: 111.071, $P = 0.001$ and Fibroblast: 291.671, $P = 0.005$. Although no significant changes were detected for the expression of endogenous Klf-4 there was a tendency for up-regulation of the transcription factor in treated BMSCs (ASCs: 1.719, $P = 0.175$; BMSCs: 1.736, $P = 0.084$ and Fibroblast: 1.857, $P = 0.399$). No statistical differences were observed between cell types.

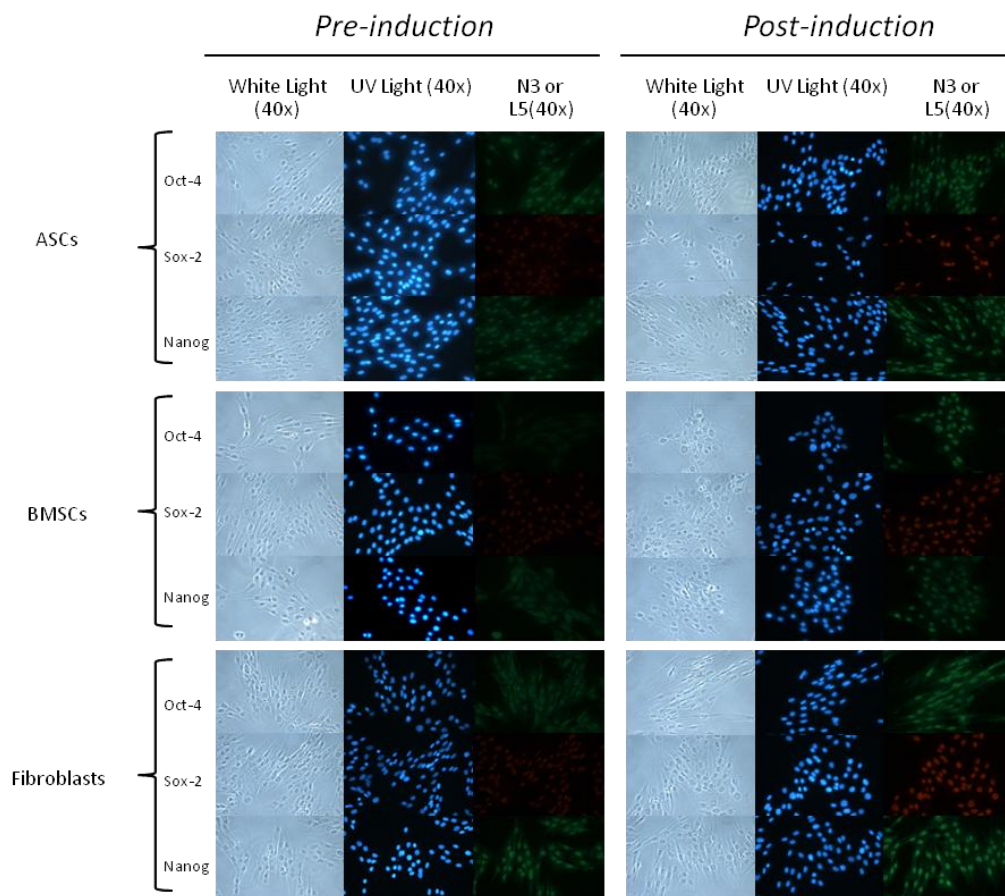


Figure 3.7 Immunocytochemistry of pluripotency factors after 7 days of treatment with synthetic mRNA to induce pluripotency

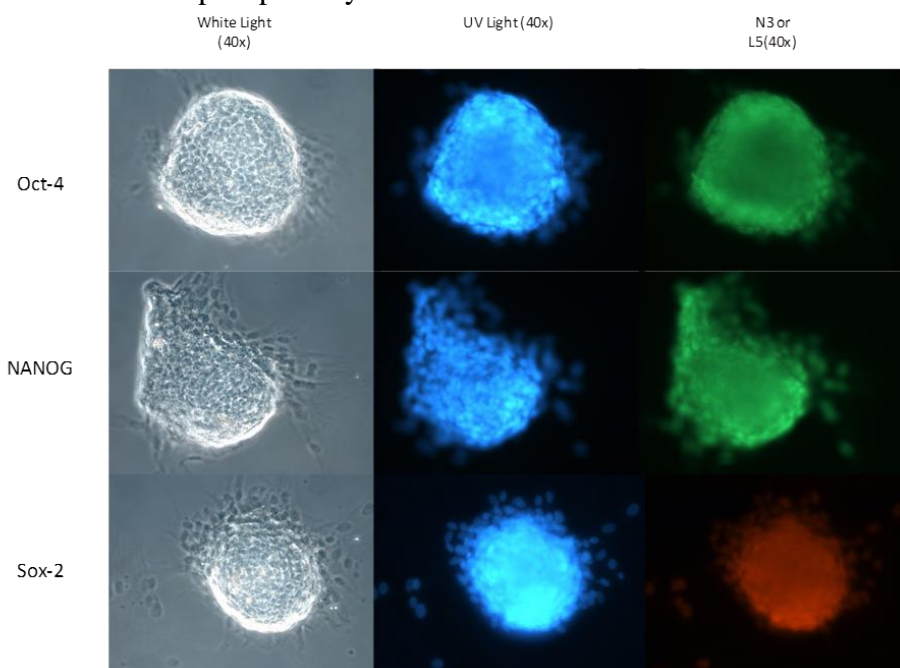


Figure 3.8 Immunocytochemistry of pluripotency factors after 14 days of treatment with synthetic mRNA to induce pluripotency

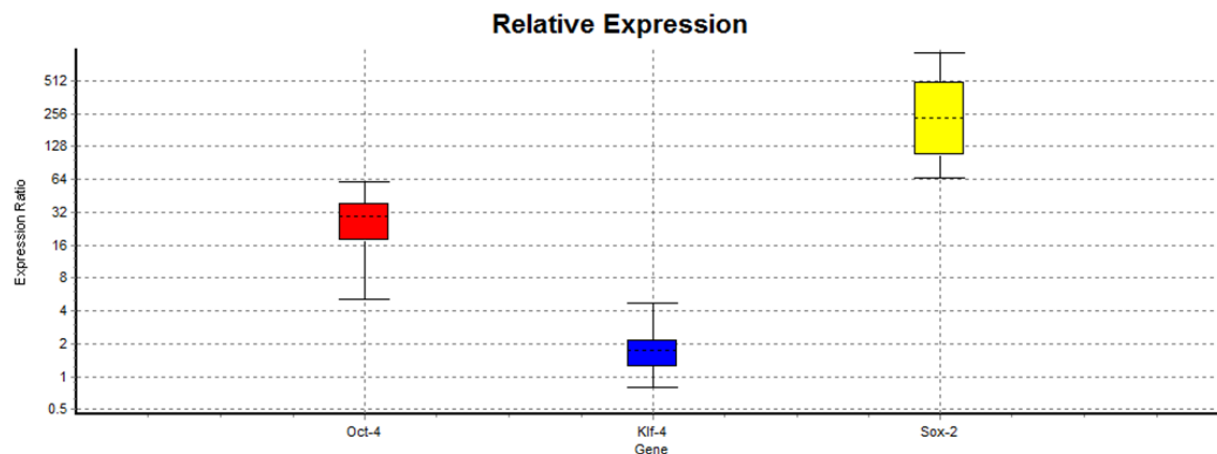


Figure 3.9 Expression ratios of pluripotency genes in ASCs determined by qRT-PCR

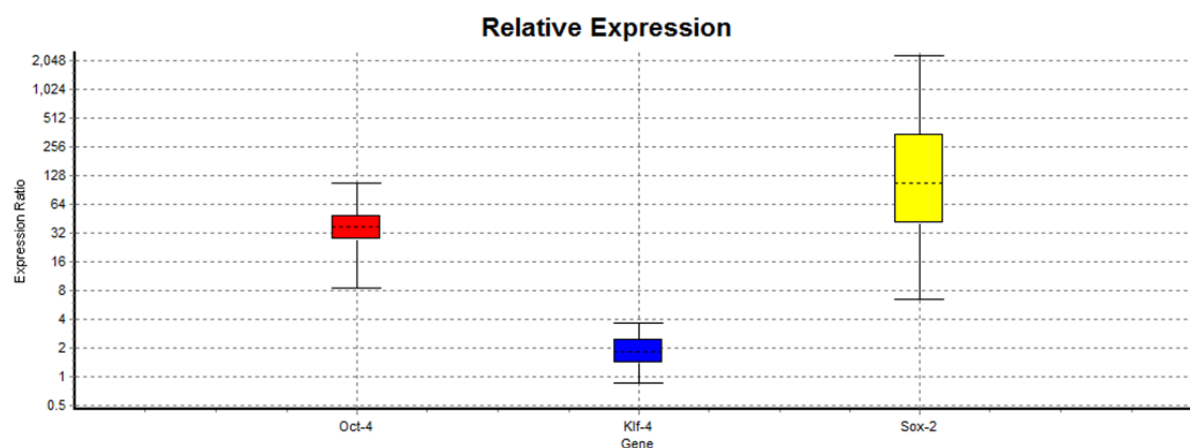


Figure 3.10 Expression ratios of pluripotency genes in BMSCs determined by qRT-PCR

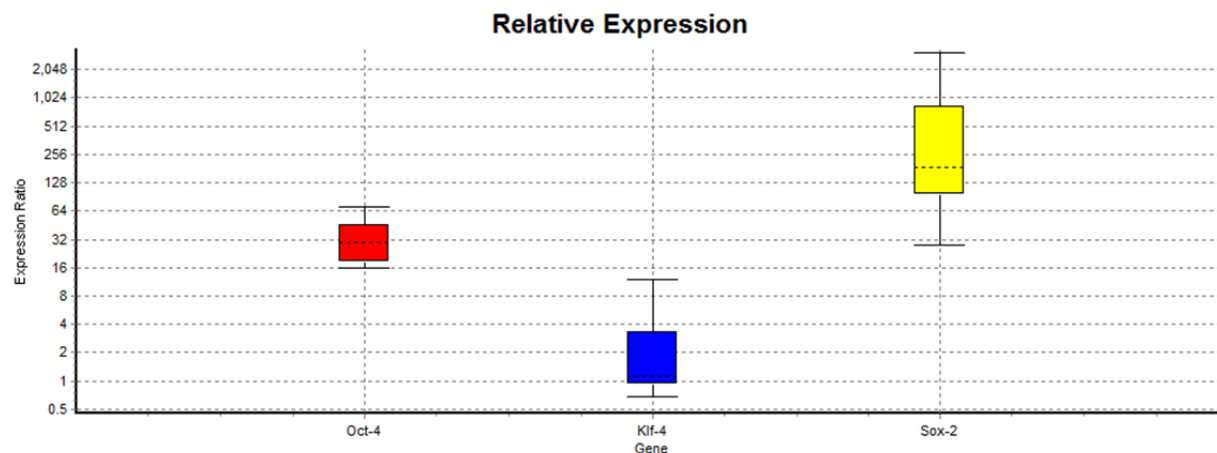


Figure 3.11 Expression ratios of pluripotency genes in fibroblast determined by qRT-PCR

Experiment 3: Evaluation of the in vitro expansion of the pluripotency induced cells

Cell growth

A standard curve between arbitrary fluorescence units and a known cell number was utilized to determine the incubation protocol with the alamarBlue® dye when the fluorescence is linear to the number of cells (Fig 3.12A). The fluorescence was linear to number of cells after 2h when cell numbers range from 1600-12800 with a regression coefficient of $r^2=0.97$ (Fig 3.12 B). No differences were observed over the time points in ASCs and fibroblast. A significant increase proliferation of the induced BMSCs was observed at day 21 when compared to the non-induced cells ($P<0.05$).

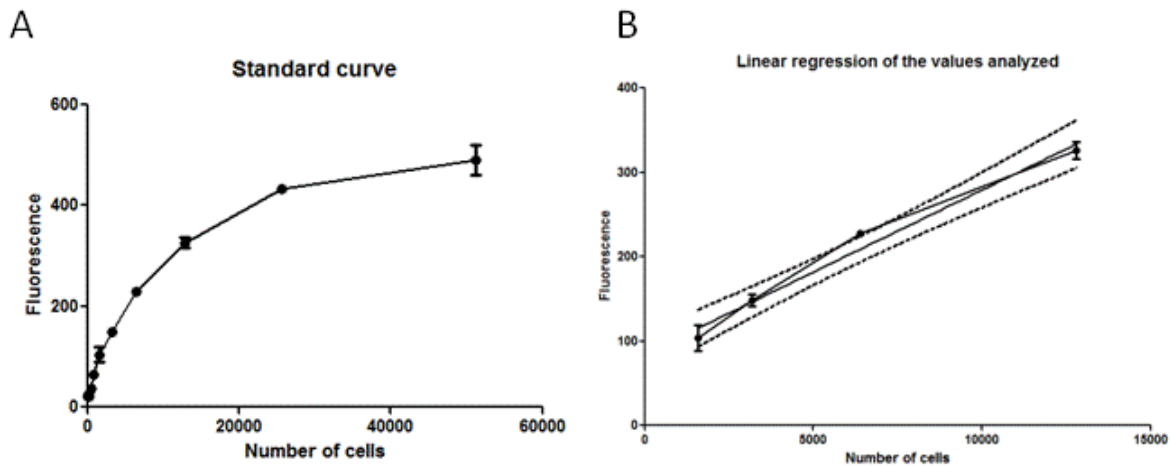


Figure 3.12(A) Standard curve between arbitrary fluorescence units and cell number (B) Linear regression between arbitrary fluorescence units and cell number

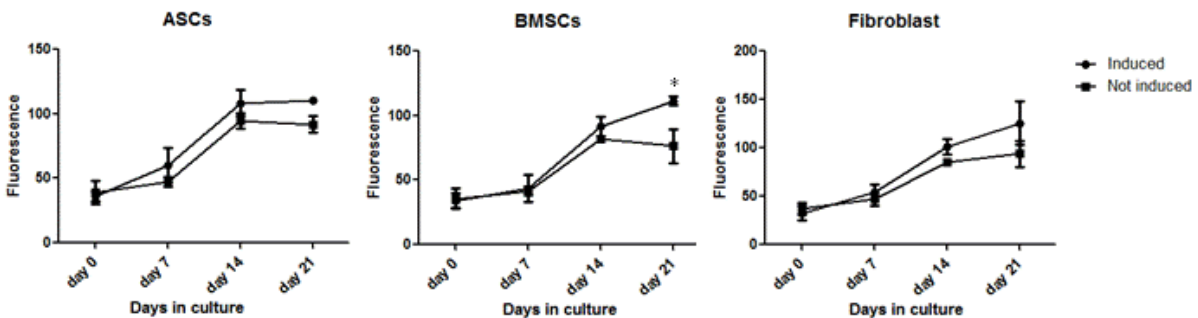


Figure 3.13 Arbitrary Fluorescence units after incubation of alamarBlue® dye for 2h with induced and not induced ASCs, BMSCs and fibroblast over a time period of 21 days

Differentiation into osteogenic, chondrogenic and adipogenic lineage

There was a reduction in the response of the non-induced cells when subjected to the differentiation media compared to induced cells. No lipids droplets, calcium deposits or presence of proteoglycans were observed in non-induced cells when differentiated into the adipogenic, osteogenic and chondrogenic lineage respectively (Fig 3.14-3.20). Cells that were induced to a pluripotent state, then expanded and differentiated into the three lineages showed the capability of producing the intra- and extracellular factors that characterize a successful adipogenic, chondrogenic and osteogenic differentiation. Non differentiated controls did not present staining.

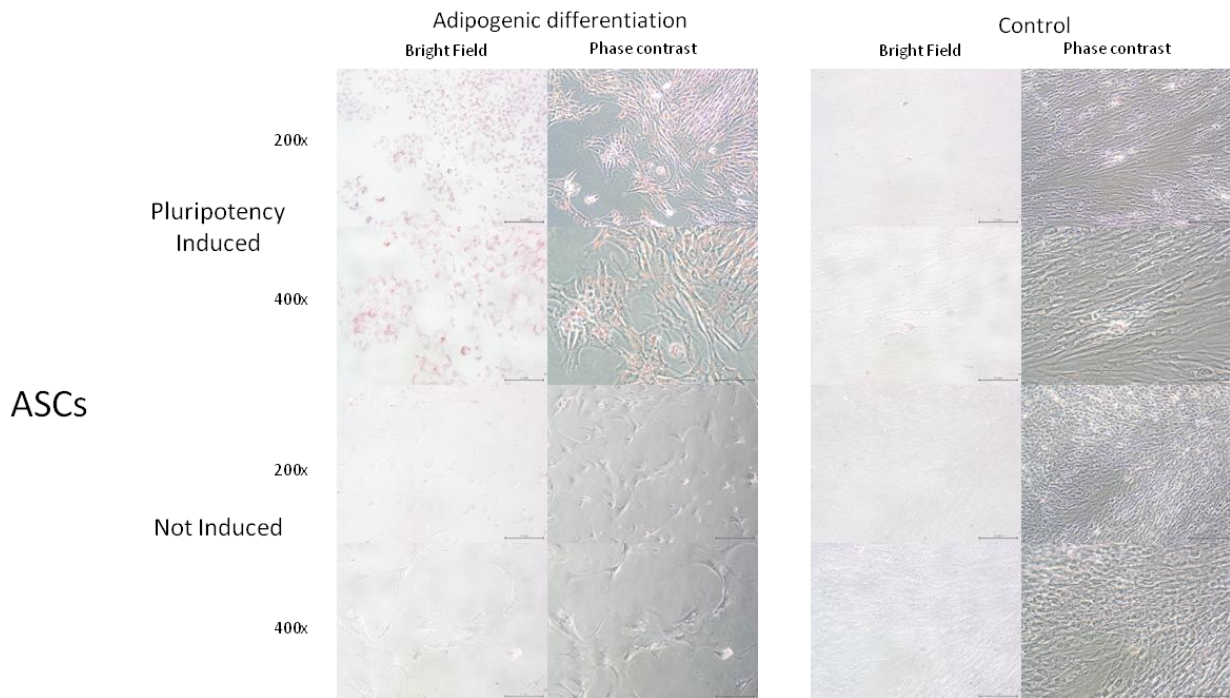


Figure 3.14 Adipogenic differentiation of induced and not induced ASCs after cell expansion

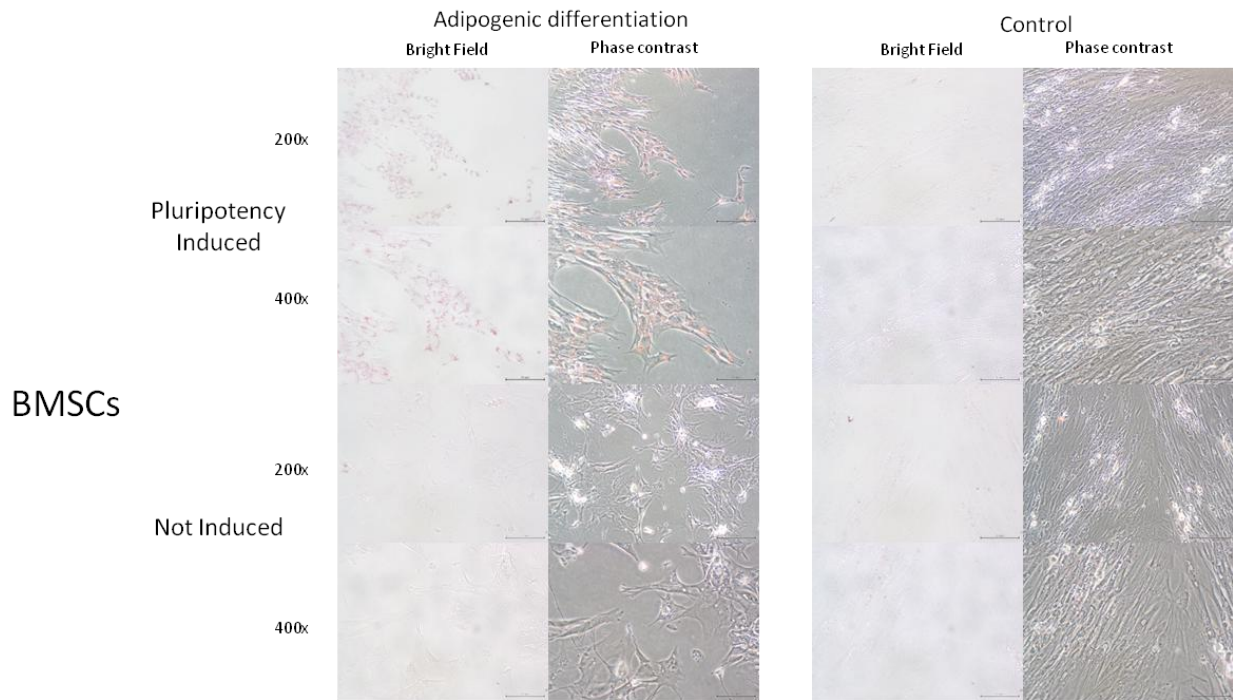


Figure 3.15 Adipogenic differentiation of induced and not induced BMSCs after cell expansion

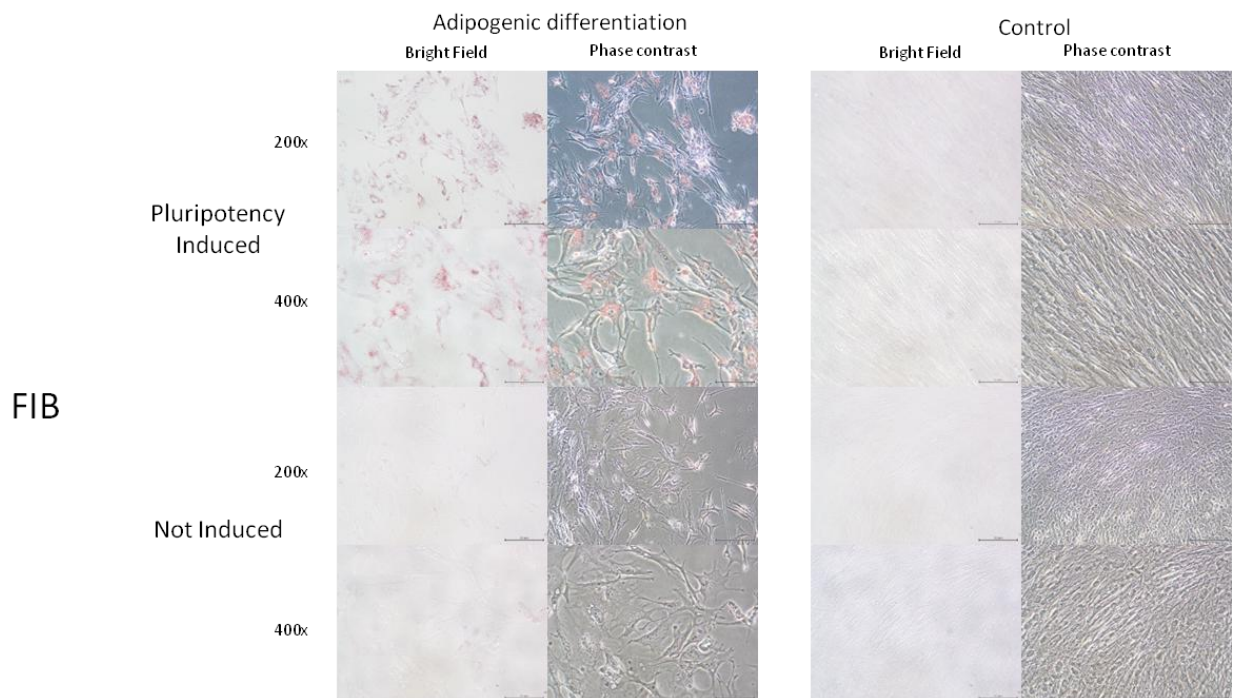


Figure 3.16 Adipogenic differentiation of induced and not induced fibroblast after cell expansion

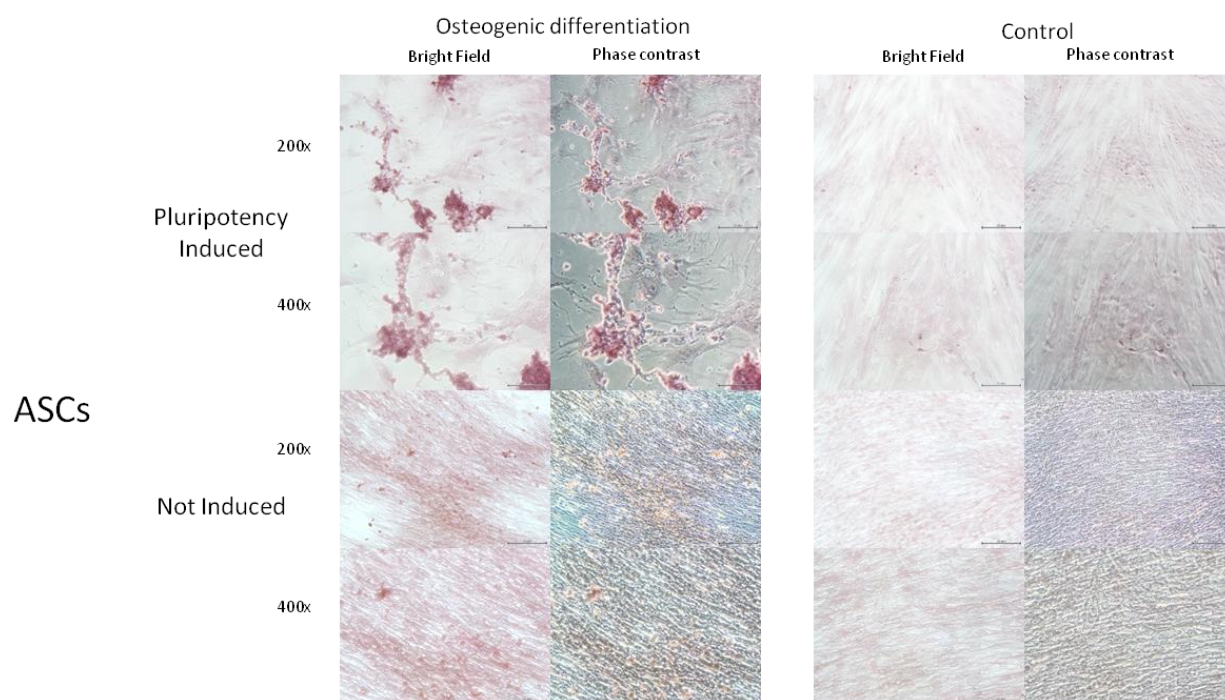


Figure 3.17 Osteogenic differentiation of induced and not induced ASCs after cell expansion

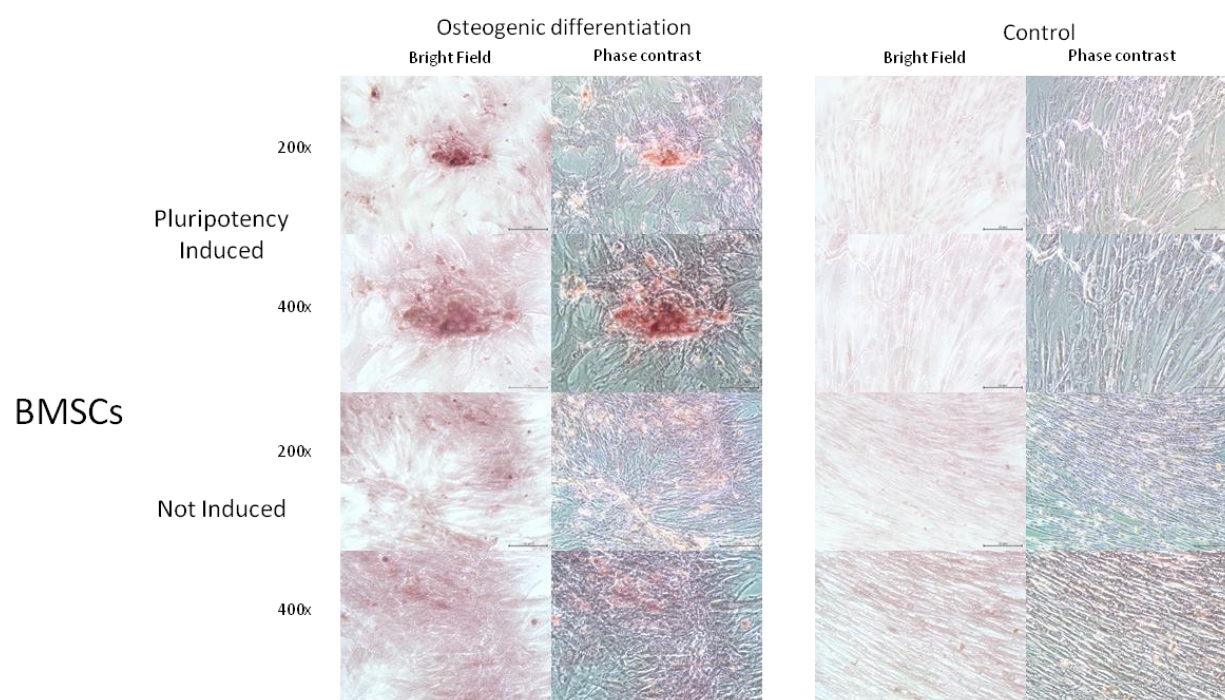


Figure 3.18 Osteogenic differentiation of induced and not induced BMSCs after cell expansion

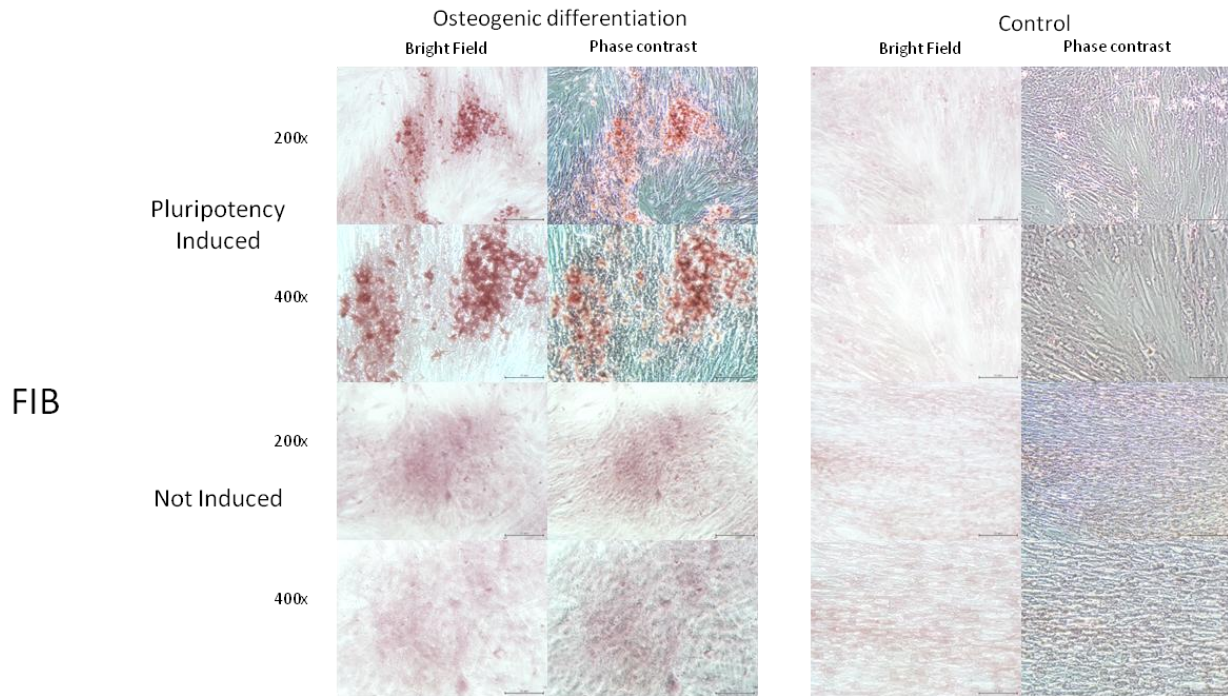


Figure 3.19 Osteogenic differentiation of induced and not induced fibroblast after cell expansion

Experiment 4: Effect of pluripotency induction to the tenogenic differentiation potential of MSCs

Evaluation of the differentiation media: Gene expression

Significant up-regulation of the tenascin gene was observed in ASCs when treated (T) v non-treated (NT) were compared (expression ratio increased compared to the average expression of the two reference genes in 2.274 folds, $P=0.025$). The mean fold change in BMSCs (fold change 0.412, $P=0.424$) and fibroblast (fold change 0.823, $P=0.668$) was not different between treated and non-treated cells.

Effect of the pluripotency treated cells in tenogenic differentiation: Gene expression

Tenascin expression in ASCs 2I (double induced cells: induced to pluripotency and later to tenogenic lineage, 2I) and fibroblast 2I compared with their respective I (only one induction: induction to pluripotency, I) were not different (ASCs: 0.285, $P=0.102$ and fibroblast: 0.344,

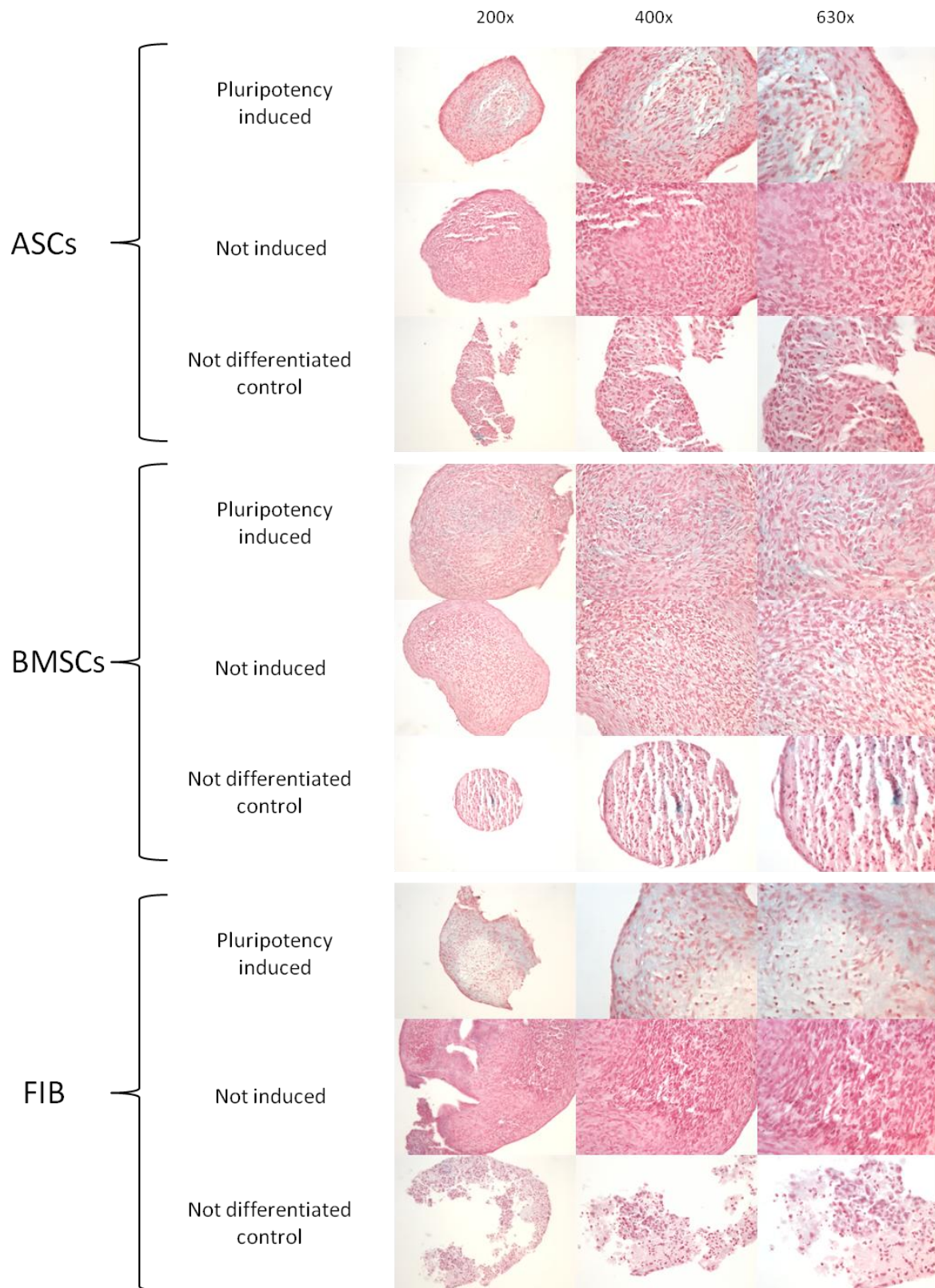


Figure 3.20 Chondrogenic differentiation of induced and not induced cells after cell expansion

P=0.084) but exhibited a tendency to be down-regulated. However, tenascin expression in BMSCs was down-regulated with a expression ratio of 0.288 (P=0.028). The expression of tenascin in ASCs T and ASC 2I (both groups were subjected to tenogenic differentiation media, but only one previously induced to pluripotency), cells that were induced to pluripotency exhibited down-regulation (Expression ratio: 0.135, P=0.008). Down-regulation of tenascin was also observed in fibroblast (fibroblast 2I v. fibroblast T, Expression ratio: 0.086, P=0.026). A tendency to be down-regulated was detected when BMSCs were evaluated (BMSCs 2I v BMSCs T, Expression ratio:0.143, P=0.063). Down-regulation of was also observed when the expression in I v NT cells was compared. Reduction in tenascin gene expression was different in BMSCs and fibroblast (BMSCs I v BMSCs NT Expression ratio:0.205, P=0.011; fibroblast I v. fibroblast NT Expression ratio: 0.207, P=0.023). In the case of ASCs there was not statistical difference (ASC I v ASCs NT Expression ratio: 1.077, P=0.699).

Discussion

A treatment with synthetic mRNA seems to be an interesting approach to overcome all limitations that traditional viral reprogramming has and different sources of cells respond similarly to this treatment. The use of MSCs had to be validated first by a screening of the typical properties presented by these SCs, namely: cell morphology, cell surface markers and trilineage differentiation. The cells used in the study present similar characteristics to those seen in previous reports of equine MSCs (Vidal et al., 2006; Vidal et al., 2007; Vidal et al., 2008).

The characteristics observed in the colonies that appear in culture after the treatment with synthetic mRNA are different to those seen in equine iPSc colonies produced by transduction which are flatter and present a defined but irregular shape when compared to mouse iPSc colonies (Takahashi and Yamanaka, 2006). We believe that this difference is due to the culturing

system used in this study without feeder layers. When these cells are cultured, they present the advantage of secreting factors such as Leukemia inhibitory factor as well as to provide a cell adhesive layer for the colonies to attach (Silva et al., 2008). Furthermore, the grade of undifferentiation reached with the mRNA treatment might have been different from those reported in equine iPSCs (Nagy et al., 2011; Khodadadi et al., 2012; Breton et al., 2013), giving another reason for the difference in colony morphology. Our approach to develop an induction of pluripotency treatment without the use of feeder-layers resides in the need to reduce any possible immunogenic agent that might be altering the cells used in regenerative therapy (Warren et al., 2012). Further studies are needed to evaluate different cultures surface and culture media that help the establishment of colonies.

Even though, the morphology of the colonies was different compared to the reported equine pluripotent cells colonies (Saito et al., 2002; Li et al., 2006; Guest and Allen, 2007; Guest et al., 2010; Nagy et al., 2011; Khodadadi et al., 2012; Breton et al., 2013), the fold change in gene expression of the pluripotency genes is similar to those detected with mRNA technique in humans (Warren et al., 2010). To date, no other report using the synthetic mRNA technique for induction of pluripotency in equine cells has been published.

Correct localization of the proteins related to a pluripotency state in the nuclei observed by the immunocytochemistry analysis determines not only that the synthetic mRNA used to treat the cells is being transcribed but also that these proteins accomplished the first step to gain their function, reach the nucleus. Plus, the stimulation of endogenous production of NANOG was detected adding more evidence that the translated proteins had normal function.

The upregulation of the expression of the endogenous pluripotency genes detected by qRT-PCR suggest that a reactivation of the equine genome took place. However, we cannot state

that the cells obtained were truly iPSCs since a further characterization (teratoma formation, chimeric embryos production and germline transmission) is needed to classify them as such. We can account for a dedifferentiation process to a higher hierarchical potency state, but not to a pluripotent state. This intermediate dedifferentiation state was previously described in the literature when mouse fibroblast were partially reprogrammed to an intermediate state of differentiation when treated with specific reprogramming small molecules (Park et al., 2011).

Not only was the gene expression modified by the treatment with synthetic mRNA, but also the ability of the cells to be expanded without losing their multipotentiality characteristics. This is an important outcome. Previous reports have stated that MSCs expansion *in vitro* reduces their multipotentiality and regenerative capacity (Zhironkina et al., 2012). A comparative study done in humans stated that BMSCs and ASCs became senescent at passage 7 and 8 respectively (Kern et al., 2006), compared to umbilical cord derived stem cells that were able to maintain their characteristics until 10 passages. In this study, ASCs and fibroblast were able to expand until passage and still have cell viability after 21 days of culture. However, BMSCs showed a significant decrease in their viability at day 21 that was not observed when the cells were previously treated with the synthetic mRNA. Moreover, the multipotency characteristics were lost when cells not subjected to the mRNA treatment were in not treated cells, while treated cells were able to maintain them. The loss of multipotency characteristics due to cell expansion to obtain enough material to treat a musculoskeletal injury in the horse might be overcome by the treatment used in this study, because trilineage differentiation and cell viability were maintained when cells previously treated to a higher potency state with synthetic mRNA were subjected to ten cell passages.

Regarding the tenogenic differentiation, the expression of tenascin a gene used as a marker of tenogenic anabolism was up-regulated when ASCs were treated with the differentiation media. However, not observing the same changes in the other cell types suggest that the tenogenic differentiation media and protocol needs to be adjusted to guarantee a fully differentiation into tendon tissue. Adjustments to the differentiation into the tenogenic lineage protocol might include the culture of cells in collagen gels subjected to a constant cycles of tensile strain in a bioreactor (Raabe et al., 2013). When the tenogenic differentiation treatment was used to evaluate the influence of a previous transfection with synthetic mRNA, different results were observed since the tenascin expression of mRNA treated ASCs differentiated into tendon (2I) did not show significant difference compared with not differentiated ones (I). This further supports the idea that the cells reached a higher potency state with the mRNA treatment and it was harder to differentiated when compare to the tenogenic differentiation of untreated ASCs. This suggest that the first upregulation of tenascin presented by the ASCs might be due to a higher level of basal expression of this gene in this cell type that would explain why was the only cell type that responded to this tenogenic differentiation media. Plus, these findings support the theory that when considering differentiation within the same germ layer (in this case mesoderm) cells with a higher potency state are harder to differentiate.

CHAPTER IV: SUMMARY AND CONCLUSION

The use of SCs in regenerative therapy is a constant expanding field not only in research but also in clinical applications. Although these cells could be obtained from different sources, none of them meet all the standards that satisfy physicians, researchers and public opinion to achieve the final goal of developing a controlled treatment with SCs. Thus the idea of developing a technique that combines the qualifying properties of MSCs and iPSCs, is appealing from every angle.

In this study, different sources of equine MSCs were subjected to an induction of pluripotency treatment with synthetic mRNA coding for pluripotency related proteins. We evaluated this treatment based on a previous study performed in humans by Warren et al. 2010 since it represents a safe alternative to the viral transfection induction of pluripotency. Our findings, suggest that this technique uses mechanisms fairly conserved across species since upregulated gene expression of the endogenous pluripotency genes OCT4 and SOX2 was detected in all the equine tissue sources evaluated.

Moreover, correct localization of these transcripts factors was also assessed with evidence of functionality since an increased expression of NANOG, the other factor self regulated by the pluripotency triad was detected by immunocytochemistry. However, further studies are needed in order to determine if these cells could be categorized as iPSCs.

Fortunately, other positive results serve us as proof that this increased gaining of potency is valuable, since the ability of the expansion of MSCs without losing their multipotentiality characteristics was assessed. This represents an important finding from a therapeutic point of view since it might solve one of the limitations of in vitro culture of MSCs. Further studies are also needed to define a more accurate tenogenic differentiation system to obtain tenocytes in a

successful manner. The addition of an increase in vitro expansion of cells after the treatment with synthetic mRNA and the ability of in vitro tendon formation might allow a sequential therapy where undifferentiated MSCs could be used in the first period of the healing process to take advantage of their immunomodulatory and cell recruiting properties followed by the addition of functional cells that might become part of the final reconstructed tissue.

This technique might be also appealing from an animal conservationism point of view, because somatic cell nuclear transfer could be benefit with the treatment with synthetic mRNA coding for pluripotency proteins. Reports in the equine specie established that incorporation of transgenes coding for the pluripotency genes increase pregnancy rates. The alternative of using non-DNA methods for increasing the expression as the one evaluated in this study might be more suitable for use in a reproductive assisted technique for the preservation of endangered animals.

Overall, this technique might represent a solution to the limitations presented with the use of SCs in regenerative therapy due to the lack of predictable outcomes. Gaining the ability of directing cell fate to our needs might provide us with enough biological material to fill the gap in a musculoskeletal injury as well as to fully characterize the cells before transplantation.

LITERATURE CITED

- Adachi, K., H. Suemori, S. Y. Yasuda, N. Nakatsuji, and E. Kawase. 2010. Role of SOX2 in maintaining pluripotency of human embryonic stem cells. *Genes to cells* 15:455-470.
- Angel, M. and M. Yanik. 2010. Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. *PloS one* 5:e11756.
- Aoi, T., K. Yae, M. Nakagawa, T. Ichisaka, K. Okita, K. Takahashi, T. Chiba, and S. Yamanaka. 2008. Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* 321:699-702.
- Aranda, P., X. Agirre, E. Ballestar, E. Andreu, J. Román-Gómez, I. Prieto, J. Martín-Subero, J. Cigudosa, R. Siebert, M. Esteller, and F. Prosper. 2009. Epigenetic signatures associated with different levels of differentiation potential in human stem cells. *PloS one* 4:e7809.
- Avilion, A., S. Nicolis, L. Pevny, L. Perez, N. Vivian, and R. Lovell-Badge. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 17:126-140.
- Bahadur, G., M. Morrison, and L. Machin. 2010. Beyond the 'embryo question': human embryonic stem cell ethics in the context of biomaterial donation in the UK. *Reprod. Biomed. Online* 21:868-874.
- Bayart, E. and O. Cohen-Haguénauer. 2013. Technological overview of iPS induction from human adult somatic cells. *Curr. Gene. Ther.* 13:73-92.
- Behfar, M., R. Hobbenaghi, N. Delirez, B. Dalir-Naghadeh, and F. Sarrafzadeh-Rezaei. 2013. Enhanced mechanical properties of rabbit flexor tendons in response to intratendinous injection of adipose derived stromal vascular fraction. *Curr. Stem. Cell. Res. Ther.* 7:173-178.
- Ben-Nun, I., S. Montague, M. Houck, H. Tran, I. Garitaonandia, T. Leonardo, Y. C. Wang, S. Charter, L. Laurent, O. Ryder, and J. Loring. 2011. Induced pluripotent stem cells from highly endangered species. *Nat. Methods* 8:829-831.
- Bjornson, C., R. Rietze, B. Reynolds, M. Magli, and A. Vescovi. 1999. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 283:534-537.
- Bourzac, C., L. C. Smith, P. Vincent, G. Beauchamp, J. P. Lavoie, and S. Lavery. 2010. Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. *Equine Vet. J.* 42:519-527.
- Boyer, L., T. Lee, M. Cole, S. Johnstone, S. Levine, J. Zucker, M. Guenther, R. Kumar, H. Murray, R. Jenner, D. Gifford, D. Melton, R. Jaenisch, and R. Young. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947-956.

- Braun, J., A. Hack, M. Weis-Klemm, S. Conrad, S. Treml, K. Kohler, U. Walliser, T. Skutella, and W. K. Aicher. 2010. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. *Am. J. Vet. Res.* 71:1228-1236.
- Brehm, W., J. Burk, U. Delling, C. Gittel, and I. Ribitsch. 2012. Stem cell-based tissue engineering in veterinary orthopaedics. *Cell Tissue Res.* 347:677-688.
- Brennand, K., A. Simone, J. Jou, C. Gelboin-Burkhart, N. Tran, S. Sangar, Y. Li, Y. Mu, G. Chen, D. Yu, S. McCarthy, J. Sebat, and F. Gage. 2011. Modelling schizophrenia using human induced pluripotent stem cells. *Nature* 473:221-225.
- Breton, A., R. Sharma, A. C. Diaz, A. G. Parham, A. Graham, C. Neil, C. B. Whitelaw, E. Milne, and F. X. Donadeu. 2013. Derivation and characterization of induced pluripotent stem cells from equine fibroblasts. *Stem Cells Dev.* 22:611-621.
- Burk, J., I. Ribitsch, C. Gittel, H. Juelke, C. Kasper, C. Staszky, and W. Brehm. 2013. Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources. *Vet. J.* 195:98-106.
- Carrade, D. D., M. W. Lamé, M. S. Kent, K. C. Clark, N. J. Walker, and D. L. Borjesson. 2012. Comparative analysis of the immunomodulatory properties of equine adult-derived mesenchymal stem cells. *Cell Med.* 4:1-11.
- Cawley, S., S. Bekiranov, H. Ng, P. Kapranov, E. Sekinger, D. Kampa, A. Piccolboni, V. Sementchenko, J. Cheng, A. Williams, R. Wheeler, B. Wong, J. Drenkow, M. Yamanaka, S. Patel, S. Brubaker, H. Tammana, G. Helt, K. Struhl, and T. Gingeras. 2004. Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell* 116:499-509.
- Chambers, I., D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, and A. Smith. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113:643-655.
- Clegg, P. 2012. Musculoskeletal disease and injury, now and in the future. Part 2: Tendon and ligament injuries. *Equine Vet. J.* 44:371-375.
- Corradetti, B., A. Lange-Consiglio, M. Barucca, F. Cremonesi, and D. Bizzaro. 2011. Size-sieved subpopulations of mesenchymal stem cells from intervacular and perivascular equine umbilical cord matrix. *Cell Proliferat.* 44:330-342.
- Crovace, A., L. Lacitignola, G. Rossi, and E. Francioso. 2010. Histological and immunohistochemical evaluation of autologous cultured bone marrow mesenchymal stem cells and bone marrow mononucleated cells in collagenase-induced tendinitis of equine superficial digital flexor tendon. *Vet. Med. Int.* 2010:250978.
- Davis, R., C. Nemes, E. Varga, C. Freund, G. Kosmidis, K. Gkatzis, D. de Jong, K. Szuhai, A. Dinnyés, and C. Mummery. 2013. Generation of induced pluripotent stem cells from

- human foetal fibroblasts using the Sleeping Beauty transposon gene delivery system. *Differentiation* 86:30-37.
- De Los Angeles, A., Y. H. Loh, P. Tesar, and G. Daley. 2012. Accessing naïve human pluripotency. *Curr. Opin. Genet. Dev.* 22:272-282.
- de Mattos Carvalho, A., A. L. G. Alves, M. A. Golim, A. Moroz, C. A. Hussni, P. c. G. G. de Oliveira, and E. Deffune. 2009. Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue. *Vet. Immunol. Immunopathol.* 132:303-306.
- De Schauwer, C., K. Goossens, S. Piepers, M. Hoogewijs, J. Govaere, K. Smits, E. Meyer, A. Van Soom, and G. Van de Walle. 2014. Characterization and profiling of immunomodulatory genes of equine mesenchymal stromal cells from non-invasive sources. *Stem Cell Res. Ther.* 5:6.
- De Schauwer, C., E. Meyer, G. Van de Walle, and A. Van Soom. 2011. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. *Theriogenology* 75:1431-1443.
- De Schauwer, C., G. R. Van de Walle, S. Piepers, M. K. Hoogewijs, J. L. Govaere, E. Meyer, and S. A. Van. 2013a. Successful isolation of equine mesenchymal stromal cells from cryopreserved umbilical cord blood-derived mononuclear cell fractions. *Equine Vet. J.* 45:518-522.
- De Schauwer, C., G. R. Van de Walle, S. A. Van, and E. Meyer. 2013b. Mesenchymal stem cell therapy in horses: useful beyond orthopedic injuries? *Vet. J.* 33:234-241.
- Devine, M., M. Ryten, P. Vodicka, A. Thomson, T. Burdon, H. Houlden, F. Cavaleri, M. Nagano, N. Drummond, J. W. Taanman, A. Schapira, K. Gwinn, J. Hardy, P. Lewis, and T. Kunath. 2011. Parkinson's disease induced pluripotent stem cells with triplication of the α -synuclein locus. *Nat. Commun.* 2:440.
- Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, and E. Horwitz. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315-317.
- Eilertsen, K. J., Z. Floyd, and J. M. Gimble. 2008. The epigenetics of adult (somatic) stem cells. *Crit Rev. Eukaryot. Gene Expr.* 18:189-206.
- Enders, A., S. Schlafke, K. Lantz, and I. K. Liu. 1993. Endoderm cells of the equine yolk sac from Day 7 until formation of the definitive yolk sac placenta. *Equine Vet. J.* 25:3-9.
- Erdö, F., C. Bührle, J. Blunk, M. Hoehn, Y. Xia, B. Fleischmann, M. Föcking, E. Küstermann, E. Kolossov, J. Hescheler, K. A. Hossmann, and T. Trapp. 2003. Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. *J. Cereb. Blood Flow Metab.* 23:780-785.

- Evans, M. and M. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.
- Ezashi, T., B. Telugu, A. Alexenko, S. Sachdev, S. Sinha, and R. Roberts. 2009. Derivation of induced pluripotent stem cells from pig somatic cells. *Proc. Natl. Acad. Sci. U. S. A.* 106:10993-10998.
- Faiola, F., X. Liu, S. Lo, S. Pan, K. Zhang, E. Lyman, A. Farina, and E. Martinez. 2005. Dual regulation of c-Myc by p300 via acetylation-dependent control of Myc protein turnover and coactivation of Myc-induced transcription. *Mol. Cell. Biol.* 25:10220-10234.
- Friedenstein, A. J., K. V. Petrakova, A. I. Kurolesova, and G. P. Frolova. 1968. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 6:230-247.
- Frisbie, D. D., J. D. Kisiday, C. E. Kawcak, N. M. Werpy, and C. W. McIlwraith. 2009. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. *J. Orthop. Res.* 27:1675-1680.
- Fusaki, N., H. Ban, A. Nishiyama, K. Saeki, and M. Hasegawa. 2009. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc. Jpn. Acad. , Ser. B, Phys. Biol. Sci.* 85:348-362.
- Giovannini, S., W. Brehm, P. Mainil-Varlet, and D. Nesic. 2008. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. *Differentiation* 76:118-129.
- Godwin, E. E., N. J. Young, J. Dudhia, I. C. Beamish, and R. K. Smith. 2012. Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. *Equine Vet. J.* 44:25-32.
- González, F., S. Boué, and J. Izpisua Belmonte. 2011. Methods for making induced pluripotent stem cells: reprogramming á la carte. *Nat. Rev. Genet.* 12:231-242.
- Gourronc, F. and A. Klingelhutz. 2012. Therapeutic opportunities: telomere maintenance in inducible pluripotent stem cells. *Mutat. Res.* 730:98-105.
- Guest, D. J. and W. R. Allen. 2007. Expression of cell-surface antigens and embryonic stem cell pluripotency genes in equine blastocysts. *Stem Cells Dev.* 16:789-796.
- Guest, D. J., M. R. Smith, and W. R. Allen. 2010. Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon. *Equine Vet. J.* 42:636-642.
- Haack-Sorensen, M., L. Bindslev, S. Mortensen, T. Friis, and J. Kastrup. 2007. The influence of freezing and storage on the characteristics and functions of human mesenchymal stromal cells isolated for clinical use. *Cytotherapy* 9:328-337.

- Han, X., J. Han, F. Ding, S. Cao, S. Lim, Y. Dai, R. Zhang, Y. Zhang, B. Lim, and N. Li. 2011. Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. *Cell Res.* 21:1509-1512.
- Hattori, N., K. Nishino, Y. G. Ko, N. Hattori, J. Ohgane, S. Tanaka, and K. Shiota. 2004. Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J. Biol. Chem.* 279:17063-17069.
- Heng, B., K. Heinimann, P. Miny, G. Iezzi, K. Glatz, A. Scherberich, H. Zulewski, and M. Fussenegger. 2013. mRNA transfection-based, feeder-free, induced pluripotent stem cells derived from adipose tissue of a 50-year-old patient. *Metab. Eng.* 18:9-24.
- Hochedlinger, K. and K. Plath. 2009. Epigenetic reprogramming and induced pluripotency. *Development* 136:509-523.
- Hou, P., Y. Li, X. Zhang, C. Liu, J. Guan, H. Li, T. Zhao, J. Ye, W. Yang, K. Liu, J. Ge, J. Xu, Q. Zhang, Y. Zhao, and H. Deng. 2013. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. *Science* 341:651-654.
- Houbaviy, H., M. Murray, and P. Sharp. 2003. Embryonic stem cell-specific MicroRNAs. *Dev. cell* 5:351-358.
- Hoynowski, S. M., M. M. Fry, B. M. Gardner, M. T. Leming, J. R. Tucker, L. Black, T. Sand, and K. E. Mitchell. 2007. Characterization and differentiation of equine umbilical cord-derived matrix cells. *Biochem. Biophys. Res. Commun.* 362:347-353.
- Huangfu, D., R. Maehr, W. Guo, A. Eijkelenboom, M. Snitow, A. Chen, and D. Melton. 2008. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotechnol.* 26:795-797.
- Israel, M., S. Yuan, C. Bardy, S. Reyna, Y. Mu, C. Herrera, M. Hefferan, S. Van Gorp, K. Nazor, F. Boscolo, C. Carson, L. Laurent, M. Marsala, F. Gage, A. Remes, E. Koo, and L. Goldstein. 2012. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. *Nature* 482:216-220.
- Keefer, C., D. Pant, L. Blomberg, and N. Talbot. 2007. Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. *Anim. Reprod. Sci.* 98:147-168.
- Kern, S., H. Eichler, J. Stoeve, H. Klüter, and K. Bieback. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem cells* 24:1294-1301.
- Khodadadi, K., H. Sumer, M. Pashaiasl, S. Lim, M. Williamson, and P. J. Verma. 2012. Induction of pluripotency in adult equine fibroblasts without c-MYC. *Stem Cells Int.* 429160.

- Kim, D., C. H. Kim, J. I. Moon, Y. G. Chung, M. Y. Chang, B. S. Han, S. Ko, E. Yang, K. Cha, R. Lanza, and K. S. Kim. 2009. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell stem cell* 4:472-476.
- Kim, J., H. Zaehres, G. Wu, L. Gentile, K. Ko, V. Sebastiano, M. Araújo-Bravo, D. Ruau, D. Han, M. Zenke, and H. Schöler. 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 454:646-650.
- Kobayashi, T., T. Yamaguchi, S. Hamanaka, M. Kato-Itoh, Y. Yamazaki, M. Ibata, H. Sato, Y. S. Lee, J. I. Usui, A. Knisely, M. Hirabayashi, and H. Nakauchi. 2010. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* 142:787-799.
- Koch, T. G., T. Heerkens, P. D. Thomsen, and D. H. Betts. 2007. Isolation of mesenchymal stem cells from equine umbilical cord blood. *BMC Biotechnol.* 7:26-29.
- Koh, S. and J. Piedrahita. 2014. From "ES-like" cells to induced pluripotent stem cells: a historical perspective in domestic animals. *Theriogenology* 81:103-111.
- Kriks, S., J. W. Shim, J. Piao, Y. Ganat, D. Wakeman, Z. Xie, L. Carrillo-Reid, G. Auyeung, C. Antonacci, A. Buch, L. Yang, M. Beal, D. Surmeier, J. Kordower, V. Tabar, and L. Studer. 2011. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480:547-551.
- Kuroda, T., M. Tada, H. Kubota, H. Kimura, S.-y. Hatano, H. Suemori, N. Nakatsuji, and T. Tada. 2005. Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol. Cell. Biol.* 25:2475-2485.
- Lakshmipathy, U. and C. Verfaillie. 2005. Stem cell plasticity. *Blood Rev.* 19:29-38.
- Lange-Consiglio, A., B. Corradetti, A. Meucci, R. Perego, D. Bizzaro, and F. Cremonesi. 2013a. Characteristics of equine mesenchymal stem cells derived from amnion and bone marrow: in vitro proliferative and multilineage potential assessment. *Equine Vet. J.* 45:737-744.
- Lange-Consiglio, A., S. Tassan, B. Corradetti, A. Meucci, R. Perego, D. Bizzaro, and F. Cremonesi. 2013b. Investigating the efficacy of amnion-derived compared with bone marrow-derived mesenchymal stromal cells in equine tendon and ligament injuries. *Cytotherapy*. 15:1011-1020.
- Lee, H. Y., P. W. Kopesky, A. Plaas, J. Sandy, J. Kisiday, D. Frisbie, A. J. Grodzinsky, and C. Ortiz. 2010. Adult bone marrow stromal cell-based tissue-engineered aggrecan exhibits ultrastructure and nanomechanical properties superior to native cartilage. *Osteoarthr. Cartil.* 18:1477-1486.
- Li, X., S. G. Zhou, M. P. Imreh, L. Ahrlund-Richter, and W. R. Allen. 2006. Horse embryonic stem cell lines from the proliferation of inner cell mass cells. *Stem Cells Dev.* 15:523-531.

- Li, Z., J. Zhao, Q. Li, W. Yang, Q. Song, W. Li, and J. Liu. 2010. KLF4 promotes hydrogen-peroxide-induced apoptosis of chronic myeloid leukemia cells involving the bcl-2/bax pathway. *Cell Stress Chaperones* 15:905-912.
- Li, Z. and T. Rana. 2012. A kinase inhibitor screen identifies small-molecule enhancers of reprogramming and iPS cell generation. *Nat. Commun.* 3:1085.
- Loh, Y. H., Q. Wu, J. L. Chew, V. Vega, W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K. Y. Wong, K. Sung, C. Lee, X. D. Zhao, K. P. Chiu, L. Lipovich, V. Kuznetsov, P. Robson, L. Stanton, C. L. Wei, Y. Ruan, B. Lim, and H. H. Ng. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* 38:431-440.
- Lyssiotis, C., R. Foreman, J. Staerk, M. Garcia, D. Mathur, S. Markoulaki, J. Hanna, L. Lairson, B. Charette, L. Bouchez, M. Bollong, C. Kunick, A. Brinker, C. Cho, P. Schultz, and R. Jaenisch. 2009. Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. *Proc. Natl. Acad. Sci. U. S. A.* 106:8912-8917.
- Mangani, D., A. Roberti, F. Rizzolio, and A. Giordano. 2013. Emerging molecular networks in Burkitt's lymphoma. *J. Cell. Biochem.* 114:35-38.
- Marfe, G., G. Rotta, M. L. De, M. Tafani, F. Fiorito, S. C. Di, M. Poletti, M. Ranalli, M. A. Russo, and A. Gambacurta. 2012. A new clinical approach: use of blood-derived stem cells (BDSCs) for superficial digital flexor tendon injuries in horses. *Life Sci.* 90:825-830.
- Martin, G. 1981. 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.* 78:7634-7638.
- Masui, S., Y. Nakatake, Y. Toyooka, D. Shimosato, R. Yagi, K. Takahashi, H. Okochi, A. Okuda, R. Matoba, A. Sharov, M. Ko, and H. Niwa. 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell Biol.* 9:625-635.
- McCarthy, H. E., J. J. Bara, K. Brakspear, S. K. Singhrao, and C. W. Archer. 2012. The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. *Vet. J.* 192:345-351.
- McLenachan, S., D. Zhang, A. Palomo, M. Edel, and F. Chen. 2013. mRNA transfection of mouse and human neural stem cell cultures. *PloS one* 8:e83596.
- Mitsui, K., Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda, and S. Yamanaka. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113:631-642.

- Miyoshi, N., H. Ishii, H. Nagano, N. Haraguchi, D. Dewi, Y. Kano, S. Nishikawa, M. Tanemura, K. Mimori, F. Tanaka, T. Saito, J. Nishimura, I. Takemasa, T. Mizushima, M. Ikeda, H. Yamamoto, M. Sekimoto, Y. Doki, and M. Mori. 2011. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell stem cell* 8:633-638.
- Molchadsky, A., N. Rivlin, R. Brosh, V. Rotter, and R. Sarig. 2010. p53 is balancing development, differentiation and de-differentiation to assure cancer prevention. *Carcinogenesis* 31:1501-1508.
- Moriyama, H., Y. Kasashima, A. Kuwano, and S. Wada. 2013. Anatomical location and culture of equine corneal epithelial stem cells. *Vet. Ophthalmol.* 7:106-112.
- Nagy, K., H. K. Sung, P. Zhang, S. Laflamme, P. Vincent, S. gha-Mohammadi, K. Woltjen, C. Monetti, I. Michael, L. Smith, and A. Nagy. 2011. Induced pluripotent stem cell lines derived from equine fibroblasts. *Stem Cell Rev. and Rep.* 7:693-702.
- Nakagawa, M., M. Koyanagi, K. Tanabe, K. Takahashi, T. Ichisaka, T. Aoi, K. Okita, Y. Mochiduki, N. Takizawa, and S. Yamanaka. 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* 26:101-106.
- Nichols, J. and A. Smith. 2009. Naive and primed pluripotent states. *Cell stem cell* 4:487-492.
- Niwa, H., J. Miyazaki, and A. Smith. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* 24:372-376.
- Obokata, H., T. Wakayama, Y. Sasai, K. Kojima, M. Vacanti, H. Niwa, M. Yamato, and C. Vacanti. 2014. Stimulus-triggered fate conversion of somatic cells into pluripotency. *Nature* 505:641-647.
- Okano, H., M. Nakamura, K. Yoshida, Y. Okada, O. Tsuji, S. Nori, E. Ikeda, S. Yamanaka, and K. Miura. 2013. Steps toward safe cell therapy using induced pluripotent stem cells. *Circ. Res.* 112:523-533.
- Okita, K., T. Ichisaka, and S. Yamanaka. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313-317.
- Okita, K., Y. Matsumura, Y. Sato, A. Okada, A. Morizane, S. Okamoto, H. Hong, M. Nakagawa, K. Tanabe, K. i. Tezuka, T. Shibata, T. Kunisada, M. Takahashi, J. Takahashi, H. Saji, and S. Yamanaka. 2011. A more efficient method to generate integration-free human iPS cells. *Nat. Methods* 8:409-412.
- Okita, K., M. Nakagawa, H. Hyenjong, T. Ichisaka, and S. Yamanaka. 2008. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 322:949-953.
- Olivera, R., R. Jordan, C. Alvarez, M. Radrizzani, and G. Vichera. 2013. Pregnancy of equine cloned embryos microinjected with pluripotency inducing genes (Oct4, Sox2, c-Myc, Klf4). *Reprod. Fertil. Dev.* 26:130-131.

- Paris, D. B. and T. A. Stout. 2010. Equine embryos and embryonic stem cells: defining reliable markers of pluripotency. *Theriogenology* 74:516-524.
- Park, J., C. Kim, Y. Tang, T. Amano, C. J. Lin, and X. Tian. 2011. Reprogramming of mouse fibroblasts to an intermediate state of differentiation by chemical induction. *Cell. Reprogram.* 13:121-131.
- Pascucci, L., G. Curina, F. Mercati, C. Marini, C. Dall'Aglio, B. Paternesi, and P. Ceccarelli. 2011. Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: Towards the definition of minimal stemness criteria. *Vet. Immunol. Immunopathol.* 144:499-506.
- Pesce, M. and H. Schöler. 2001. Oct-4: gatekeeper in the beginnings of mammalian development. *Stem cells* 19:271-278.
- Polak, J. and A. Bishop. 2006. Stem cells and tissue engineering: past, present, and future. *Ann. N. Y. Acad. Sci.* 1068:352-366.
- Raabe, O., K. Shell, D. Fietz, C. Freitag, A. Ohrndorf, H. Christ, S. Wenisch, and S. Arnhold. 2013. Tenogenic differentiation of equine adipose-tissue-derived stem cells under the influence of tensile strain, growth differentiation factors and various oxygen tensions. *Cell Tissue Res.* 352:509-521.
- Radtke, C. L., R. Nino-Fong, B. P. Esparza Gonzalez, H. Stryhn, and L. A. McDuffee. 2013. Characterization and osteogenic potential of equine muscle tissue- and periosteal tissue-derived mesenchymal stem cells in comparison with bone marrow- and adipose tissue-derived mesenchymal stem cells. *Am. J. Vet. Res.* 74:790-800.
- Ranera, B., A. R. Remacha, S. varez-Arguedas, T. Castiella, F. J. Vazquez, A. Romero, P. Zaragoza, I. Martin-Burriel, and C. Rodellar. 2013. Expansion under hypoxic conditions enhances the chondrogenic potential of equine bone marrow-derived mesenchymal stem cells. *Vet. J.* 195:248-251.
- Ranera, B., A. R. Remacha, S. varez-Arguedas, A. Romero, F. J. Vazquez, P. Zaragoza, I. Martin-Burriel, and C. Rodellar. 2012. Effect of hypoxia on equine mesenchymal stem cells derived from bone marrow and adipose tissue. *BMC. Vet. Res.* 8:142.
- Ray, A., M. Alalem, and B. Ray. 2013. Loss of epigenetic Kruppel-like factor 4 histone deacetylase (KLF-4-HDAC)-mediated transcriptional suppression is crucial in increasing vascular endothelial growth factor (VEGF) expression in breast cancer. *J. Biol. Chem.* 288:27232-27242.
- Reed, S. A. and S. E. Johnson. 2008. Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types. *J. Cell Physiol.* 215:329-336.

- Reed, S. A. and S. E. Johnson. 2014. Expression of scleraxis and tenascin C in equine adipose and umbilical cord blood derived stem cells is dependent upon substrata and FGF supplementation. *Cytotechnology* 66:27-35.
- Rodda, D., J. L. Chew, L. H. Lim, Y. H. Loh, B. Wang, H. H. Ng, and P. Robson. 2005. Transcriptional regulation of nanog by OCT4 and SOX2. *J. Biol. Chem.* 280:24731-24737.
- Rowland, B., R. Bernards, and D. Peeper. 2005. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat. Cell Biol.* 7:1074-1082.
- Saito, S., H. Ugai, K. Sawai, Y. Yamamoto, A. Minamihashi, K. Kurosaka, Y. Kobayashi, T. Murata, Y. Obata, and K. Yokoyama. 2002. Isolation of embryonic stem-like cells from equine blastocysts and their differentiation in vitro. *FEBS Lett.* 531:389-396.
- Sang-Bum, P., S. Min-Soo, K. Jun-Gu, C. Joon-Seok, and K. Kyung-Sun. 2011. Isolation and characterization of equine amniotic fluid-derived multipotent stem cells. *Cytotherapy* 13:341-349.
- Seo, J. P., N. Tsuzuki, S. Haneda, K. Yamada, H. Furuoka, Y. Tabata, and N. Sasaki. 2013. Comparison of allogeneic platelet lysate and fetal bovine serum for in vitro expansion of equine bone marrow-derived mesenchymal stem cells. *Res. Vet. Sci.* 95:693-698.
- Shimada, H., A. Nakada, Y. Hashimoto, K. Shigeno, Y. Shionoya, and T. Nakamura. 2010. Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. *Mol. Reprod. Dev.* 77:2.
- Siddappa, R., R. Licht, B. C. van, and B. J. de. 2007. Donor variation and loss of multipotency during in vitro expansion of human mesenchymal stem cells for bone tissue engineering. *J. Orthop. Res.* 25:1029-1041.
- Silva, J., O. Barrandon, J. Nichols, J. Kawaguchi, T. Theunissen, and A. Smith. 2008. Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS biology* 6:60253.
- Spaas, J. H., S. Broeckx, G. R. Van de Walle, and M. Poletini. 2013a. The effects of equine peripheral blood stem cells on cutaneous wound healing: a clinical evaluation in four horses. *Clin. Exp. Dermatol.* 38:280-284.
- Spaas, J. H., C. De Schauwer, P. Cornillie, E. Meyer, A. Van Soom, and R. V. de Walle. 2013b. Culture and characterisation of equine peripheral blood mesenchymal stromal cells. *Vet. J.* 195:107-113.
- Spencer, N. D., J. M. Gimble, and M. J. Lopez. 2011. Mesenchymal stromal cells: past, present, and future. *Vet. Surg.* 40:129-139.

- Srivastava, A., S. Shenouda, R. Mishra, and E. Carrier. 2006. Transplanted embryonic stem cells successfully survive, proliferate, and migrate to damaged regions of the mouse brain. *Stem cells* 24:1689-1694.
- Stadtfield, M., M. Nagaya, J. Utikal, G. Weir, and K. Hochedlinger. 2008. Induced pluripotent stem cells generated without viral integration. *Science* 322:945-949.
- Stewart, A. A., J. G. Barrett, C. R. Byron, A. C. Yates, S. S. Durgam, R. B. Evans, and M. C. Stewart. 2009. Comparison of equine tendon-, muscle-, and bone marrow-derived cells cultured on tendon matrix. *Am. J. Vet. Res.* 70:750-757.
- Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861-872.
- Takahashi, K. and S. Yamanaka. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663-676.
- Tapia, N. and H. Schöler. 2010. p53 connects tumorigenesis and reprogramming to pluripotency. *J. Exp. Biol.* 207:2045-2048.
- Terskikh, A., P. Bryant, and P. Schwartz. 2006. Mammalian stem cells. *Pediatr. Res.* 59:13-20.
- Thomson, J., J. Kalishman, T. Golos, M. Durning, C. Harris, R. Becker, and J. Hearn. 1995. Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U. S. A.* 92:7844-7848.
- Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall, and J. M. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.
- Toupadakis, C. A., A. Wong, D. C. Genetos, W. K. Cheung, D. L. Borjesson, G. L. Ferraro, L. D. Galuppo, J. K. Leach, S. D. Owens, and C. E. Yellowley. 2010. Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. *Am. J. Vet. Res.* 71:1237-1245.
- Tryon, R., M. Penedo, M. McCue, S. Valberg, J. Mickelson, T. Famula, M. Wagner, M. Jackson, M. Hamilton, S. Nooteboom, and D. Bannasch. 2009. Evaluation of allele frequencies of inherited disease genes in subgroups of American Quarter Horses. *J. Am. Vet. Med. Assoc.* 234:120-125.
- Vidal, M. A., G. E. Kilroy, J. R. Johnson, M. J. Lopez, R. M. Moore, and J. M. Gimble. 2006. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. *Vet. Surg.* 35:601-610.
- Vidal, M. A., S. O. Robinson, M. J. Lopez, D. B. Paulsen, O. Borkhsenius, J. R. Johnson, R. M. Moore, and J. M. Gimble. 2008. Comparison of chondrogenic potential in equine

- mesenchymal stromal cells derived from adipose tissue and bone marrow. *Vet. Surg.* 37:713-724.
- Vidal, M., G. Kilroy, M. Lopez, J. Johnson, R. Moore, and J. Gimble. 2007. Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Vet. Surg.* 36:613-622.
- Wakayama T. 2007. Production of cloned mice and ES cells from adult somatic cells by nuclear transfer: how to improve cloning efficiency? *J Reprod Dev* 53:13-26.
- Warren, L., P. Manos, T. Ahfeldt, Y. H. Loh, H. Li, F. Lau, W. Ebina, P. Mandal, Z. Smith, A. Meissner, G. Daley, A. Brack, J. Collins, C. Cowan, T. Schlaeger, and D. Rossi. 2010. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell stem cell* 7:618-630.
- Warren, L., Y. Ni, J. Wang, and X. Guo. 2012. Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. *Scient. Report.* 2:657-664.
- Watts, A. E., J. ckerman-Yost, and A. Nixon. 2013. A comparison of three-dimensional culture systems to evaluate in vitro chondrogenesis of equine bone marrow derived mesenchymal stem cells. *Tissue Eng Part A* 19:2275-2283.
- Wei, Z., Y. Yang, P. Zhang, R. Andrianakos, K. Hasegawa, J. Lyu, X. Chen, G. Bai, C. Liu, M. Pera, and W. Lu. 2009. Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. *Stem cells* 27:2969-2978.
- Williams, R., L. Harkins, C. Hammond, and J. Wood. 2001. Racehorse injuries, clinical problems and fatalities recorded on British racecourses from flat racing and National Hunt racing during 1996, 1997 and 1998. *Equine Vet. J.* 33:478-486.
- Yakubov, E., G. Rechavi, S. Rozenblatt, and D. Givol. 2010. Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. *Biochemical and biophysical research communications* 394:189-193.
- Yamanaka, S. 2012. Induced pluripotent stem cells: past, present, and future. *Cell stem cell* 10:678-684.
- Zaidi, N. and A. J. Nixon. 2007. Stem cell therapy in bone repair and regeneration. *Ann. N. Y. Acad. Sci.* 1117:62-72.
- Zhao, S., J. Nichols, A. Smith, and M. Li. 2004. SoxB transcription factors specify neuroectodermal lineage choice in ES cells. *Mol. Cell. Neurosci.* 27:332-342.
- Zhao, T., Z. N. Zhang, Z. Rong, and Y. Xu. 2011. Immunogenicity of induced pluripotent stem cells. *Nature* 474:212-215.

- Zhironkina, O., I. Shipounova, A. Bigildeev, N. Sats, N. Petinati, and N. Drize. 2012. Proliferative potential of multipotent mesenchymal stromal cells from human bone marrow. *Bull. Exp. Biol. Med.* 152:543-547.
- Zhu, F., M. Gamboa, A. Farruggio, S. Hippenmeyer, B. Tasic, B. Schüle, Y. Chen-Tsai, and M. Calos. 2013. DICE, an efficient system for iterative genomic editing in human pluripotent stem cells. *Nucleic Acids Res.* 10:1-13.

APPENDIX A

Plasmid Preparation

Stab cultures containing plasmids encoding desired sequences were obtained from Addgene (Cambridge, Massachusetts). The plasmids used were: 26816pcDNA3.3_OCT4, 26817pcDNA3.3_SOX2 and 26815pcDNA3.3_KLF4. The cultures were streaked on agar plates containing ampicillin 100ug/ml concentration. Clones were chosen from each plate by inserting a pipette tip into a single isolated colonies and inserting it into 3ml of Terrific Broth containing ampicillin 100ug/ml in a 15 ml culture tube. The selected clones were cultured overnight at 37° C shaking at 225 RPM in C24 Incubator Shaker (New Brunswick Scientific, Classic Series).

Mini Prep Isolation

Tubes containing a cloudy media post-incubation (indicating growth of the selected clones) were used for Miniprep isolation. Minipreps were performed on using PureLink® Quick Plasmid Miniprep Kit (Invitrogen, K2100-10). Cells were pelleted from 1.5 ml of previous overnight culture for 1 minute at 12,000 x g in table top centrifuge (Thermo, Heraeus Pico 17 Centrifuge). Supernatant was discarded and cells were resuspended by pipetting up and down in 250 µl of Resuspension Buffer provided in the kit. The cells were then lysed with the addition of 250 µl of Lysis Solution and inverting the tubes gently to mix. The tubes were then allowed to equilibrate for ≤ 5 minutes. The cleared lysate was then prepared for isolation with the addition of 350 µl of Precipitation buffer and inverted 4-6 times to mix. The tubes were then centrifuged at maximum speed of 12,000 x g for 10 minutes. The binding column was prepared by adding 500 µl of Wash Buffer and centrifuged at 12,000 x g for 1 minute. The cleared lysate was then transferred to the prepared binding columns to bind the plasmid DNA for isolation. The column containing the cleared lysate was centrifuged for 1 minute at 12,000 x g and the flow-through

was discarded. Contaminants were removed by adding 750 µl of Wash Buffer to the column followed by centrifugation at 12,000 x g for one minute. The flow through was discarded and was centrifuged for an additional minute to remove to dry the column. Each column was transferred to a new collection tube and the plasmid DNA was eluted in 75ul of molecular grade water and was stored at -20°C.

Clone Selection

Diagnostic cuts in the DNA were performed with restriction enzyme XbaI (Invitrogen , 15226-012) on each clone to determine the quality of the isolated plasmids. A master mix was prepared (buffer provided by manufacturer, molecular grade water, XbaI restriction enzyme, Invitrogen, Grand Island, New York) for all mini-prep isolations. The master mix was then divided and 85ul was placed in 1.5 ml centrifuge tubes. Then, 15ul of purified DNA of each clone was added to the tube and digestion was performed in a hot water bath of 37°C for 1 hour and 15 minutes. The tubes were removed from the water bath and 15ul of loading buffer was added to each tube and mixed gently by pipetting up and down. A 1% agarose gel was prepared and 25 µl of each digest were loaded into the wells for electrophoresis evaluation. The bands were evaluated with the additional 1 kilobase (1Kb) and 100 base pair (100bp) ladders added to the outside lanes. Clones presenting only appropriate cut by the restriction enzyme were selected to synthesize.

mRNA in vitro Synthesis

In order to synthesize the desired mRNA molecules a series of steps are needed starting from the purified plasmids: Linearization of the plasmid, Capped Transcription Reaction, Poly(A) tail synthesis and Recovery of the RNA.

Linearization of the Plasmids

Plasmids containing template sequence were linearized using restriction enzyme XbaI. The restriction digest was terminated with the addition of 1/10th the volume of 5 M NH₄ acetate and 2 volumes of ethanol. The solution was mixed well and stored in -20°C for 15 minutes. The DNA was pelleted by centrifugation at 13,000 x g for 15 minutes. The supernatant was removed and the tube centrifuged again, and any residual fluid was removed with a fine-tipped pipet. The pellet was resuspended in 6 µl DNase free water and was carried over into the mMESSAGE mACHINE[®] T7 transcription Kit (Invitrogen, AM1344) for the Capped Transcription Reaction, following the next protocol:

mMessage mMachine[®] t7 Transcription Kit Protocol

1. Thaw frozen reagents. Place RNA Polymerase Enzyme Mix on ice. Vortex the 10X Reaction Buffer and 2X NTP/CAP to ensure contents are resuspended and place the 2X NTP/CAP on ice. Centrifuge in microfuge briefly prior to removing caps.
2. Assemble transcription reaction at room temperature. A cocktail of the modified bases was prepared containing 15mM of 5-methylcytidine-5'-triphosphate (5-Methyl-CTP, TriLink Biotechnologies, N-1014) 15mM pseudouridine-5'-triphosphate (Pseudo-UTP, TriLink Biotechnologies, N-1019), 15mM ATP, 3mM GTP and 12mM N7-Methyl-3'-O-Methyl-Guanosine-5'-Triphosphate-5'-Guanosine (Anti reverse Cap analog, ARCA, TriLink Biotechnologies, N-7003). Add the water and ribonucleotides to the tube prior to adding the 10X Reaction Buffer, followed by the addition of linear DNA template and Enzyme Mix: 10 µl of modified bases cocktail, 2 µl 10X Reaction Buffer, 6 µl (0.1-1ug) Linear DNA template, 2 µl Enzyme Mix.

3. Mix thoroughly by gently flicking the tube or pipetting up and down. Centrifuge briefly to bring the reaction to the bottom of the tube.
4. Incubate at 37°C for 2 hours for maximum yield.
5. Reaction goes immediately into Poly(A) Tailing Kit.

The previously completed 20ul reaction was carried over to the Poly (A) Tailing Kit (Applied Biosystems AM1350) for further RNA packaging.

Ambion® Poly(a) Tailing Kit Protocol

1. Begin with a completed, room temperature, mMessage mMachine reaction (20 µl in 1.5ml tube).
2. Kept at room temperature, add the following reagents in the order they are listed to the 20 µl mMessage mMachine reaction: 20 µl mMessage mMachine reaction, 36 µl Nuclease-free water, 20 µl 5X E-PAPBuffer, 10 µl 25 mM MnCl₂, 10 µl 10 mM ATP.
4. Add 4 µl of E-PAP and mix gently. The final reaction should be 100ul.
5. Incubate for 1 hour at 37°C.
6. Reaction can go directly into the MEGAclean™ Kit procedure or can be placed on ice or stored at -20°C until for later use.

The tailing reaction was purified using MEGAclean Kit (Applied Biosystems AM1908).

Megaclean™ Kit Procedure

1. Begin with 100 µl Poly(A) Tailing Reaction (or bring RNA sample up to 100 µl by adding Elution Solution and mix gently).
2. Add 350 µl of Binding Solution Concentrate and mix gently by pipetting.
3. Add 250 µl of 100% ethanol and mix gently by pipetting.
4. Insert a filter cartridge into a Collection and Elution tube supplied by the manufacturer.

5. Apply the RNA sample to the filter cartridge.
6. Centrifuge at 10,000-15,000 x g for 1 minute with the cap of the tube open to pass mixture through the filter.
7. Discard flow-through and reuse the Collection and Elution Tube during the following wash steps.
8. Be sure ethanol has been added to the wash solution before use. Apply 500 µl Wash Solution and centrifuge at 10,000-15,000 x g for 1 minute to pass the Wash Solution through the filter.
9. Repeat with a second 500 µl Wash Solution.
10. Discard flow-through and centrifuge for 30 seconds at 10,000-15,000 x g to remove any Wash Solution.
11. Put the cartridge in a new Collection Tube. Elute RNA with 50 µl of TE: Pre-heat 110 µl of TE to 95°C. Apply 50 µl of pre-heated Elution Solution to the center of the filter and close the cap. Centrifuge for 1 minute at 10,000-15,000 x g. Repeat the elution to maximize recovery with a second pre-heated 50 µl of TE into the same tube.

The synthetic mRNA was then quantified using NanoDrop 1000 Spectrophotometer using TE as the blank for the machine. The solution was then diluted with an adequate volume of TE to obtain a working concentration of 100 ng/ml.

Cell Collection

Cells from different types of tissue were collected from 4 euthanized horses due to clinical reasons. Collections of MSCs were performed from the Bone Marrow (from the sternbrae) and Adipose tissue (from the base of the tail) following the next detailed protocols. Fibroblasts were cultured from biopsied dermis tissue. They were cultured in stromal media containing: HyClone Ham's F12/DMEM (HyClone, SH30023.01) medium containing 10% FBS (Atlanta Biologicals,

S12450) and 2% antibiotic/antimycotic solution (MP Biomedicals, 1674049) in 5% CO₂ in humidified air at 38°C. The primary culture was replicated before cells reached confluence with 0.25% Trypsin (Gibco®, 25200-0569 until P2 and frozen in 90% FBS with 10% DMSO and stored in cryogenic vials at -80°C. Vials were revitalized within a month of cryopreservation. After revitalization all plates were seeded at a number of 5000cells/cm². All cells were further culture in 5% CO₂ in humidified air at 38°C, except during the pluripotency induction treatment when cells were place in a modular incubator containing 5% CO₂, 5% O₂ and 90%N₂ at 38°C.

Isolation of Adipose Derived MSCs

1. Use aseptic technique to collect the sample. Clip the area around the base of the tail and make three washes with 3% Clorexidine in distilled water and three washes with 70% ethanol in distilled water.
2. Transport in PBS supplemented with 5% FBS and penicillin and streptomycin (1%) to the lab in order to process.
3. Wash the tissue collected 3 times with PBSs and put in Erlenmeyer or 50 ml centrifuge tube.
4. Mince the tissue with a scalpel blade to loosen the structure of the fat.
5. Add 25ml of 0.25% Collagenase I (Worthington, 4197) solution in PBS with 0.25% Bovine Serum Albumin (Sigma, 85040C).
6. Put the Erlenmeyer or tube in shaker at 200 rpm for 2 hours at 37°C.
7. If you use an Erlenmeyer place everything in a 50 ml tube. Add 15 ml of PBS. Shake the tube vigorously with your hand for 1-2 minutes.
8. Centrifuge at 1,600 rpm for 5 minutes.

9. In case oil has been separated (the top interface) from the rest of the sample (fat tissue and cells detached), remove it by aspiration (taking care not to aspirate the floating tissue) shake again the tube vigorously with your hand for 1-2 minutes.
10. Centrifuge at 1,600 rpm for 5 minutes.
11. Take out all the floating and big pieces of tissue with a sterile needle or pliers.
12. Shake again for 1 minute (not vigorously) just to homogenize the pellet with the solution.
13. Filter the solution with a disposable 100 µm filter
14. Centrifuge at 1,600 rpm for 5 minutes.
15. Discard everything keeping the pellet.
16. Resuspend in culture media and place in T12.5 flask or 10 cm diameter Petri dish.
17. Change the media 24 hours later. Viable cells should be attached at that time.

Isolation of Bone Marrow Derived MSCs

1. Routine procedure is used to harvest sternal bone marrow with a 8.89 cm 10 gauge bone marrow Jamshidi® needle in a sterile manner.
2. Samples are collected with a 50ml Luer lock syringe containing 2 ml of heparin solution (300 IU/ml).
3. Immediately after collection put the sample into a 50 ml conical centrifugation tube.
4. Add approximately 20 to 25 ml of stromal medium to each 50 ml centrifugation tubes containing the bone marrow and mix gently.
5. Prepare one 50 ml centrifugation tube containing 15ml of Ficoll-Paque® PLUS (Stem Cell Technologies, 07975).
6. Layer bone marrow cell suspension mixture carefully over the Ficoll without disrupting the interface.

7. Centrifuge the bone marrow aspirate/Ficoll gradient for 30 minutes at 1400 rpm. Set the centrifuge brake to 1 so that interface does not disrupt when slowing down.
8. Harvest the faint layer of nucleated cells just above the Ficoll layer using a sterile pipette until a sharp contrast between the Ficoll layer and overlying supernatant layer becomes apparent.
9. Pool the cells collected from above the Ficoll into a sterile 50 ml centrifuge tube containing 15ml of stromal media and centrifuge at 1200 rpm for 10 minutes to sediment the cells.
10. Plate the cells in T75 flask or 10cm diameter Petri
11. Incubate at 37°C in 5% CO₂ humidified atmosphere.
12. Allow the mesenchymal stromal cells (stem cells) to adhere to the plastic during a delayed culture period of 5- 6 days duration.
13. On day 5 or 6 remove 70 to 80 % of the culture media and replace with fresh stromal medium.

Lipofectamine Transfections

Cells plated in a 24w plate at 70% confluency were used for transfection. Specific synthetic mRNA was diluted in Opti-MEM I Medium (Invitrogen, 31985-062) in 15ml conical tubes at a final amount of 2pmol of RNA per well and the Lipofectamine RNAiMAX Reagent (Invitrogen, 13778-150) was prepared following manufacturer protocol with slight modifications. At day 7 and 14 cells were trypsinized and a sample of 40,000 cells was taken to seed the Chamber slide for immunocytochemistry.

Lipofectamine® RNAiMax Reagent Transfection Protocol

1. Plate target cells 24-hours prior to transfection in 500 µl growth medium each well of a 24 well plate. Cell should be 50-70% confluent for transfection.

2. Dilute synthetic mRNA in 50 µl of Opti-MEM® I Medium without serum per well (always make for an extra well to account for pipetting error) in a 15 ml conical tube and mix gently. Label as Tube 1.
3. Gently mix Lipofectamine® RNAiMAX Reagent prior to use. Dilute 1 µl of Lipofectamine® RNAiMAX Reagent in 50 µl of Opti-MEM® I Medium per well in a separate 15 ml conical tube. Label as Tube 2.
4. Combine the contents of Tube 2 into the contents of Tube 1 and mix gently. Incubate for 15 minutes at room temperature.
5. Add adequate amount of RNA-Lipofectamine® complexes to each well (100 µl) and mix gently by rocking the plate back and forth several times. The total volume in the well should be roughly 600 µl.
6. Incubate in CO₂ incubator at 37°C.
7. Repeat transfection 24 hours later, everyday for 14 days. Change medium before making a new transfection.

Evaluation of Induction of Pluripotency Treatment

To determine if the treatment with synthetic mRNA was successful, quantitative and qualitative assessments of gene expression and protein localization were performed.

Concentration of endogenous equine transcripts was determined with qRT-PCR with specific primers design and PCR conditions set to detect the activation of the equine endogenous pluripotent genes and not the human exogenous sequences. Treated and controls cells were subjected to the RNA isolation procedure, cDNA synthesis and qRT-PCR.

RNA Isolation

Cells were trypsinized from each well and two replicates of each group were combined (approximately 1×10^6 cells) into one 1.5ml tube each for each group. The cells were pelleted and subjected to the mRNA isolation protocol using Dynabeads[®]mRNA DIRECT[™] Kit (Ambion, 61011) following the “standard” procedure. The mRNA was eluted in 30 μ l of DNase free water.

Dynabeads[®]mRNA Direct[™] Kit Protocol

1. Harvest cells from culture well and pellet as per standard procedure
2. Add 1250 μ l of Lysis/Binding buffer (100 mM Tris-HCL, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8, 1% LiDS, 5 mM dithiothreitol) to a fresh cell pellet with repeated passage of the solution through a pipette tip to obtain complete lysis. The formation of a viscous solution confirms complete lysis.
3. Perform DNA-shearing step forcing the lysate strip cells using a 21-gauge needle 5 times using a 1–2 mL syringe. The reduction in viscosity should be noticeable. Repeated shearing causes foaming of the lysate due to detergent in the buffer, however, this should not affect the mRNA yield. The foam can be reduced by a 30 second centrifugation. The lysate can be frozen and stored at -80°C for later use.
4. Resuspend Dynabeads[®] Oligo(dT)₂₅ thoroughly before use.
5. Transfer 250 μ l Dynabeads[®] Oligo (dT)₂₅ stock tube to an RNase-free 1.5-mL microcentrifuge tube and place the tube on a magnet . After 30 seconds (or when the suspension is clear), remove the supernatant. Remove the tube from the magnet and wash the beads by resuspending in an equivalent volume of fresh Lysis/Binding Buffer.

6. Remove the Lysis/Binding Buffer from the prewashed Dynabeads® Oligo(dT)₂₅ by placing on the magnet for 30 seconds, or until the suspension is clear and discard the supernatant.
7. Remove the microcentrifuge tube from the magnet and add the 1250 µl of sample lysate.
8. Resuspend beads completely into sample lysate and incubate with continuous mixing (rolling mixer) for 5 minutes at room temperature. Rotate the tube after the first 2.5 minutes of mixing. This allows the polyA tail to hybridize to the beads.
9. Place the vial on the magnet for 10 minutes and remove supernatant.
10. Wash beads/mRNA complex twice in 1000 µl Washing Buffer A (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS) at room temperature. Place on magnet to separate beads from solution between each wash step.
11. Wash beads/mRNA complex in 1000 µl Washing Buffer B (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 mM EDTA) at room temperature. Transfer to a new tube. Use the magnet to separate the beads from the solution.
12. Wash again beads/mRNA complex with 1000µl Washing Buffer B. Use the magnet to separate the beads from the solution.
13. Elute the RNA from the beads by adding 27 µl of nuclease-free water, incubate at 75°C for 2 minutes. Immediately place tube on magnet, and transfer supernatant to a new RNase-free tube.
14. Take 2 µl of the sample to measure mRNA concentration and purity in the NanoDrop 1000.
15. Use the isolated RNA directly into cDNA protocol or keep them at -80°C.

Conversion of mRNA into cDNA

To perform quantitative PCR all of the samples were converted to cDNA with the two step reverse transcription kit of QIAGEN (Quantitect® Reverse transcription Kit, 205310) that

eliminates genomic DNA. Conversion to cDNA was performed in a bench-top Bio-Rad MJ Mini Personal Thermocycler

Quantitect[®] Reverse Transcription Kit Protocol

1. Use fresh RNA or thaw template RNA on ice . Thaw gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room temperature (15–25°C). Mix each solution by vortexing the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then store on ice.

2. Prepare the genomic DNA elimination reaction on ice: Add 2 µl gDNA Wipeout Buffer 7x to 12 µl of RNA sample (check that the final amount of RNA should not exceed 1ug per reaction, if it does use less volume of the sample and complete with RNase-free water). Mix and then store on ice.

3. Incubate for 2 minutes at 42°C. Then place immediately on ice.

Note: Do not incubate at 42°C for longer than 10 minutes.

4. Prepare the reverse-transcription master mix on ice: Quantiscript Reverse Transcriptase 1 µl, Quantiscript RT Buffer 5x 4 µl and RT Primer Mix 1 µl per reaction tube. Prepare one reaction more for pipetting error. Add 6 µl of the mix to the 14 µl from step 3, obtaining a final volume of 20 µl.

5. Incubate for 30 minutes at 42°C to increase cDNA yields.

6. Incubate for 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase.

Calibrator Development

To evaluate the gene expression of the pluripotency genes with qRT-PCR primers need to be validated (see Appendix B) for what is required a positive control to develop the standard curves. The positive control is also going to be use on every qRT-PCR to asses that the reactions

during amplification occurred normally. This positive control was developed by mixing mRNA extracted from horse day 7 blastocysts and mRNA from all the cell types used in the experiment. This mRNA mixture was then transformed into cDNA as described previously. The positive control was then “spiked” with purified PCR product (QIAquick PCR Purification Kit) of the specific genes, leading to the formation of a “calibrator”. The genes include both the genes of interest, OCT4, SOX2, and KLF-4, as well as housekeeping genes polyadenylate polymerase and hypoxanthine phosphoribosyltransferase-1. The calibrator contains genes of interest at a final concentration of 2 pg/μl and the housekeeping genes at a final concentration of 0.2 pg/μl. The same procedure was performed for the development of a calibrator to detect the expression of the tenogenic related genes. In this case the positive control was mRNA extracted from a section of 5 mg of the superficial digital flexor tendon of a horse mixed with mRNA from all the cell types used in the experiment. Validation of the tenascin primers was developed using the same procedures (see Appendix B). In order to develop this calibrator mRNA extraction using the RNeasy® Plus Micro kit (QIAGEN, 74004) and RT-PCR were performed following these detailed protocols.

RNeasy® Plus Mini Kit (QIAGEN)

1. Different procedures were performed to obtain an homogenized lysate depending on the sample type.

For processing the tendon: In order to grind the tendon a TissueRuptor was used. The TissueRuptor is a rotor–stator homogenizer that thoroughly disrupts and simultaneously homogenizes single tissue samples in the presence of 350 μl Buffer RLT Plus in 2 minutes.

For processing the blastocysts: Disrupt cells with the addition of 75 μl Buffer RLT Plus. Mix by vortexing or pipetting up and down. Centrifuge for 5 minutes at 10,000 rpm to remove the

capsule. Transfer the supernatant to a new tube. Adjust the volume to 350 µl of Buffer RLT Plus. Vortex for 1 minute to homogenize.

For processing the cell cultures: Harvest cells by trypsinization and pellet. Completely remove supernatant. Disrupt cells with the addition of 350 µl Buffer RLT Plus. Mix by vortexing or pipetting up and down. Homogenize lysate by passing it through a 20-gauge needle 5 times in an RNase-free 1 ml syringe.

2. Transfer homogenized lysate to a gDNA Eliminator spin column placed within 2 ml collection tube. Centrifuge 30 seconds at 10,000 rpm and discard column. Collection tube should contain flow-through to be used in following steps.

3. Add 350 µl of 70% ethanol to the flow-through in the collection tube. Mix by pipetting up and down.

4. Transfer 700 µl of sample to RNeasy® spin column placed within a 2 ml collection tube and close lid. Centrifuge for 15 seconds at 10,000 rpm. Discard flow-through.

5. Add 700 µl Buffer RW1 to the RNeasy® spin column placed into previous collection tube. Close lid and centrifuge for 15 seconds at 10,000 rpm. Discard flow-through.

6. Add 500 µl Buffer RPE to RNeasy® spin column and close lid. Centrifuge for 15 seconds at 10,000 rpm. Discard flow-through.

7. Add 500 µl Buffer RPE to RNeasy® spin column and close lid. Centrifuge 2 minutes at 10000 rpm to ensure removal of ethanol. Remove column carefully not coming in contact with flow-through.

8. Place RNeasy® spin column into a new 1.5 ml collection tube and add 17 µl RNase-free water directly to spin column membrane. Close lid and centrifuge for 1 minute at 10,000 rpm for RNA elution. Repeat with an additional 17 µl to maximize recovery.

Retro Transcriptase Polymerase Chain Reaction

RT-PCR was used to determine the correct size amplicon produced by the primers designed (see Appendix B), to perform a temperature gradient to evaluate the annealing temperature in order to detect the endogenous expression of equine pluripotency genes (see Appendix B), and to have enough amplicon to make the “calibrator”. Each PCR reaction consisted of 25 µl JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma Aldrich, P0982), 4 µl forward primer (5 mM), 4 µl reverse primer (5 mM), 2 µl cDNA, and 15 µl dH2O for a total of 50 µl. The PCR were carried out in a Bio-Rad MyiQ thermocycler. The final PCR products were evaluated by gel electrophoresis using a 2% agarose gel with 1µl of propidium iodide (PI, Sigma, P4864) including a 100bp ladder (New England Bio Labs, N3231L) on the outside lanes. The gel was observed under UV light using BIO-RAD Universal Hood II and pictures were taken with Quantity One Analysis Software.

RT-PCR Protocol

1. Each reaction is carried out in a total of 50 µl. Mix 25 µl Jumpstart Red Mix, 2 µl of (10 mM) forward primer, 2 µl of (10 mM) reverse primer, 11 µl of water, and 10 µl of sample cDNA. Prepare master mixes prior to avoid repeated pipetting.

2. Place tubes into thermocycler with the following program:

Step 1: Hotstart: 94°C for 2 minutes

Step 2: 30 repeats of: Denaturation: 94°C for 30 seconds

Annealing: 60°C for 30 seconds

Extension: 72°C for 1 minute

Step 3: Final Extension: 72°C for 5 minutes

Step 4: Hold: 4°C indefinitely

To determine the annealing temperature that only amplified the equine sequence the temperature gradient used was: 60°C, 62.1°C, 63.9°C, 66.4°C, 68.3°C and 70°C

3. Remove tubes from thermocycler and add 25 µl of each PCR product to a 2% agarose gel for electrophoresis.

Purification of PCR Products

The “calibrator” used to construct the standard curves for qRT-PCR was spiked with PCR amplicons. The PCR products were purified to eliminate all those reagents that were used for RT-PCR (purified from primers, nucleotides, polymerases and salts) with QIAquick PCR Purification Kit (QIAGEN, 28104) following the next protocol.

QIAquick PCR Purification Kit Protocol:

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. (Normally, add 100 µl of Buffer PB to 20 µl PCR sample).
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge at 10,000 rpm for 60 seconds.
4. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge at 10,000 rpm for 60 seconds.
6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column at 10,000 rpm for an additional 1 minute.
7. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

8. To elute DNA, add 30 µl of RNase free water (pH 7.0–8.5) to the center of the QIAquick membrane, let the column stand for 1 minute, and then centrifuge at 10,000 rpm for 1 minute.

qRT-PCR

Quantitative PCR was used to evaluate gene expression in the target cells. Total RNA was extracted from cells in all wells using Dynabeads[®] mRNA DIRECT[™] Kit as described previously. The final elution into 27 µl of RNase free water was used to determine the concentration and purity of the sample (2 µl of sample), to generate cDNA with the Quantitect[®] Reverse transcription Kit (12 µl of sample) and the rest (13 µl of sample) was stored at -80°C as backup. The quantitative PCR reactions were performed in a final volume of 20 µl containing 10 µl of Bio-Rad SsoFast[™] EvaGreen[®] Supermix (Bio-Rad, 172-5200) 2 µl of forward primer, 2 µl of reverse primer 1 µl of cDNA sample and 5 µl of nuclease-free water. Master mixes were prepared per primer sets and per sample triplicate. A no-template reaction was used as a negative control, replacing the sample with nuclease-free water. The “calibrator” was used as positive control. Amplification, standard curves and gene expression efficiency were normalized against housekeeping genes GAP and HPRT-1 as internal controls. The qRT-PCR were carried out in a Bio-Rad MyiQ Single color Real-time PCR detection system with a Bio-Rad MyiQ Optical System Software v1.1. Gene expression

qRT-PCR Protocol

1. Set up plate template.
2. Prepare master mixes for each gene. Each reaction is carried out in a 20 µl reaction (10 µl of SsoFast[™] EvaGreen Supermix (Bio-Rad Laboratories, Inc. Hercules, CA, USA), 5 µl of nuclease-free water, 2 µl of (5 mM) forward primer, 2 µl of (5 mM) reverse primer, and 1 µl of sample cDNA, calibrator cDNA, or water as negative control.

3. Add 20 µl of each reaction into a single well of a 96-well plate with the designated plate set-up. Cover with sealing tape supplied by plate manufacturer. Be sure to completely seal each well using the rubber scraper.

4. Place 96-well plate into thermocycler and run the following program:

Step 1: Hotstart: 95°C for 1 minute

Step 2: 40 repeats of: Denaturation: 94°C for 5 seconds

Annealing and extension: 60°C for 30 seconds

Step 3: 95°C for 1 minute

Step 4: 55°C for 1 minute

Step 5: 80 repeats of increasing 0.5°C starting on repeat 2 55°C for 10 seconds.

Step 6: Hold: 4°C indefinitely

Data collection and real time analysis were carried out in Step 2. Melt curve data collection and analysis were carried out in Step 5.

Different annealing temperatures were used depending on the gene evaluated: OCT4 67°C, KLF-4 65°C, SOX-2 60°C, HPRT-1 60°C, PAP 60°C and TNC 60°C. Each plate was run for a specific gene.

5. Evaluate post-run data for analysis and melting curve. If a calibrator dilution was performed, evaluate Standard Curve Efficiency, PCR Amp/Cycle graph, and Melt Curve Analysis.

Immunocytochemistry

Specific localization of the pluripotency related proteins was assessed with Immunocytochemistry using the Fluorescent Human ES/iPS cell characterization kit (Milipore, scr078) for detection of OCT4, Sox-2 and NANOG expression following the next protocol for cells cultured in a Chamber slide (Glass chamber Slide system NUNC, 4wells, 0.5–0.9 ml/well

of media, 9 x 20mm, 1.8 cm², cat# 177399). The same procedure was done to evaluate cell morphology with the 488 phalloidin F-Actin (Molecular Probes®, A12379) staining, except that the Blocking step was omitted and the incubation with the antibody was replaced with incubation with 488 phalloidin F. Nuclei staining were performed with DAPI (contained in Human ES/iPS cell characterization kit) or Hoechst 33258 (Sigma, 94403) Avoid confluence, since cells should be spaced enough to identify each cell, but culture them for enough time to have some cells close to one another to take pictures. Pictures were taken using a Leica DM4500B microscope with a Leica DFC480 camera using a fluorescence lamp Leica EL6000.

Immunocytochemistry Protocol

1. Aspirate culture media. Wash 3 times with PBS to remove culture media. (Aspirate with vacuum from one the side to avoid touching the cells on the bottom of the slide)
2. Fix cells in 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature.
3. Aspirate solution and Wash 3 times with PBS (5 minutes per wash).
4. Permeabilization: Add approximately 300 µl (to cover the entire well) to each well of Permeabilization solution (0.2% Triton X-100, and 0.05% NaN₃ in 1X PBS). Incubate for 20 minutes at room temperature.
5. Aspirate solution and Wash 3 times with PBS (5 minutes per wash).
6. Blocking: Add 300 µl to each well of Blocking solution (3% Normal Goat or Donkey Serum, 0.2% Triton X-100, and 0.05% NaN₃ in 1X PBS). Incubate at 37°C for 30 minutes.
7. Before the end of the incubation time, prepare 1:100 dilutions of the conjugated antibodies in t blocking buffer (protected from light).
8. Aspirate solution and Wash 3 times with PBS (5 minutes per wash).

9. Add the 1:100 diluted antibodies to the designated well. Incubate for 2 hours at room temperature. Cover the Chamber slides with tin foil to protect from the light. Prepare to use 300 μ l of antibody solution for each well.
10. Aspirate solution and Wash 3 times with PBS (5 minutes per wash).
11. Prepare the DAPI dye. Dilute the DAPI in 1X PBS at 1:1000 dilution.
12. Counterstaining: Add 300 μ l of the DAPI solution and incubate at room temperature for 5 minutes covered from light.
13. Aspirate solution and Wash 3 times with PBS (3 minutes per wash).
14. Removal of the chamber and mounting: Remove the chamber from the slide using the “lifting device”. Be sure that the device is free from any rubber from previous detachments. Pressure the device between the chamber and the slide firmly and slide it to the other end just to lift the plastic chamber. In order to remove the sealing rubber is better to grab it with a mouse tooth forceps from one end and lift the rubber firmly while holding down the slide (wear goggles). Make sure to remove all the rubber that is sealing the chamber (if not it would make difficult the mounting of the cover slide). To remove the extra rubber attached to the slide, use a pliers and a scalpel blade to peel it off. Use the vacuum system with a tip to remove the extra PBS in the slide. Do it from the sides setting the slide in a vertical position over the longest border of it.

To mount the cover slide, put a drop (10 μ l) of glycerol in the area where each of the wells was (4 drops in total). Place the slide in the counter and gently put the slide on top. The weight of the cover slide should be enough pressure to avoid bubble formation

Flow Cytometry

The multipotentiality of the cells used in the different experiments was assessed in a first instance with Flow cytometry using specific antibodies against cell surface markers: α CD44 clone:HR-BAG40a (Washington State University, Monoclonal antibody center cat#:HR-BOV2037), α CD105-PE clone:SN6.(eBioscience, cat#12-1057), α CD90-PE clone:YKIX337.217 (eBioscience, cat#12-5900) and α CD29 clone:18/CD29 (BD Transduction Laboratories, cat#610468). For those unconjugated antibodies a secondary antibody was used (anti-mouse IgG whole molecule FITC conjugated, Sigma, F9006). For each sample/antibody at least 2×10^5 cells at P2 were used. The flow tubes used were polystyrene round bottom 5ml (BD Falcon 352054).

Flow Cytometry Protocol

1. Trypsinize the cells (200.000 cells/sample-antibody). Add PBS to dilute the trypsin.
2. Transfer everything to 15 ml centrifuge tubes
3. Centrifuge 10 minutes 1,400 rpm. Discard supernant (Gently pour instead of aspirating)
4. Resuspend in PBS. The volume will depend on how many samples (antibodies to test). Usually 200 μ L/sample
5. Aliquot cells in flow tubes (it is good practice to have the tubes uncap and the antibodies in order. Cap them when you put the antibody, just to keep track of what you are doing)
6. Centrifuge with a quick spin the whole tube of antibody.
7. Add 1 μ L of (primary) antibody in each sample. Vortex (10-20sec). Incubate for 30 minutes in the dark at room temperature.
8. Add PBS: 3 ml/sample.
9. Centrifuge 10 minutes 2,000 rpm.

10. Observe the pellet on the bottom of the tube.
11. Discard supernant (Gently pour instead of aspirating. There might be a leftover of PBS that is enough to resuspend the cells with formalin or add secondary antibody).
12. If no secondary antibody is needed add 30-50 μL of 1-2% PFA in PBS, Vortex and keep it in 4°C until flow cytometry. The samples will be good for up to one week.
13. If secondary antibody is needed, add 1 μL in each sample. Vortex. Incubate for up to 30 minutes in the dark. Do not incubate longer than that.
14. Add PBS: 3 ml/sample.
15. Centrifuge 10 minutes 2,000 rpm.
16. Observe the pellet on the bottle of the tube.
17. Discard supernant (Gently pour instead of aspirating. There might be a leftover of PBS that is enough to resuspend the cells with formalin) add 30-50 μL of 1% PFA in PBS, Vortex and keep it in 4°C until flow cytometry.

Differentiation into Specific Lineages

Cells used in the experiments were further characterized with trilineage differentiation induced with media supplementation at P2. Furthermore, assessment of maintenance of the multipotency characteristics after induction of pluripotency treatment was performed following the same protocol. In this last case cells were expanded until P10. Control cells were culture with stromal media. Differentiation into osteogenic, adipogenic and chondrogenic lineages were evaluated with Oil Red O staining, Alcian Blue staining and von Kossa staining respectively. Furthermore, differentiation into the tenogenic lineage was induce and evaluated with qRT-PCR as described previously. Before induction of differentiation into the specifics lineages evaluated, cells were cultured until 80% confluence in a 12 well plate for the adipogenic, osteogenic and

tenogenic differentiation, and in form of a pellet in 15ml tubes for the chondrogenic differentiation.

For adipogenic differentiation the induction media consisting of: DMEM-Ham's F12, 10% FBS, 33 μ M biotin (Sigma, B4639), 17 μ M pantothenate (Sigma, P5155), 2 μ M insulin (Sigma, I5500), 1 μ M dexamethasone (Sigma, D4902), 500 μ M isobutylmethylxanthine (Sigma, I5789), and 5 μ M rosiglitazone (AK Scientific, 70325) was used during 7 days in cells in a monolayer. Then, the cells were cultured 14 days more in the same media without isobutylmethylxanthine and rosiglitazone. For osteogenic cells were cultured in monolayer for 21 days in this differentiation medium: DMEM-Ham's F12, 10% FBS, 10 mM β -glycerol phosphate (USB Corporation, 21655), 100 nM dexamethasone and 0.25 mM ascorbic acid 2-phosphate. For tenogenic lineage cells in monolayer were differentiated with a two step protocol. First, cells were incubated for 24 hours with stromal media supplemented with 10 ng/ml of BMP-12 (BioVision, 4572) reported to augment the formation of tendon-like tissue in adult multipotent stromal cells in human. Then, cells were cultured in DMEM, 15% FBS, 0.34 mM proline and 0.17 mM ascorbic acid (tenogenic differentiation media) for 21 days. For chondrogenic differentiation pellet cultures were made by centrifuging aliquots of 2.5×10^5 cells. Cells were resuspended in 1ml of pre-warmed stromal media and centrifuge at 1,500 rpm (200 x g) for 5 minutes at room temperature. Media was discarded and the pellet was resuspended cells in 500 μ l of chondrogenic differentiation media. The tube cap was loosen to allow gas exchange and incubate at 37°C and 5% CO₂. After 1-2 days the cell pellet formed a round ball of approximately 1-2 mm in diameter. Caution is needed when replacing the medium to avoid aspirating the pellet. The chondrogenic differentiation media used to maintain the pellets for 21 days was DMEM high glucose supplemented with 100 nM dexamethasone, 0.25 mM ascorbic

acid 2-phosphate (Sigma, A8960), 40 µg/ml proline (Sigma, P0380) and 10 ng/ml TGF-β (R&D System, 243-B3). All the cell culture differentiation media were renewed every 3-4 days. Control cells were cultured in stromal media for the same period and stained as described previously.

Evaluation of Trilineage Differentiation

Evaluation of the differentiation into the three lineages (osteogenic, adipogenic and chondrogenic) was performed with specific staining following the next detailed protocols. Pictures were taken under a Olympus CKX41 stereoscopic microscope using a Olympus DP21 camera incorporated.

Osteogenic Differentiation: Alizarin Red Staining Protocol

1. Rinse with 150 mM NaCl three times each well that is going to be stained (approximately 1 ml per rinse per well). Do not use PBS at any time during this protocol; it will create an artifact in your data.
2. Fix in ice cold 70% ethanol. Place plate for 1 hour at 4°C.
3. Prepare 2% alizarin red solution in distilled water (0.2 g Alizarin Red, Sigma A5533, in 10 ml distilled water). Adjust pH to 4.1 to 4.3 with dilute NaOH. Filter the solution in a two step procedure. First, through a 100 µm filter and then through a 0.22 µm syringe filter. This can be stored at room temperature covered with aluminum foil.
4. Remove ethanol from the well. Rinse with water three times. Cover the well with 2% alizarin red solution; use 1 ml per well on a 12 well plate. Allow to stain for 10 minutes at room temperature. Observe under a microscope to determine extent of staining.
5. Rinse the wells 5 times with water. Rinse a final (6th) time for 15 minutes in distilled water. Remove this last rinse and add 1 ml of distilled water to take pictures.
6. Photograph immediately at this step for documentation at selected magnification.

Adipogenic Differentiation: Oil Red O Staining Protocol

1. Prepare stock solution of Oil Red O. Weigh out 0.5 grams Oil Red O (Sigma, O0625). Dissolve in 100 ml isopropanol. Filter the solution in a two step procedure. First, through a 100 μm filter and then through a 0.22 μm syringe filter. Store at room temperature as stock solution.
2. Take 6 ml of Oil Red O stock solution. Add 4 ml of distilled water. Stand 1 hour at room temperature. Filter through 0.2 μm filter.
3. Rinse the 12 well plate three times with PBS.
4. Fix cells by adding 1ml of 1% PFA in PBS. Place plate at 4° C for 1 hour.
5. Remove all formaldehyde from well. Well should be dry after fixation and removal. Let sit the plate under the hood until the surface of the well is dry.
6. Add 1ml of Oil Red O to each well for 15 minutes at room temperature.
7. Rinse three times or more (the rinse should become completely clear) with 1 ml distilled water. Remove the last rinse and add 1 ml of distilled water to take pictures.
8. Photograph immediately at this step for documentation at selected magnification.

Chondrogenic Differentiation: Paraffin Embedding and Alcian Blue Staining Protocol

1. Remove the chondrogenic differentiation media from the 15 ml tube and fix the sample by adding 500 μl of 4% PFA. Store at 4°C overnight.
2. Turn on all equipment of the tissue embedding station the day before to melt the paraffin beads.
3. Label plastic cassettes with pencil and label paper strip in pencil making a circle where the sample is going to be placed in.
4. To remove the pellet from the 15ml tube use a 1000 μl pipette to aspirate it. Hold the pipette vertically and watch the pellet go to the tip falling by gravity. Then, just touch the paper in the drawn circle while making only a small discharge with the pipette. Avoid discarding a lot of PFA

since it might move the pellet from the center of the drawn circle. It is advisable to wear goggles and to place several tissue papers under the paper strip to absorb any excess of PFA.

5. Fold the paper to make sure pellet will not come out of it and put it in a labeled cassette.

6. Place the cassettes in a beaker and start with the dehydration process.

<u>Reagent</u>	<u>Manual time</u>
a. 70% Ethyl alcohol	1 hour
b. 80% Ethyl alcohol	30 minutes
c. 95% Ethyl alcohol	30 minutes
d. 95% Ethyl alcohol	30 minutes
e. 100% Ethyl alcohol	30 minutes
f. 100% Ethyl alcohol	30 minutes
g. 100% Ethyl alcohol	30 minutes
h. Xylene	30 minutes
i. Xylene	30 minutes
j. Paraffin Ca 58°C (m.p. = 56°C)	30 minutes
k. Paraffin Ca 58°C (m.p. = 56°C)	30 minutes
l. Paraffin Ca 58°C (m.p. = 56°C)	30 minutes
m. Paraffin under vacuum, 58°C	Overnight

7. Remove cassette from paraffin using hot forceps and place it on the warm plate of the tissue embedding station.

8. Fill a metal mold with paraffin and leave it in the plate of the tissue embedding station. Make sure that the mold is small, in order to easily track the pellet in the center of the mold for later paraffin sections.

9. Remove the folded paper strip from cassette, place it in the warm plate of the tissue embedding station and carefully open it using hot forceps.

10. Place the pellet inside of the paraffin-filled mold by using the tip of a scalpel blade (previously warmed).

11. Move the mold to the cold plate of the tissue embedding station. Let it cool but not completely harden before putting the back of the cassette on top of the mold. Fill with more

paraffin until the whole back of the cassette is covered in paraffin and let it harden on the cold plate for around 1 hour.

12. Once hardened, remove the cassette from the mold and cut around the edges with a razor blade to remove excess of paraffin. Paraffin blocks can be stored at 4°C until sections are done.

13. Make sure that the blade on the microtome is sharp. When cutting a large number of samples, the blade may need to be changed as often as once a day. Never change the angle of the blade once it is set. Clamp sample into the block on the microtome. Set thickness to 5-7 microns. Make at least 5 sections to place in one slide.

14. Deparaffinize and hydrate to distilled water: Place the slides in a rack, and perform the following washes:

a) Xylene: 2 x 3 minutes

b) 95% ethanol: 3 minutes

c) 70 % ethanol: 3 minutes

d) 50 % ethanol: 3 minutes

e) Running cold tap water to rinse (3 minutes)

15. Mordant the sections in 3% acetic acid in distilled water. Drain the slides. Do not rinse.

16. Place the slides in a solution containing 1% Alcian Blue (Acros Organic, 400460100) in 3% acetic acid (Adjust pH to 2.5) for 30 minutes

17. Running cold tap water to rinse (2 minutes).

18. Counter stain with Nuclear Fast Red (Leica, 38016SS3B) for 3 minutes.

19. Running cold tap water to rinse (2 minutes).

20. Dehydrate by moving the slides 4 times in four different 100% ethanol solutions. Move the slides three times in 3 different Xylene solutions. Leave the slides in the last one until mounting.

21. Without rinsing the Xylene from the slide mount them with Permount (Fisher, SP15-500) by placing a small drop in the center of the slide. Apply a cover slide on top. Microwave for 1 minute to seal the Permount.

Cell Proliferation Assay

In order to evaluate cell proliferation after induction of pluripotency and expansion until P10 the alamarBlue® (Molecular Probes®, DAL1025) dye was employed. The alamarBlue® assay is quantitative with respect to time and dose as seen by the ability of metabolically active cells to convert the reagent into a fluorescent and colorimetric indicator. First, a standard curve was developed to determine the time of incubation of the dye with the cells. This was necessary to establish when the fluorescence intensity of alamarBlue® is directly proportional to a cell number range. When a short incubation time is used, a high resolution for a large number of cells (around 50,000 cells) is obtained. To gain more resolution for detecting the influence of a few cells (as low as 50) a longer incubation time is needed. Based on an estimated number of cells in one well of a 96 well plate was expected to be between 1,500-12,000 according to the seeding density ($1,000/\text{cm}^2$) and the duration of this assay (21 days), the time when the fluorescent intensity and this cell number range were linear was 2 hours incubation. The measurements for each time point were taken in three independent replicates on days 0, 7, 14 and 21 of culture. Although the dye allowed repeated measures in the same well, to avoid the influence of not independent measurements new wells were measured on each time point. Every well contained 100 μl of stromal media and 10 μl of the alamarBlue® dye was added. Measurements were detected in a Bio-Tek FLx800 Synergy HT plate reader with a KC4 v2.7 software.

APPENDIX B

Table B.1 PCR Primers

Primer	Sequence (5'-3')	Amplicon Length	Accession #
OCT4 Forward	GTGAAGGTGGACAAGGAGAAG	194	XM_001490108.3
OCT4 Reverse	CGGCAGATGGTCGTTTGG		
KLF4 Forward	GTTCTCATCTCAAGGCACACC	157	XM_001492956.3
KLF4 Reverse	TCGCACTTCTGGCACTGG		
SOX2 Forward	ATGGGTTCGGTGGTCAAGTC	202	XM_003363345.1
SOX2 Reverse	GCCGCTCTGGTAGTGCTG		
TNC Forward	TAGTAACGGTGGTGGATTCTGG	185	XM_001916622.3
TNC Reverse	CGGCTCGGCTTCTGTAACA		
PAP Forward	TGTGGGTGATTGGGTTAGTG	193	XM_001489078.3
PAP Reverse	TGATTAGGTAGTAGTTGATGGAGT		
HPRT-1 Forward	CGAGATGTGATGAAGGAGATG	233	XM_001490189.2
HPRT-1 Reverse	TTTCCAGTTAAAGTTGAGAGGTC		

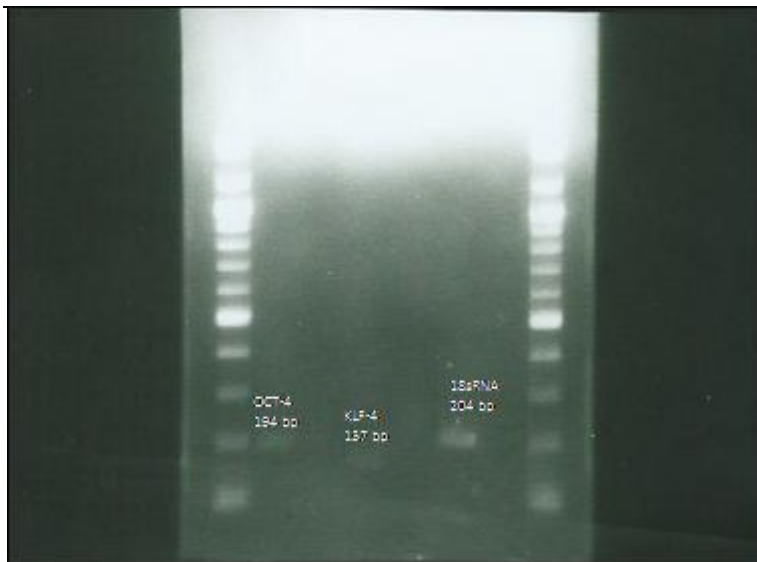


Fig B.1 Amplicon size validation for OCT4, KLF4 and 18SrRNA (not used in further experiments)

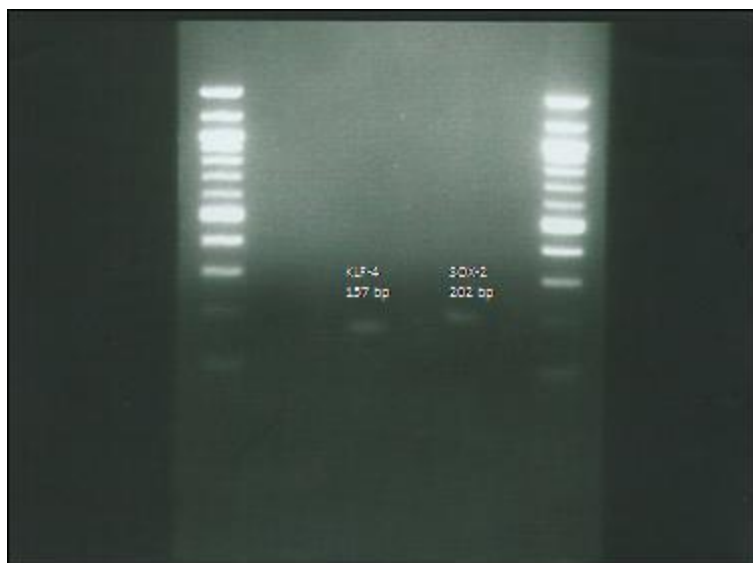


Fig B.2 Amplicon size validation for KLF4 and SOX2.



Fig B.3 Amplicon size validation for TNC, PAP and HPRT-1.

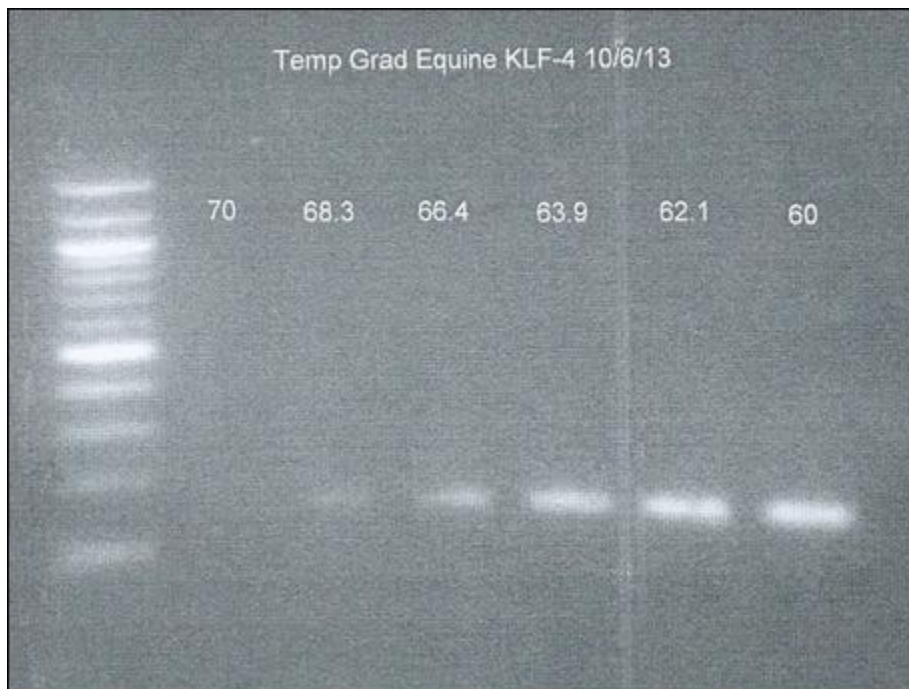


Fig B.4 Temperature gradient for the KLF4 primer tested against equine sequence

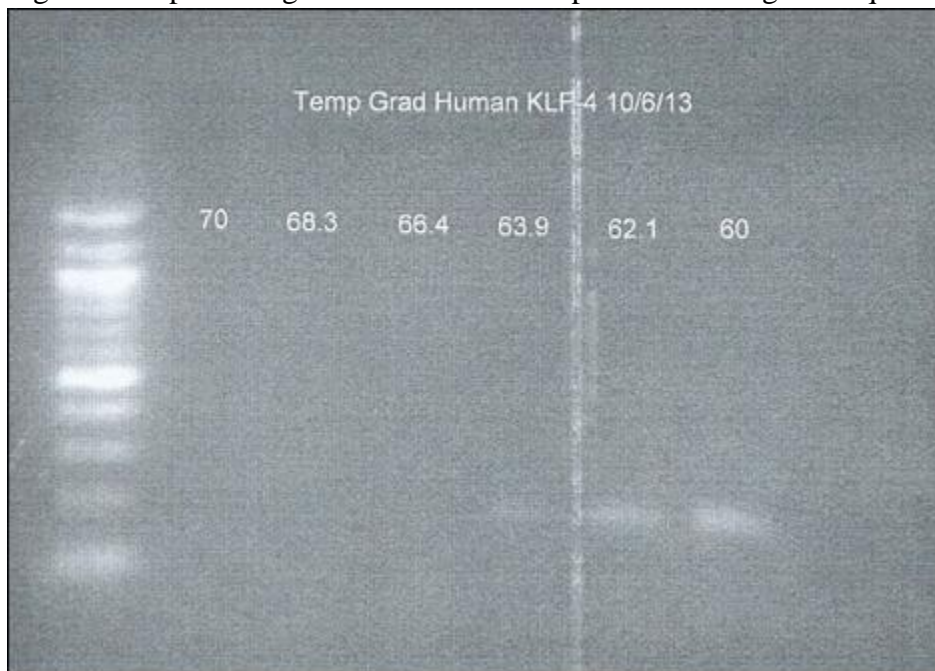


Fig B.5 Temperature gradient for the KLF4 primer tested against human sequence

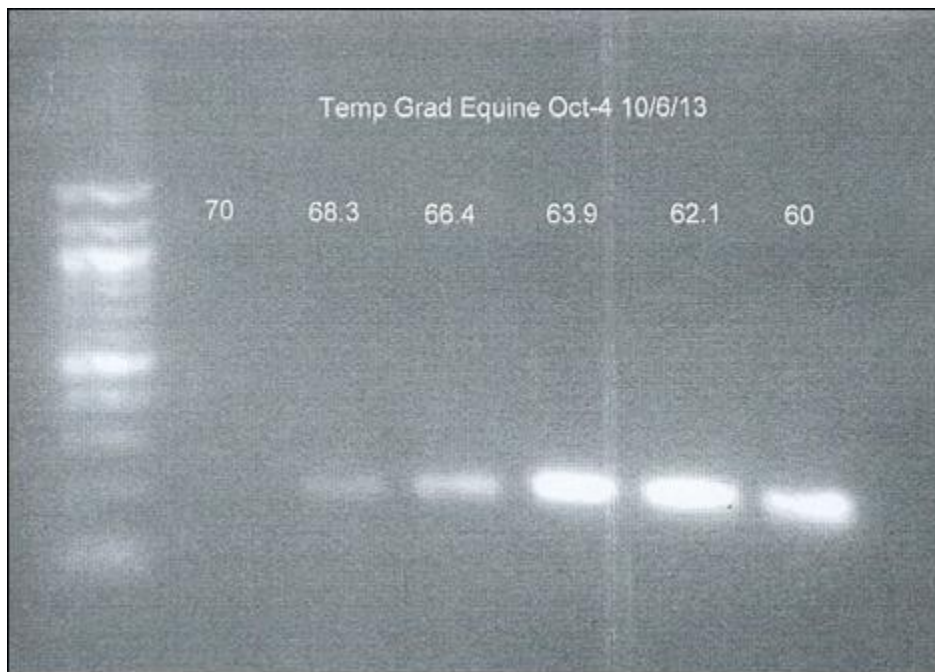


Fig B.6 Temperature gradient for the OCT4 primer tested against equine sequence

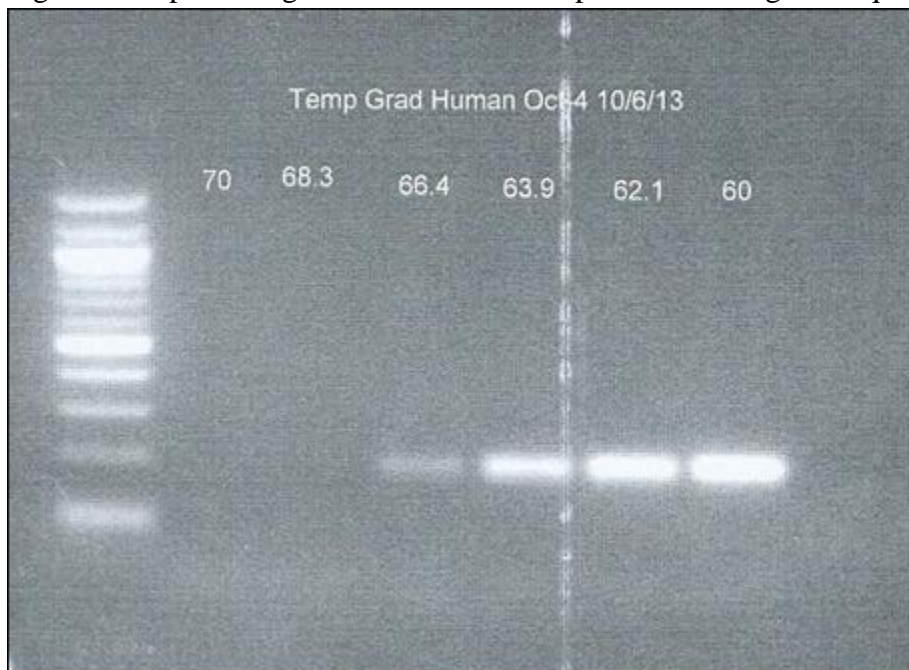


Fig B.7 Temperature gradient for the OCT4 primer tested against human sequence

OCT4 Primer and Calibrator Evaluation

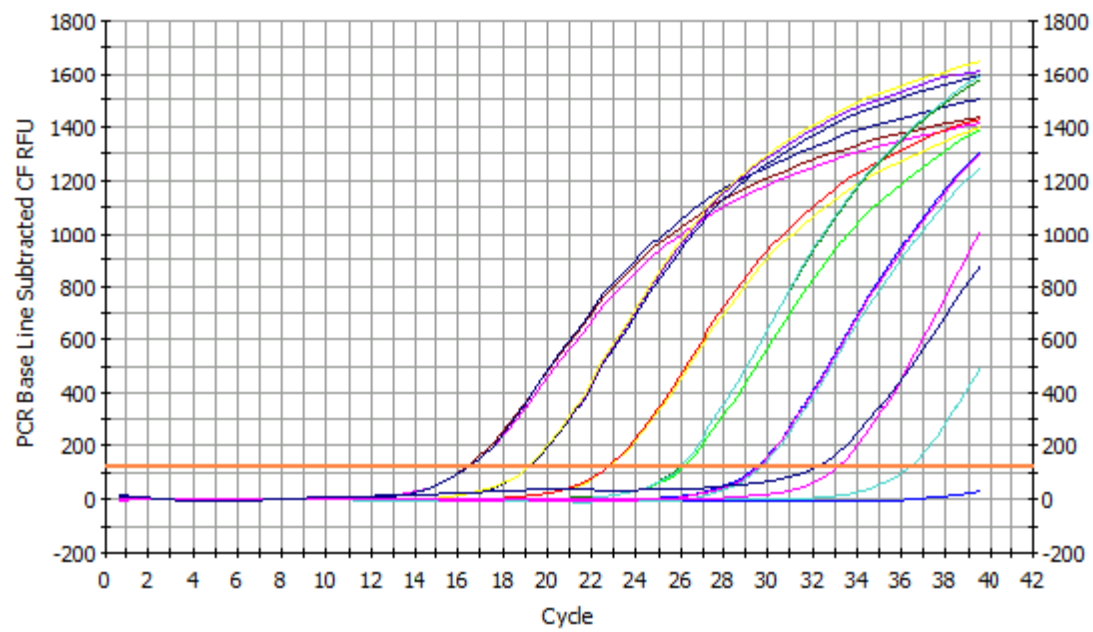


Fig B.8 OCT4 qRT-PCR Amplification/Cycle graph

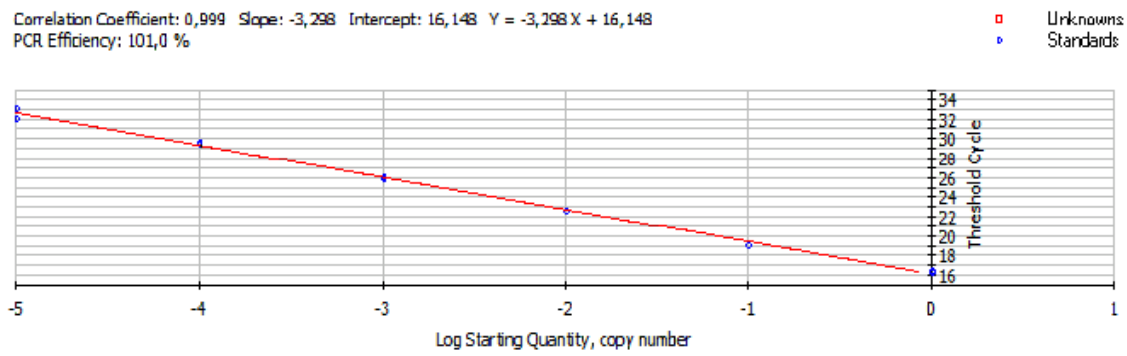


Fig B.9 OCT4 Standard curve

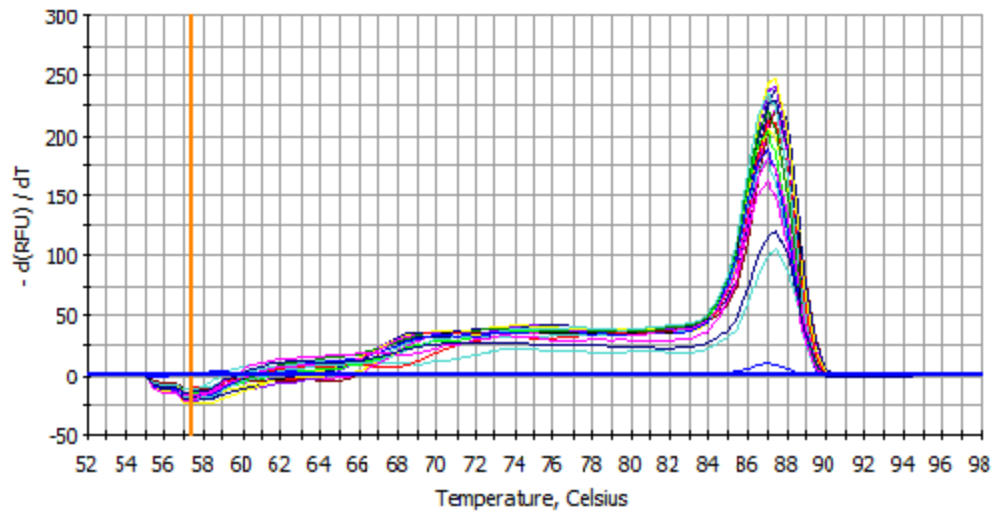


Fig B.10 OCT4 Melting curve

KLF4 Primer and Calibrator Evaluation

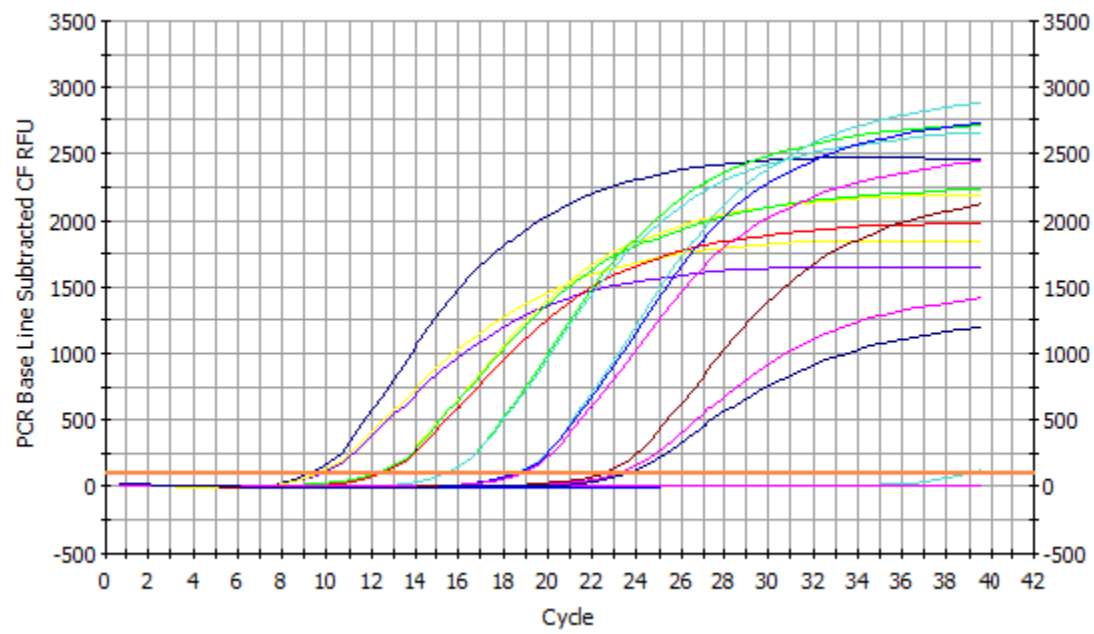


Fig B.11 KLF4 qRT-PCR Amplification/Cycle graph

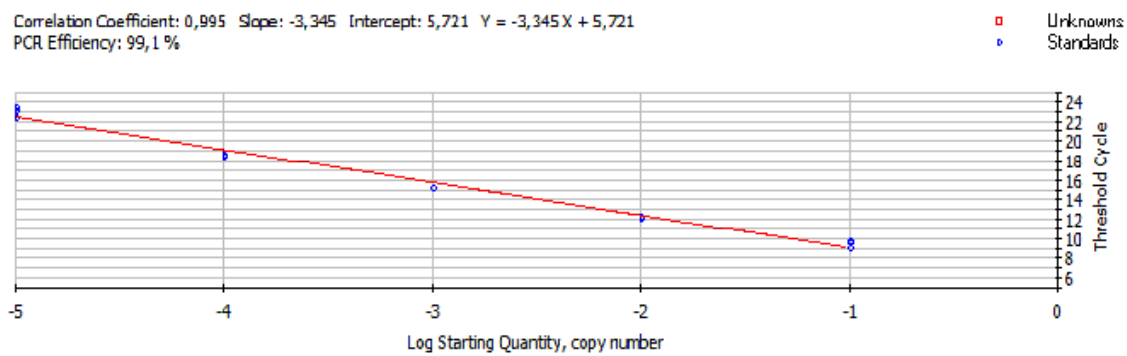


Fig B.12 KLF4 Standard curve

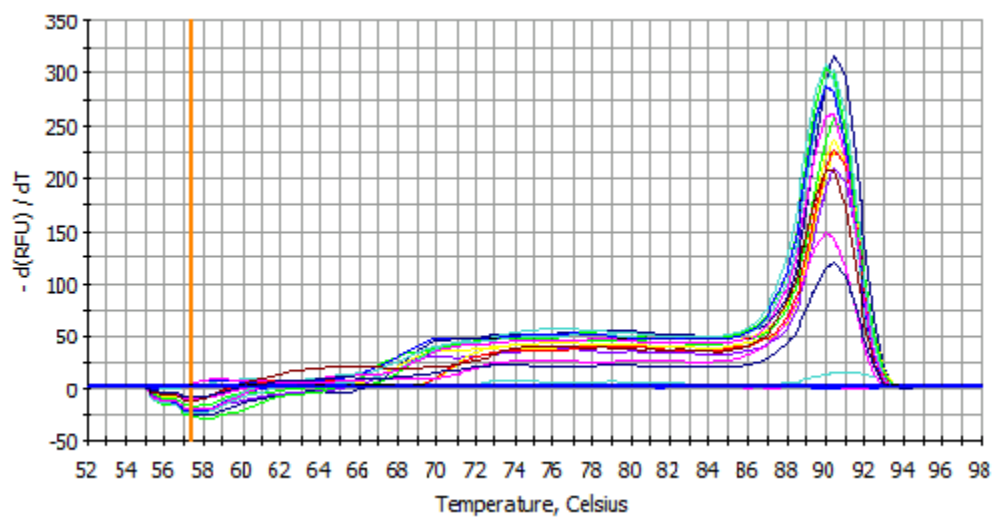


Fig B.13 KLF4 Melting curve

SOX2 Primer and Calibrator Evaluation

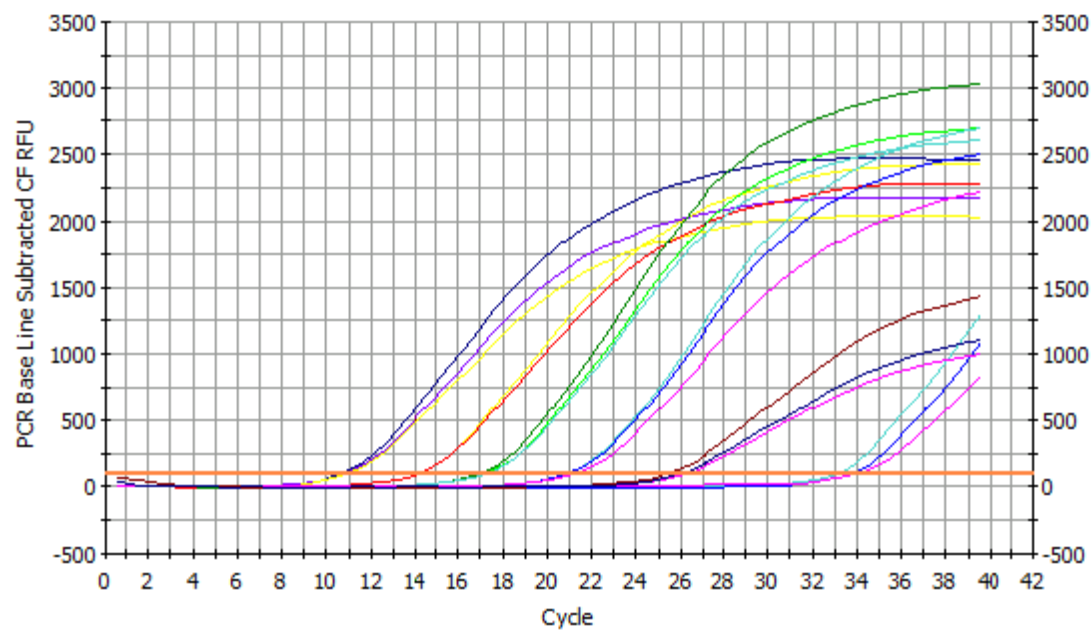


Fig B.14 SOX2 qRT-PCR Amplification/Cycle graph

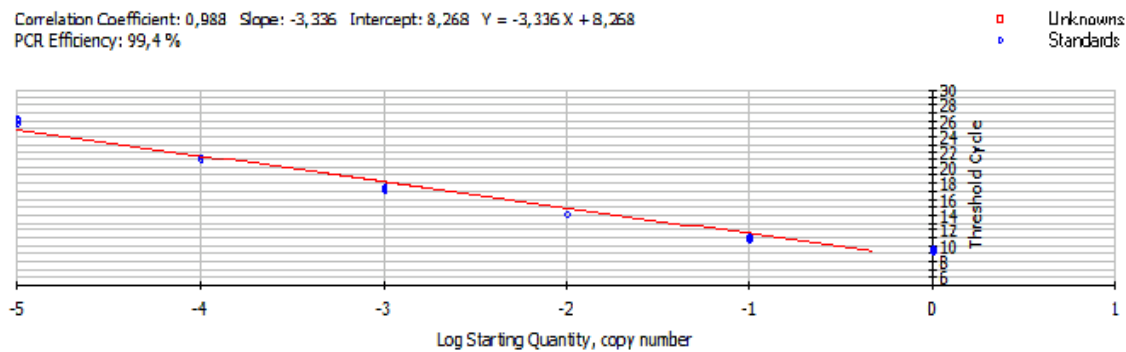


Fig B.15 SOX2 Standard curve

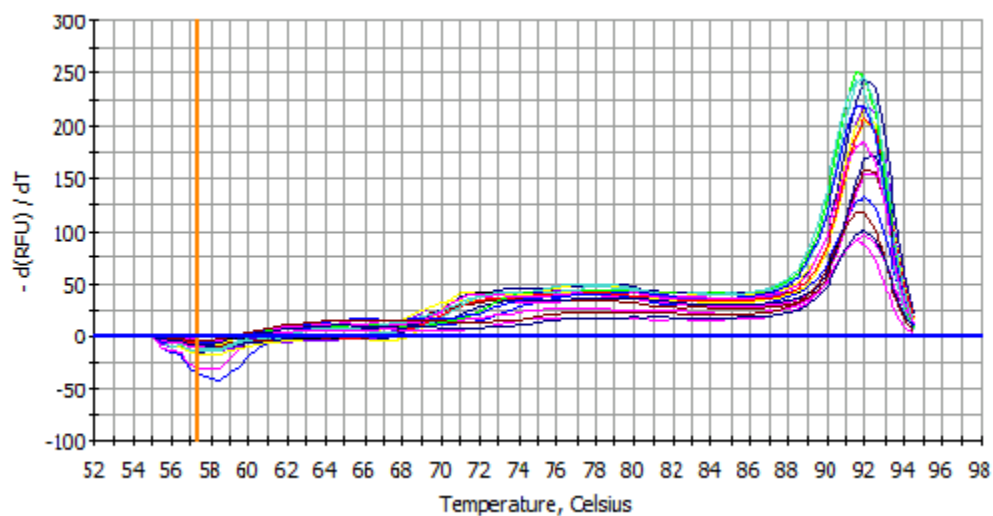


Fig B.16 SOX2 Melting curve

PAP Primer and Calibrator Evaluation

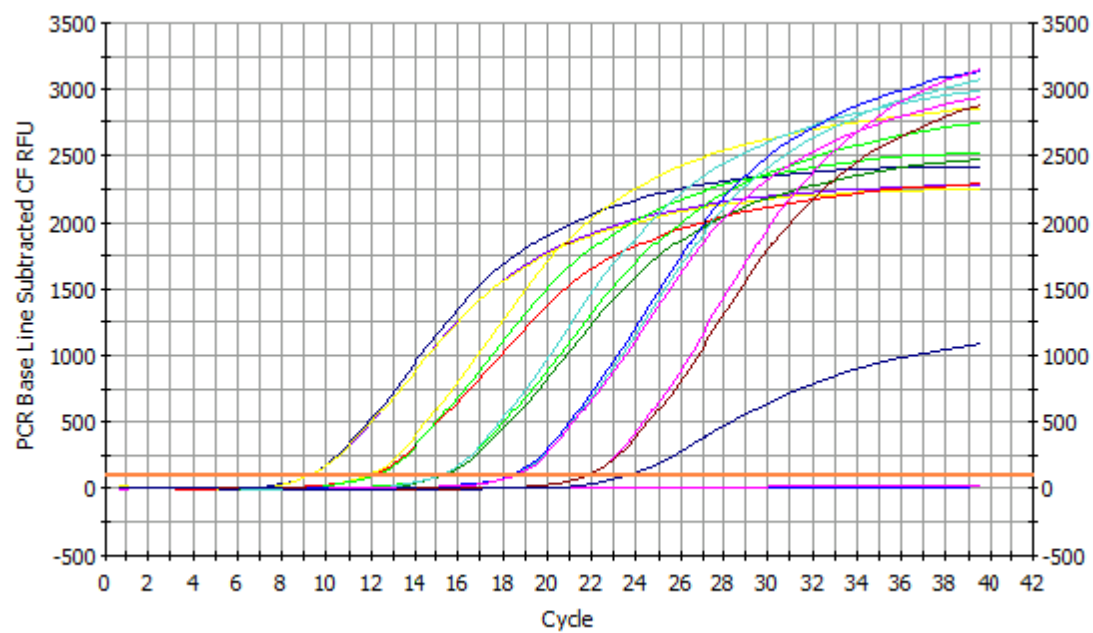


Fig B.17 PAP qRT-PCR Amplification/Cycle graph

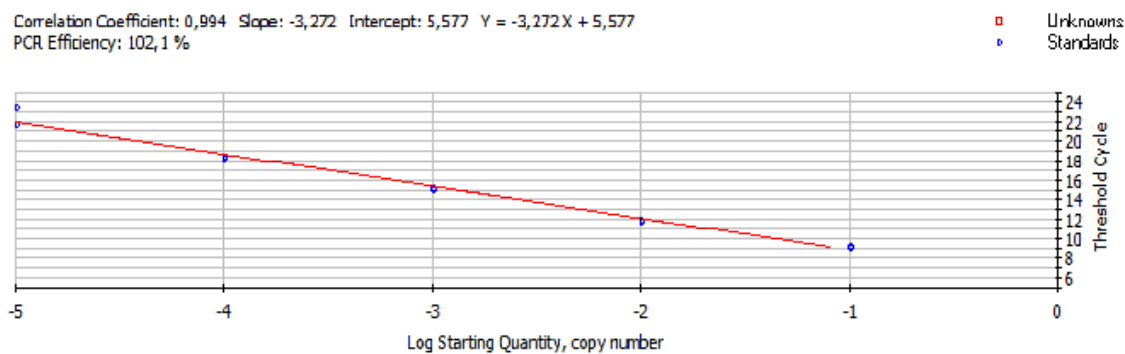


Fig B.18 PAP Standard curve

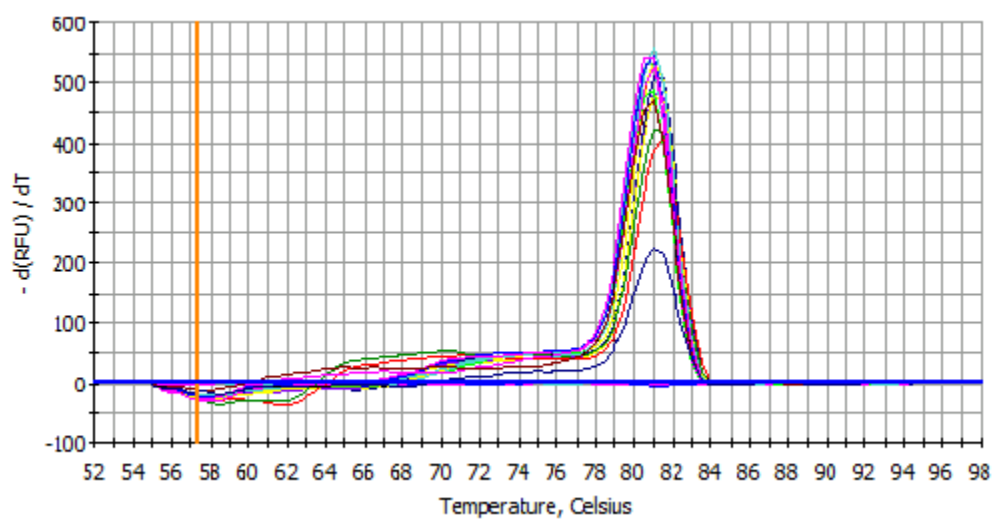


Fig B.19 PAP Melting curve

HPRT-1 Primer and Calibrator Evaluation

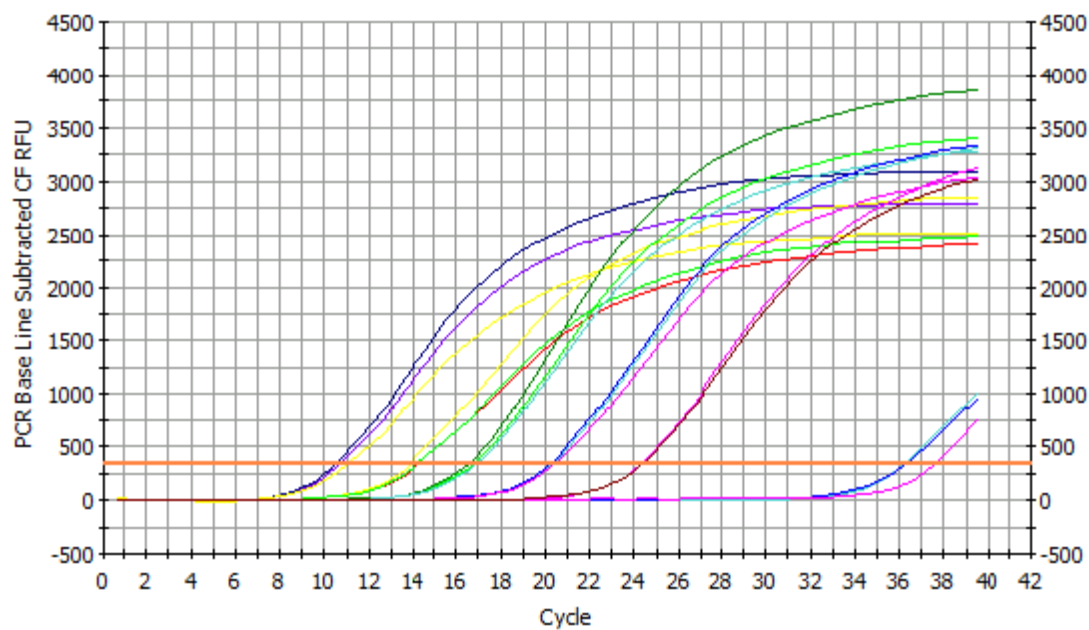


Fig B.20 HPRT-1 qRT-PCR Amplification/Cycle graph

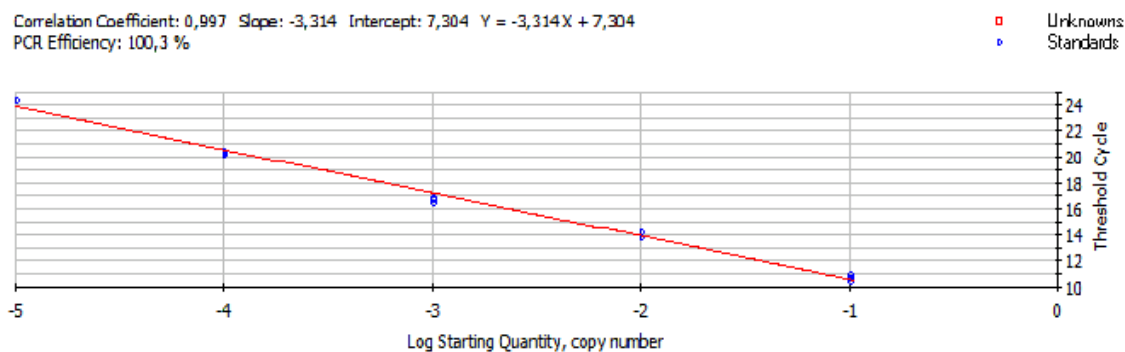


Fig B.21 HPRT-1 Standard curve

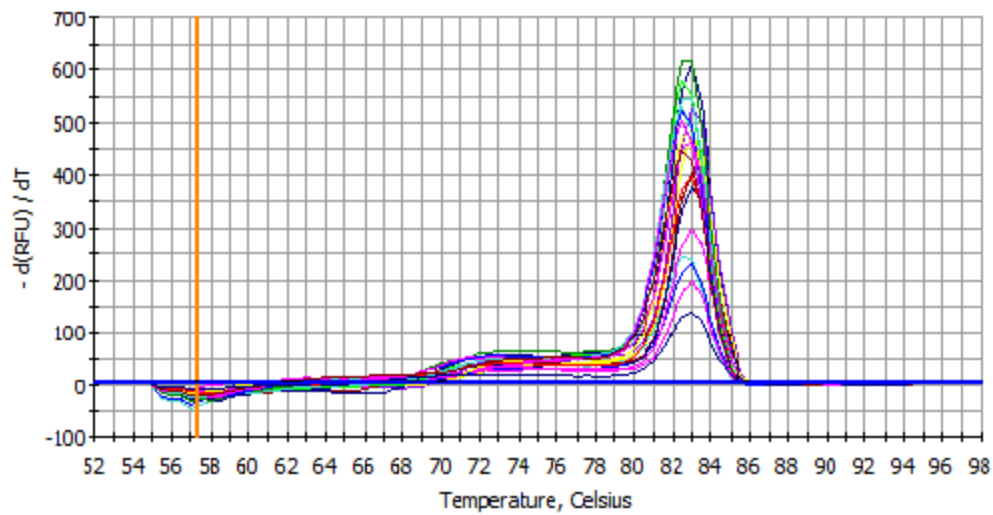


Fig B.22 HPRT-1 Melting curve

TNC Primer and Calibrator Evaluation

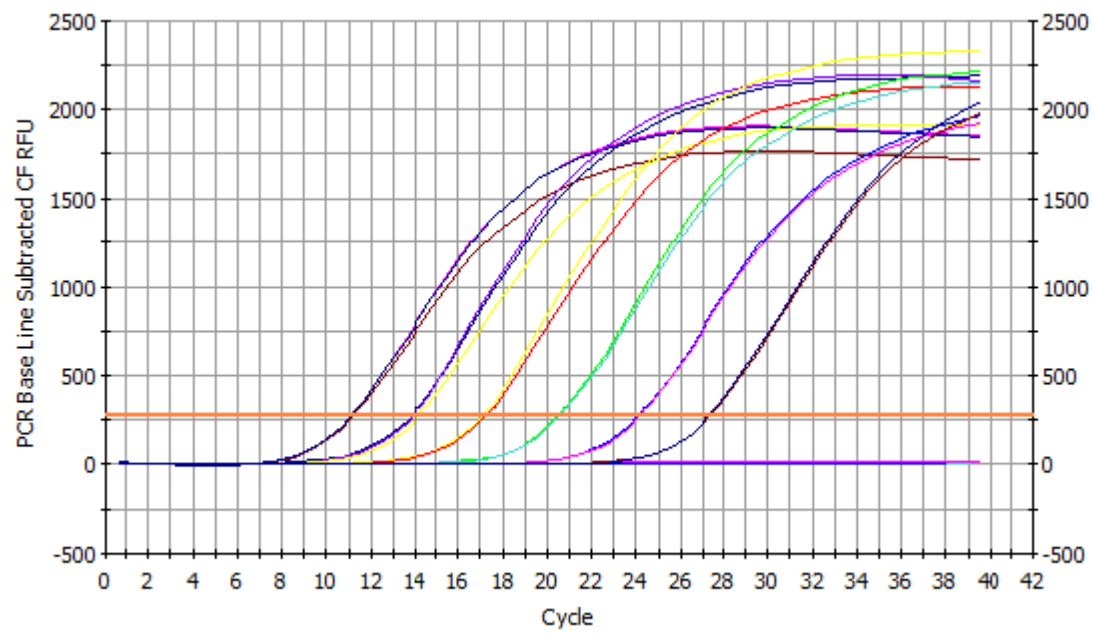


Fig B.23 TNC qRT-PCR Amplification/Cycle graph

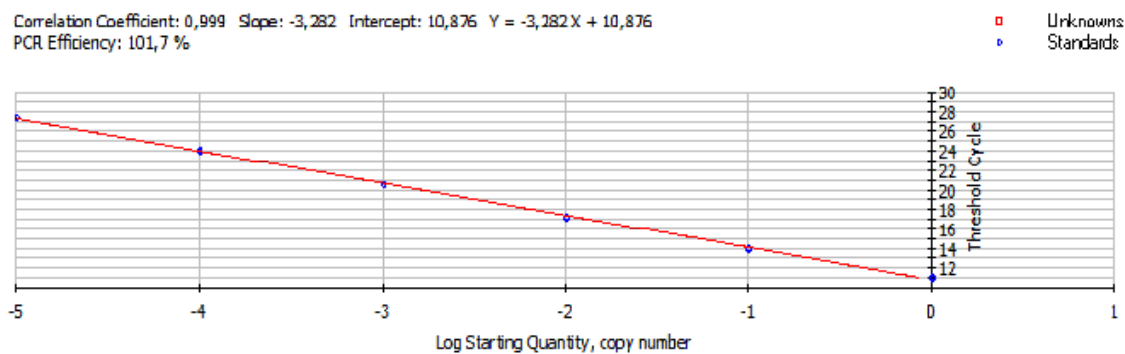


Fig B.24 TNC Standard curve

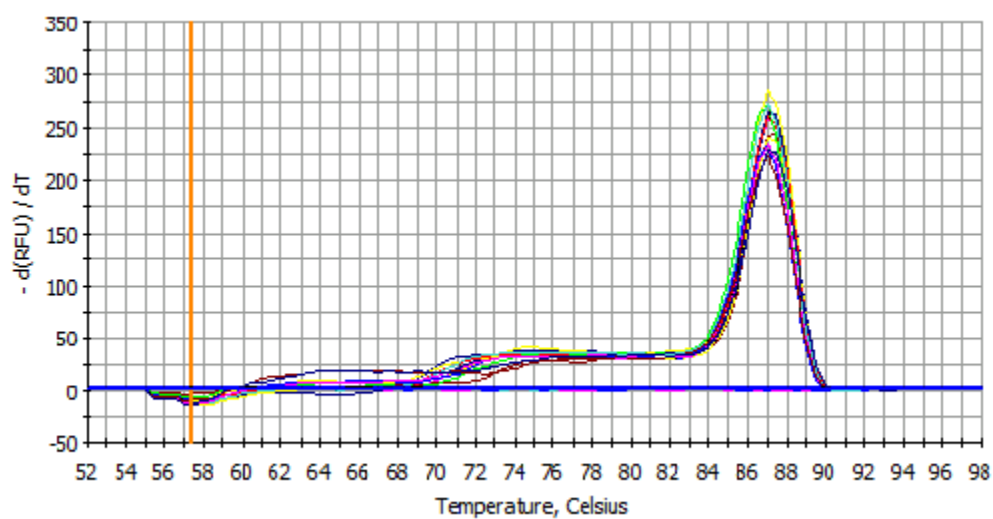


Fig B.25 TNC Melting curve

VITA

Javier Jarazo was born on July 1985 to Gabriel Agustin Jarazo and Graciela Beatriz Moschini in Buenos Aires, Argentina. In 2002, he graduated with honors from Instituto La Salle Florida in Buenos Aires, Argentina.

Following high school, he pursued his Doctor in Veterinary Medicine degree at the University of Buenos Aires, Argentina obtained in October 2010. During his undergraduate studies he became interested in assisted reproductive technique in domestic animals and joined Dr. Daniel Salamone's Laboratory of Animal Biotechnology at the University where he worked as a research assistant from 2006 to 2012. In this lab he became involved in techniques related to embryo culture, somatic cell nuclear transfer and other assisted reproductive procedures in different species focusing his laboratory work in several species, mainly feline and equine obtaining the first healthy cloned foal in Latin America. It was during his participation in those projects that he decided to continue his studies in stem cell therapy for the regeneration of damaged tissue.

Javier joined the graduate program at Louisiana State University in the summer of 2012 under the direction of Dr. Glen Gentry and is now a candidate for the degree of Master of Science in reproductive physiology in the School of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.