DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXVII

Settore Concorsuale di afferenza: 07/H5

Settore Scientifico disciplinare: VET/10

MESENCHYMAL STROMAL CELLS (MSCs) AND INDUCED PLURIPOTENT STEM CELLS (iPSCs) IN DOMESTIC ANIMALS: CHARACTERIZATION AND DIFFERENTIATION POTENTIAL

Presentata da: Barbara Rossi

Coordinatore Dottorato	**Relatore**
Chiar.mo Prof. C. Tamanini	Chiar.mo Prof. C. Galli

Esame finale anno 2015

TABLE OF CONTENTS

LIST of ABBREVIATION

α–SMA: Alpha-Smooth Muscle Actin

ACTH: adrenocorticotropic hormone

AF: amniotic fluid

AFSCs: amniotic fluid stem cells

AM: amniotic membrane

AP: alkaline phosphatase

AT: adipose tissue

bFGF: basic Fibroblast Growth Factor

bAFSCs: bovine amniotic fluid stem cells

BM: bone-marrow (BM)

BSA: bovine serum albumin

CDs: cell-doubling number

CD: Cluster of differentiation

CiPSCs: Chemically induced Pluripotent Stem Cells

Coll I: collagen type I

Coll II: collagen type II

DAPI: 49-6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle Medium

Dox: Doxycycline

DPBS: Dulbecco's Phosphate Buffer Solution

DT: Cell-doubling time

E-cells: cuboidal epitheloid

EBs: Embryoid Bodies

ESCs: embryonic stem cells

EpiSCs: Epiblast Stem Cells

F-cells: spindle-shaped fibroblastic cells

FBS: fetal bovine serum

fi: fibroblastoid cells

FITC: Fluorescein isothiocyanate

FGF5: Fibroblast Growth Factor 5

GFP: Green fluorescent protein

GMEM: Glasgow Minimum Essential Medium

HBSS: Hank's Balanced Salt Solution

HEK293: Human Embryonic Kidney Cells

HLA class II: Human Leukocyte Class II

h: hour(s)

hSCF: human Stem Cell Factor

hTGF-β1: human transforming growth factor β1

ICC: Immunicytochemistry

ITS: Insulin Transferrin Selenite

ICM: inner cell mass

KLF4: Kruppel-like factor 4

KO-DMEM: KnockOut DMEM

KSR: KnockOut Serum Replacement

LF: large and flat (LF)

LIF: Leukemia inhibitory factor

MEFs: Mouse Embryonic Fibroblasts

MSCs: mesenchymal stem/stromal cells

MHC I: major histocompatibility complex class I

MHC II: major histocompatibility complex class II

MGN: Myogenin

MYF5: Myogenic factor 5

MYOD: Myogenic Differentiation

MX: mixed samples (or mixed population)

NEAA: non-essential amino acids

OCT4: octamer-binding transcription factor 4

OPN: Osteopontin

OSKM: OCT4, SOX2, KLF4, C-MYC

OSKMN: OCT4, SOX2, KLF4, C-MYC, NANOG

OSKMLN: OCT4, SOX2, KLF4, C-MYC, LIN28, NANOG

P: passage of culture

PAX7: Paired box 7

PB: Phosphate buffer

PB2: Phosphate Buffer with 0.2 % of bovine albumin and 0.05 % of saponin

PB transposon: PiggyBac transposon

PBS: Phosphate-buffered saline

PEFs: Porcine Embryonic Fibroblasts

PFA: paraformaldehyde

piPSCs: porcine induced Pluripotent Stem Cells

POU5F1: POU domain, class 5, transcription factor 1

PPARγ: Peroxisome proliferator-activated receptor gamma

qRT-PCR: quantitative Reverse Transcriptase – polymerase chain reaction

rpm: revolutions per minute

R-cells: round cells

RS: round-shaped population

RT: room temperature

SCNT: somatic cell nuclear transfer

SKM: SOX2, KLF4, C-MYC, NANOG

SS: spindle-shaped

SSEA1: Stage-Specific Embrynic Antigen 1

SSEA4: Stage-Specific Embrynic Antigen 4

STAP: Stimulus Triggered Acquired Pluripotency

STEMCCA: Stem Cell Cassette

SV40 large T: Simian Vacuolating Virus 40 TAg

TCM199: Tissue Culture Medium-199

TERT: Telomerase Reverse Transcriptase

TRITC: Tetramethylrhodamine

UC: umbilical cord

UCB: umbilical cord blood

VPA: Valproic Acid

WJ: Wharton's jelly

ABSTRACT

Derivation of stem cell lines from domesticated animals has been of great interest as it benefits translational medicine, clinical applications to improve human and animal health and biotechnology. The main types of stem cells studied are Embryonic Stem Cells (ESCs), induced Pluripotent Stem Cells (iPSCs) and Mesenchymal Stem/Stromal Cells (MSCs). This thesis had two main aims:

(I) The isolation of bovine MSCs from amniotic fluid (AF) at different trimesters of pregnancy and their characterization to study pluripotency markers expression. Stemness markers were studied also in MSCs isolated from equine AF, Wharton's jelly (WJ) and umbilical cord blood (UCB) as continuation of the characterization of these cells previously performed by our research group;

(II) The establishment and characterization of iPSCs lines in two attractive large animal models for biomedical and biotechnology research such as the bovine and the swine, and the differentiation into the myogenic lineage of porcine iPSCs.

It was observed that foetal tissues in domestic animals such as the bovine and the horse represent a source of MSCs able to differentiate into the mesodermal lineage but they do not proliferate indefinitely and they lack the expression of many pluripotency markers, making them an interesting source of cells for regenerative medicine, but not the best candidate to elucidate pluripotency networks. The protocol used to induce pluripotency in bovine fibroblasts did not work, as well as the chemical induction of pluripotency in porcine fibroblasts, while the reprogramming protocol used for porcine iPSCs was successful and the line generated was amenable to being differentiated into the myogenic lineage, demonstrating that they could be addressed into a desired lineage by genetic modification and appropriated culture conditions. Only a few cell types have been

differentiated from domestic animal iPSCs to date, so the development of a reliable directed-differentiation protocol represents a very important result.

Keywords: Mesenchymal Stem Cells; Induced Pluripotent Stem Cells; Mesodermal Differentiation; Cattle; Pig; Horse

CHAPTER 1: INTRODUCTION

1.1 Stem cells in veterinary medicine

Stem cells are a special kind of cells that have a unique ability to self-renew and give rise to specialized cell types. Stem cells are uncommitted until they receive signals to develop into specialized cells that make up tissues and organs of the body.

Stem cells are mostly classified in terms of how committed they are to become any particular type of cells. According to this feature, stem cells could be classified into four main groups (Malgieri et al. 2011) (Fig. 1.1):

- Totitpotent stem cells: These cells could potentially give rise to any cell of the body.
- Pluripotent stem cells: A single pluripotent stem cell has the ability to give rise to types of cells that develop from the three germ layers (mesoderm, endoderm and ectoderm) from which all the cells of the body arise.
- Multipotent stem cells: These are cells isolated from adult tissues and have the same basic features of stem cells but are essentially committed to produce specific cell types, so their differentiation ability is limited.
- Adult stem cells: These are multipotent cells present in the adult body that have the function to replace cells that have died or lost function.

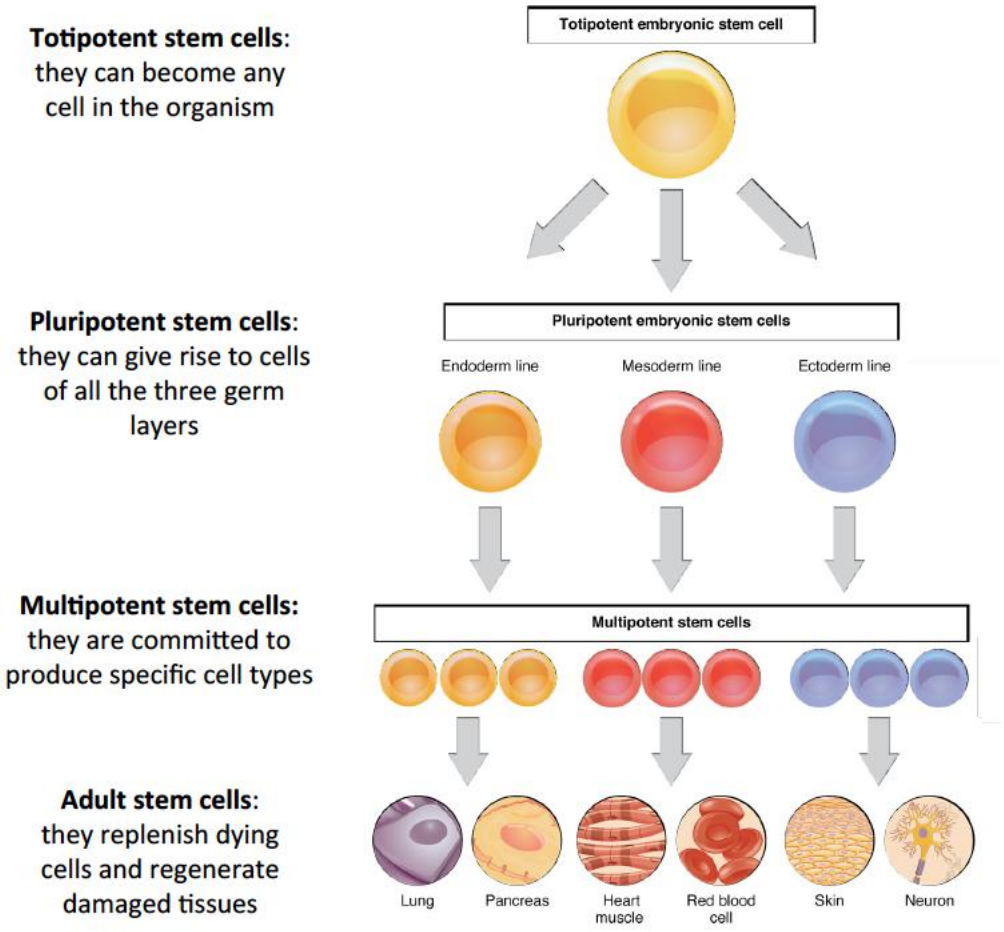


Fig. 1.1: Types of stem cells (modified from http://cnx.org/contents/966c32cc-3d6f-4f4e-af4f-ea0c975e825c@4/Cellular_Differentiation)

In veterinary medicine, as for human, the attention has been focused on three main types of stem cells: Embryonic Stem Cells (ESCs), Mesenchymal Stem Cells (MSCs) and induced Pluripotent Stem Cells (iPSCs). Each cell type has its strengths and weaknesses and they show different potency degrees (Fig. 1.2).

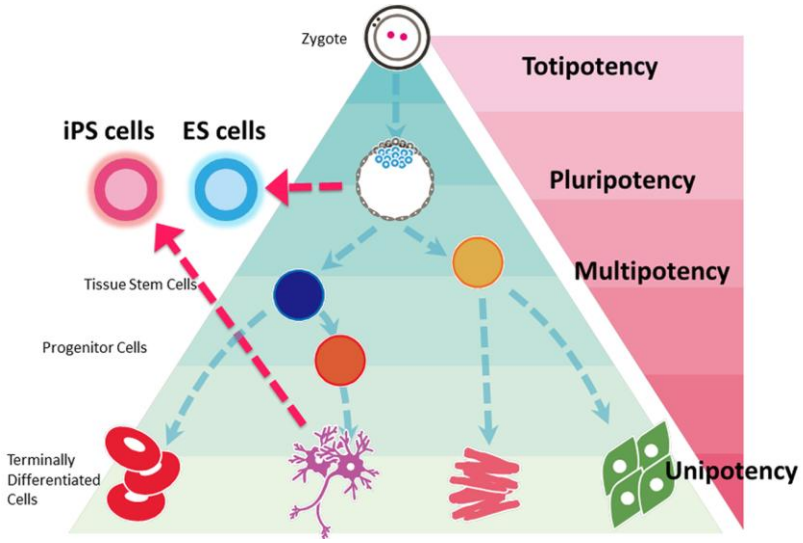


Fig. 1.2: Hierarchy of potency of stem cell development (from Sugawara et al. 2012)

1.1.1 Embryonic Stem Cells (ESCs)

ESCs are pluripotent cells derived from the inner cell mass (ICM) of blastocyst-stage embryos. These cells can form all the structures in the adult body and belong to the pluripotent stem cells class. Because of these features, ESCs have drawn the attention as model to study the mechanism underlying pluripotency to better understand biochemical pathways that drive differentiation and senescence, and in consequence the comprehension of developmental biology. More recently, ESCs have been sub-categorized into "true" ESCs, that show *naïve* pluripotency and are able to contribute to chimeric offspring, and Epiblast Stem Cells (EpiSCs), that are further advanced on the path of differentiation and that are named as *primed* pluripotent (Ernst et al. 2015). While ESCs are isolated form ICM, EpiSCs derived form post-implantation stage embryos (Koh and Piedrahita 2014). Focusing on domestic animals, although the field has progressed over the last 30 years, there are still not *bona fide* ESCs from any domesticated species that can be considered practical for the generation of transgenic animals (Koh and Piedrahita 2014). Despite two

decades of efforts, establishment of pluripotent ESCs from ungulates such as cattle and pig has remained an elusive goal, with true ESCs only successfully isolated from rodents and primates (Telugu et al. 2010). Moreover, the generation of ESCs requires the destruction of an embryo making the use of ESCs for stem cell research ethically controversial, and these issues restrict the development of ESCs for cell therapy (Jung et al. 2014). All these aspect taken together made the scientific community to focus their attention more on other stem cells sources, like mesenchymal stem cells (MSCs) and, since 2006 when they have been discovered, induced pluripotent stem cells (iPSCs).

1.1.2 Mesenchymal Stem/Stromal Cells (MSCs)

Mesenchymal stem/stromal cells (MSCs) are multipotent progenitor cells that were originally identified in the bone marrow stroma (Friedenstein et al. 1987). These cells were first described as an adherent fibroblast-like subset of the bone-marrow (BM) microenvironment called the “marrow stromal cells” and they have been later identified in other anatomical locations, although their physiological roles remain unclear. Their accessibility from several sources makes them a promising tool for cell therapies and among these alternative sources an important role is played by adipose tissue (AT). AT-derived MSCs have a higher proliferation potential compared to BM-MSCs and AT is accessible in higher amounts. Anyway, for both MSCs sources, an invasive procedure is required and there is a large variability in the cell yield related to the donor. For these reasons, researchers drew later their attention toward alternative sources, such as foetal adnexa and fluids like umbilical cord blood (UCB) and matrix (Wharton’s jelly, WJ), amniotic fluid (AF) and amniotic membrane (AM), because of their many advantages: they represent discarded material with noninvasive harvesting for the patient, they are easy to

obtain and they are free from ethical considerations (Iacono et al. 2015). Furthermore, cells derived from foetal adnexa and fluids are known to preserve some of the characteristics of the primitive embryonic layers from which they originate and many research groups found these cells to express pluripotent markers, such as the pluripotency key factor OCT4 (octamer-binding transcription factor 4, protein involved in ESCs self-renewal). About MSCs isolated form foetal adnexa in domestic animals, their unequivocal immunophenotyping is hampered by the lack of a single specific marker and conflicting data are reported regarding the expression of embryonic markers (De Schauwer et al. 2011), and their collocation into the hierarchy of pluripotency is controversial (Fig. 1.3). This discrepancy is likely due to the absence of a well-defined culture protocol and the limited availability of monoclonal species-specific antibodies (Iacono et al. 2015).

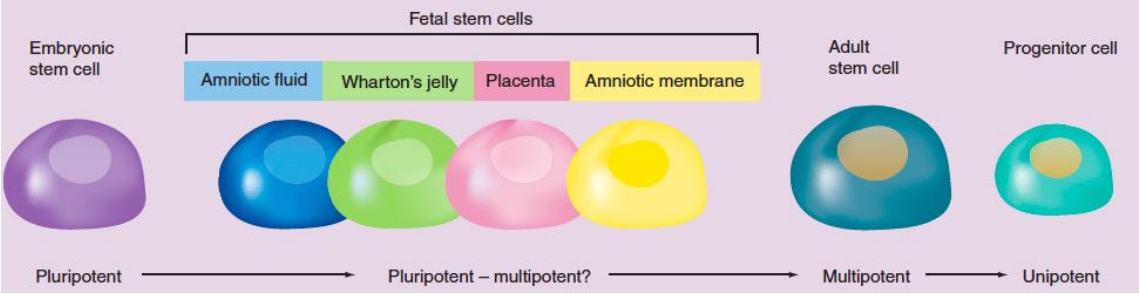


Fig. 1.3: Stemness marker expression in MSCs form foetal adnexa is debated so their collocation into the hierarchy of pluripotency is not clear (from Pappa and Anagnou 2009)

In this thesis, MSCs will be named *stem* or *stromal* referring to the same type of cells. *Stromal* is referred to their first source of isolation, *stem* is referred to their potential, even if debated, pluripotency.

In 2006 the Interational Society for Cellular Therapy (ISCT) defined a specific set of criteria for human MSCs:

(I) Adherence to plastic;

(II) Expression of CD73, CD90 and CD105 surface antigens and lack of the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II;

(III) Multipotent differentiation potential *in vitro* towards osteogenic, chondrogenic and adipogenic lineages (Dominici et al. 2006).

Despite these criteria, considering that the surface antigens listed are not specific only for MSCs, until now there is not a clear panel of univocal markers to define these cells.

An important feature of MSCs is that they have been considered safe as they do not show tumor formation after transplantation (Bauer et al. 2008) and systemic administrations of allogeneic MSCs do not cause any adverse effects, because they possess a privileged immunogenic status related to the low expression or even absent levels of MHC I and MHC II (Hu et al. 2013).

1.1.3 Induced Pluripotent Stem Cells (iPSCs)

It is less than 10 years since Takahashi and Yamanaka (2006) reported the derivation of murine iPSCs by reprogramming mouse fibroblasts into pluripotent stem cells, findings that led Professor Yamanaka to win the Nobel Prize in 2012. Induction of pluripotency was achieved through the expression of a combination of four transcription factor (OCT4, SOX2, KLF4 and c-MYC - OSKM) delivered to the cells by retroviral transduction (Fig. 1.4).

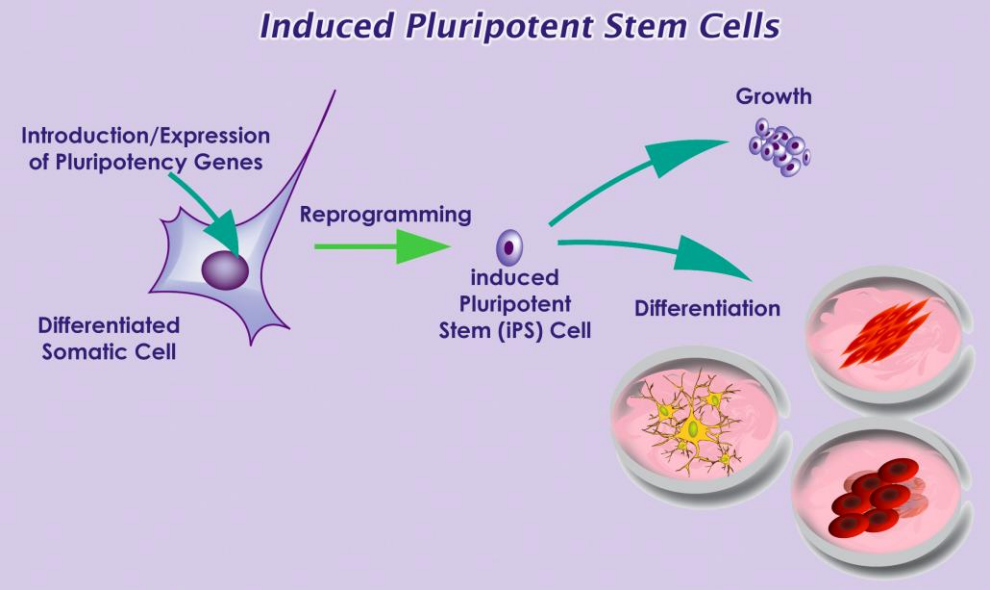


Fig. 1.4: Induced Pluripotent Stem Cells (iPSCs) technology is represented by the reprogramming of differentiated somatic cells through a combinations of factors delivered by a viral vector. When reprogrammed, iPSCs are able to proliferate indefinitely or differentiate into cells belonging to all the three germ layers if cultured in proper conditions (taken from http://galleryhip.com/induced-pluripotent-stem-cells.html).

Induced pluripotent stem cells possess a similar degree of potency compared to ESCs and the ability to differentiate into all cell types of body tissues. In fact, iPSCs and ESCs showed many genetic and functional similarities such as patterns of gene expression, cell division rate, the ability to form embryoid bodies, (EBs, multicellular structures reminescent of embryonic development), teratomas (tumors composed of the endodermal, ectodermal and mesodermal layers observed during embryonic development), and chimeras (Heffernan et al. 2009). The field is developing with a tremendous speed and, to date, iPSCs have successfully been established from several species. Initially, iPSCs were reported from humans (Aasen et al. 2008; Nakagawa et al. 2008), non-human primates (Liu et al. 2008), mice (Gonzalez et al. 2009; Hamilton et al. 2009), and rats (Liao et al. 2009).

The production of iPSCs was then extended to large-animal species such as pig, cow, sheep, goat and horse. Generation of iPSCs from large-domestic animals is reported in Table 1.1. There are technical and safety concerns associated with the production of iPSCs that currently preclude their use for clinical applications. For example, the yield using standard procedures is very low, around 0.2% of the induced cells (Liao et al. 2008). In addition, there is a risk of tumor formation after transplantation and some of the transcription factors used to generate iPSCs are oncogenic. On the other side, iPSCs, as well as ESCs, provide great potential as cell sources for gene editing to generate genetically modified animals, and also in the field of regenerative medicine. The resulting iPSCs did not have identical phenotypes in terms of surface markers, even if derived in the same species, and this is surely due to the variables in reprogramming, sources of cell induced and culture conditions (Table 1.1), since optimal protocols for iPSCs have not been clearly defined (Okada et al. 2010).

Progress in generation of iPSCs lines has been rapid and the technology has been modified in a variety of ways to make it potentially more efficient through the use of different gene combinations and potentially safer for future transplant studies by altering the delivery of the reprogramming factors to the cells (Telugu et al. 2010). Factor-based reprogramming techniques are marked with drawbacks such as slow reprogramming efficiencies, time-consuming protocols, incomplete reprogramming of somatic cells into iPSCs and the potential of spontaneous oncogenesis. Moreover, the continued expression of pluripotent genes may limit the differentiation potential of iPSCs (Telugu et al. 2010). In order to make iPSCs, these inserted transgenes will have to be either deleted or effectively silenced after the cells have been reprogrammed. These aspects have fueled studies exploring new strategies of reprogramming like the use of non-integrating vectors, transposones or

recombinant protein.

In 2013, among promising ways to avoid the viral-transduction arose the use of chemical compounds that can replace exogenous reprogramming factors (Ma et al. 2013). Chemical compounds are advantageous because they are readily accessible to the cells, they are cost-effective and they can be easily manipulated. Some studies have shown that mouse or human somatic cells can be reprogrammed by OCT4 and chemical compounds (reviewed by Masuda et al. 2013) and later it was demonstrated how murine cells could be reprogrammed with chemicals only (chemically induced pluripotent stem cells, CiPSCs; Hou et al. 2013). Although this transgenes-free approach has no risk of insertional mutagenesis, it is important to know whether chemical cocktails induce genetic instability during reprogramming process that compromise genetic integrity of resultant CiPSCs. Furthermore, *in vitro* differentiation capacity still needs to be evaluated. The human field, as well as for domestic animals, lags far behind in this regard but the optimization of a transgene-free induction protocol is for sure the new challenge in this field.

Table 1.1: iPSCs generated from large-domestic animals (modified from Koh and Piedrahita 2014)

Species	Cell sources	Feeder layer	Media supplement	Reprogramming	Reference
Pig	Foetal fibroblasts	MEFs	bFGF	Lentivirus (human OSKM)	(Ezashi et al. 2009)
	Embryonic fibroblasts (D37)	MEFs	bFGF or mLIF	Retrovirus (human/mouse OSKM)	(Esteban et al. 2009)
	Primary ear fibroblasts; primary bone marrow (10 weeks)	MEFs	Need continuous Dox	Dox-inducible lentivirus (human OSKM)	(Wu et al. 2009)
	MSCs	Matrigel	-	Lentivirus (human OSKMLN)	(West et al. 2010)
	Embryonic fibroblasts (D26-30)	MEFs	bFGF	Pantropic retrovirus (OSKM)	(Rua et al. 2011)
	Embryonic fibroblasts	MEFs	bFGF/LIF, PD0325901, CHIR99021, VPA	Episomal plasmid (human OSKMLN)	(Telugu et al. 2011)
	Ear fibroblasts (6 months)	MEFs/gelatine	bFGF, LIF	Retrovirus (mouse OSKM)	(Montserrat et al. 2011)
	Ear fibroblasts (6 months)	MEFs/gelatine	bFGF, LIF	Retrovirus (mouse SKM)	(Montserrat et al. 2012)
	Fetal fibroblasts	MEFs	mLIF, N2B27, PD0325901, CHIR, PD173074, need continuous Dox	Dox-inducible lentivirus (human OSKM)	(Rodriguez et al. 2012)
	Fetal fibroblasts	MEFs	bFGF	Dox-inducible lentivirus (human OSKMN)	(Hall et al. 2012)
	Embryonic fibroblasts	Collagen I	pLIF, forskolin	Retrovirus (human OSKM)	(Fujishiro et al. 2013)
	Embryonic fibroblasts	MEFs	bFGF, hSCF	Transfection (mouse OSKM)	(Park et al. 2013)

Cow	Fetal fibroblasts	MEFs	bFGF	Retrovirus (bovine OSKM; bovine OSKMLN; human OSKM)	(Han et al. 2011)
	Skin fibroblasts	MEFs	Bovine FGF, hLIF	Retrovirus (human OSKMN)	(Sumer et al. 2011)
	Fetal fibroblasts	MEFs	hLIF, PD0325901, CHIR99021	Episomal virus-free poly-promoter vector (bovine OSKM)	(Huang et al. 2011)
	Skin fibroblasts (2.5 – 4 months)	MEFs	LIF, bFGF	Lentivirus (human O and bovine SKM)	(Cao et al. 2012)
Sheep	Primary ear fibroblasts	MEFs	Need continuous Dox	Lentivirus (human OSKMLN + SV40 large T and TERT	(Bao et al. 2011)
	Fetal fibroblasts	MEFs	hFGF2, need continuous Dox	Lentivirus (mouse OSKM)	(Li et al. 2011)
	Embryonic fibroblasts	MEFs	bFGF	Retrovirus (mouse OSKM)	(Sartori et al. 2012)
Goat	Embryonic fibroblasts	MEFs	N2, B27, ascorbic acid, PD99023, CHIR99021, LIF, bFGF	Lentivirus (human OSKM)	(Chu et al. 2014)
	Ear fibroblasts	MEFs	Need continuous Dox	Lentivirus (OSKMLN + SV40 large T and TERT))	(Ren et al. 2011)
Horse	Fetal fibroblasts	MEFs	LIF, bFGF, CHIR99021, PD0325901, A83-01, Thiazovivin, SB431542 (only from day 8)	PB transposon (OSKM)	(Nagy et al. 2011)

1.2. Aim of the thesis

This thesis had two main goals:

(I) The isolation of bovine MSCs from amniotic fluid at different trimesters of pregnancy and their characterization, in order to study a model for human medicine, since bovine pregnancy has the same lenght, and also to study pluripotency marker expression of these cells. Stemness markers were later studied also in MSCs isolated from equine amniotic fluid, Wharton's jelly and umbilical cord blood as continuation of the characterization of these cells previously performed in our laboratory (Iacono et al. 2012a);

(II) The assessment of iPSCs lines in two attractive large animal models for biomedical and biotechnology research such as the bovine and the swine, and the differentiation into the musculo-skeletal lineage of porcine iPSCs. The study of differentiation into the myogenic lineage in the swine is important both for its similarity in size and physiology to humans, and for agricultural purposes like the increase of the muscle mass of the pig itself.

CHAPTER 2

Isolation, differentiation and characterization of mesenchymal stem cells isolated from bovine amniotic fluid at different gestational age

2.1 Introduction

The amniotic fluid (AF) represents a protective liquid layer that surrounds the foetus during its development. It gives mechanical support and nutrients for foetal growth. It is mainly composed by water and the production of AF is determined by excretion of foetal urine and oral, nasal, tracheal and pulmonary fluids, so AF overall composition changes with gestational ages (Underwood et al. 2005). Most importantly, it was discovered that AF contains a heterogeneous mixture of stem cell populations (AFSCs) deriving from both the foetus and the surrounding amniotic membrane. In the veterinary field, as in the human, AF contains a heterogeneous population of cells: cuboidal epitheloid (E-cells), round (R-cells) and spindle-shaped fibroblastic (F-cells) cells. E-cells probably derive from foetal skin and urine and they are lost during the first passages of culture; R-cells are supposed to originate from foetal membranes and trophoblasts; F-cells are generated from mesenchymal tissues and are supposed to be the MSC population of the AF (Klemmt et al. 2011). Some research groups found both populations persisting through passages, while Roubelakis and co-workers (Roubelakis et al. 2011) reported the prevalence of round-shaped (RS) population over the spindle-shaped (SS). In veterinary medicine the predominant AFSCs population found in different species is represented by SS cells. AFMSCs generally express MSC markers and they are also known to express mesodermal markers like Vimentin and α-Smooth Muscle Actin (αSMA) (Kaviani et al. 2001, Sartore et al. 2005). It is also

important to underline that human AF-MSCs have not shown tumorigenicity and they showed immunomodulatory properties (Yi and Song 2012), making them good candidate for potential therapeutic use as well as their migration potential and the expression of adhesion molecules in common with leukocytes like CD44, CD24, CD29 and CD18 (Burk et al. 2013; Kavanagh et al. 2014). The bovine is an important agricultural species with significant commercial value and it is also an attractive large animal model for biomedical and biotechnology research. Bovine pregnancy lasts about 280 days, like the human one, so the cow represents a good model to understand differences in cells composition among trimesters. The development of large animal experimental models, including cattle, may open alternative strategies for investigating MSCs physiology and potential application for human and veterinary regenerative medicine. For this reason, it could be interesting to identify a cell population from bovine amniotic fluid that would display a high proliferative ability and a longer lifespan than a normal differentiated cell line in order to allow gene targeting experiments and a successful use for somatic cell nuclear transfer. So the aim of this study was to investigate and characterize bovine AFSCs (bAFSCs) populations by analysing phenotype, gene expression profile, growth curves and differentiation potential and evaluating the AF cells yield in different gestational periods.

2.2 Materials and Methods

2.2.1 Samples harvesting and cell isolation

All chemical were obtained from Sigma-Aldrich and plastic dishes and tubes from Sarstedt Inc., unless otherwise indicated.

Bovine amniotic fluid samples were harvested at the slaughterhouse from pregnant uteri of 39 Holstein cows at different gestational ages. Bovine pregnancy was divided into 3 trimesters, as done in humans (Ashwood et al. 2006), and gestational periods were estimated by measuring crown-rump length of the foetuses (Fig 2.1).

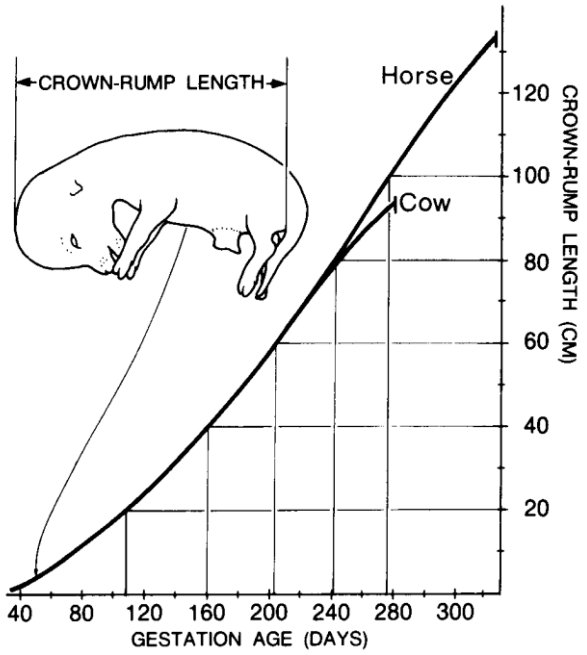


Fig. 2.1: Gestational periods were estimated using this graph (Noden and De Lahunta, 1985) by measuting the crown-rump length of the foetuses.

AF was collected into 50 ml sterile syringes (IMI) and transported to the laboratory at 4 °C within 1 h. AF samples were then diluted with Dulbecco's Phosphate Buffer Solution DPBS (1:1, v/v) containing 100 IU/ml penicillin and 100 mg/ml streptomycin and centrifuged (Heraeus Megafuge 1.0R; rotor: Heraeus #2704; ThermoFisher Scientific Inc.) for 15 min at 1500 rpm. Pellets were suspended in 5 ml of culture medium (DMEM/M199) containing DMEM and TCM199 (1:1), 10 % (v/v) FBS (Life Technologies), 100 IU/ml

penicillin and 100 mg/ml streptomycin and centrifuged for 15 min at 1500 rpm. Supernatant was discarded and cells were re-suspended in 1 ml of culture medium and counted in a Neubauer chamber.

2.2.2 Cell Culture and Population Doublings

Bovine AFSCs were seeded in 25 cm^2 flasks at the density of 5×10^3 cells/cm^2. Cells were cultured in DMEM/M199 in a humidified atmosphere with 5 % CO_2 at 38.5 °C. Different cell populations found at P0 were classified on the basis of their morphology: spindle-shaped cells (SS), round-shaped (RS), fibroblastoid (fi) and large and flat (LF). Samples which presented both SS population and RS population were defined as mixed samples (MX). Different morphologies are reported in the Fig. 2.2. When cultures reached about 80–90 % confluence, cells were dissociated with 0.25 % (w/v) trypsin solution, counted and re-seeded to the subsequent passage, named "Passage 1" or P1.

Cell-doubling time (DT) and cell-doubling numbers (CD) were calculated through the following formulae (Rainaldi et al. 1991):

$$CD = \ln(N_f/N_i) / \ln(2)$$

where N_f is the final number of cells and N_i the initial number of cells;

$$DT = CT/CD$$

where CT is the cell culture time.

Growth curves of different cell populations persisting through passages (SS, RS and MX)

were compared to understand if DT and CD differ in relation to the cell type.

2.2.3 Adhesion and migration assay

In order to define differences between SS and RS populations, a spheroid formation-and-migration test was performed to evaluate differences in adhesion and migration. Cells from each sample type were cultured in 'hanging drops' (5,000 cells/drop) for 24 h, until spheroid formation was observed. Spheroid areas were determined, then spheroids were cultured under standard conditions to allow their adhesion and the migration of cells from the spheroids themselves (Burk et al. 2013). The migration rate was calculated after 24 h of incubation, comparing images taken immediately before and after this incubation period. The pre-adhesion dimension of the spheroids gave also information about the adhesion ability of the cells: spheroid area and adhesion capability are inversely proportional. The migration capability of both population types was also evaluated by a scratch assay (also known as Wound-Healing assay), as previously described (Liang et al. 2007). Briefly, at 80–90 % confluence the cell monolayer was scraped using a p1000 pipet tip. After washing twice with PBS, the dish was incubated for 24 h at 38.5 °C and 5 % CO_2 in a humidified atmosphere. Images were acquired both immediately after the tip-scratch (time 0, T0) and after the incubation period (last time point or time 1, T1), and the distances of each scratch closure were calculated by ImageJ software. The migration percentages were calculated using the following formula:

[(distance at T0 − distance at T1)*100] / distance at T0

2.2.4 In vitro differentiation

Bovine AFSCs were expanded in culture at P3 and then their ability to differentiate into mesenchymal lineages was tested. Each experiment consisted of a treated group, cultured in specific inducing medium, and a control group, maintained in standard culture conditions. For differentiation, cells were seeded at 5×10^3 cells/cm^2 in 6-well plates.

2.2.4.1 Adipogenic differentiation

In order to test their ability to differentiate into adipocytes, bAFSCs were cultured in DMEM/M199 plus rabbit serum (15 %), dexamethasone (1 μM), 3-isobutyl-1-methylxanthine (0.5 mM), ITS (Insulin Transferrin Selenite, 10 μg/ml), and indomethacin (0.2 μM). After 3 days of culture, 3-isobutyl-1-methylxanthine was removed and after 3 additional days dexamethasone was also removed and cells were cultured for additional 15 days (Iacono et al. 2012a). To test adipogenic differentiation, cells were stained with Oil Red O. Briefly, cells were washed with PBS and then fixed with 2 % paraformaldehyde (PFA) for 30 min at room temperature (RT). The PFA was removed and cells were washed with 60 % isopropanol. Cells were finally covered with Oil Red O solution (0.3 % in 60 % isopropanol) to make lipid vacuoles appear red. After 30 min at RT, cells were rinsed with distilled water (Mizuno and Hyakusoku 2003).

2.2.4.2 Osteogenic differentiation

Osteogenic differentiation consisted of culturing cells in culture medium supplemented

with dexamethasone (0.1 μM), beta-glycerophosphate (10 mM) and ascorbic acid-2-phosphate (50 μM) (Mizuno and Hyakusoku 2003). Cells were cultured in a humidified atmosphere of 5 % CO_2 at 38.5 °C and the differentiation medium was changed every 3 days for 21 days. Under these culture conditions, cells are expected to produce a mineralized matrix. To reveal it, they were fixed with 10 % formalin for 1 h at RT and then stained with Von Kossa staining. Briefly, formalin was removed and cells were washed five times with distilled water. One ml of 5 % (w/v) silver nitrate was added and cells were exposed to yellow light for 45 min. After that, cells were washed 5 times with distilled water. Calcium phosphate deposits stained black.

2.2.4.3 Chondrogenic differentiation

For the purpose to stimulate the bAFSCs to differentiate into the chondrogenic lineage, they were cultured in DMEM/M199 supplemented with FBS (1 %), ITS (6.25 μg/ml), dexamethasone (0.1 μM), ascorbic acid-2- phosphate (50 nM) and human transforming growth factor (hTGF)-β1 (10 ng/ml), as previously described (Kim et al. 2007; Iacono et al. 2012a; Iacono et al. 2012b; Filioli Uranio et al. 2011). Cells were cultured in a humidified atmosphere of 5 % CO_2 at 38.5 °C and the medium was changed every 3 days for 3 weeks. To assess chondrogenic differentiation, cells were fixed with 10 % formalin for 1 h at room temperature, then stained with Alcian Blue solution (1 % in 3 % acetic acid, pH 2.5) for 15 min at RT. Alcian Blue stains polysaccharides like glycosaminoglycans.

2.2.5 Flow cytometry

Bovine AFSCs of different gestational trimester at passages 3 (P3) and P7 were immunophenotyped by flow cytometric analysis. Cells were fixed and permeabilised at the concentration of about 10^6 cells/ml using Reagent 1 of Intraprep Kit (Beckman Coulter), according to manufacturer's instructions. As previously reported, the International Society for Cytotherapy declared that human MSCs must be CD73, CD90 and CD105 positive and CD14, CD34 and CD45 negative (Dominici et al. 2006). Flow cytometry analysis was performed using anti-human antibodies routinely used. Cells were labelled with the following monoclonal antibodies: CD105, CD45, CD90, CD44, CD34, CD14 and CD73 (all from Beckman Coulter). To verify cross-reactivity, circulating bovine lymphocytes were used as control.

2.2.6 Immunocytochemistry

Cultured SS, RS and MX samples at P3 and P7 were washed with DPBS, fixed with 4 % PFA for 20 min at RT and then washed in phosphate buffer (PB). Cells were blocked in goat serum 10 % (Sigma) for 1 h and incubated overnight with primary antibodies against Oct4 (Abcam, ab18976, 1:50), SSEA4 (Hybridoma Bank, 1:50), and alpha-SMA (Gene Tex, 1:500). Cells were washed in PB2 (Phosphate Buffer with 0.2 % of bovine albumin and 0.05 % of saponin) and incubated with anti-mouse- or anti-rabbit- FITC conjugated secondary anti-bodies for 1 h. After that, nuclei were labelled using Hoechst 33342. The excess of secondary antibody and Hoechst was removed by 3 washes in PB2. Bovine blastocysts and bovine fibroblasts were used respectively as positive and negative control. In order to define a profile of SS and RS cells, the expression of Vimentin, N-cadherin

(mesenchymal markers), E-cadherin and Cytokeratin (epithelial markers) was analysed. The following primary antibodies were used: Vimentin clone 9 (DAKO, 1:100), N-Cadherin (Biorbyt, 1:100), E-Cadherin (Cell Signaling Technologies #, 1:200), pan-Cytokeratin (Chemicon international, Millipore, 1:250). With the purpose to test the cross-reactivity of our antibodies, the following types of cells were used as positive controls: bovine fibroblasts for Vimentin, bovine epithelial cells for pan-Cytocheratin, and bovine embryos for E-cadherin and N-Cadherin. Images were obtained with a Nikon Eclipse E400 microscope at a 40X magnification using the software Nikon NIS-Elements. Percentages of positive cells were obtained evaluating ratio between positive cells and total nuclei.

2.2.7 Molecular characterization

In order to evaluate differences in markers expression between the two types of bAF-cells, qRT-PCR for the pluripotency genes OCT4, NANOG and SOX2 was performed on SS and RS cells both at P3 and at P7. Gene expression in the samples was normalized on bovine blastocysts, used as positive control, while bovine adult fibroblasts were used as negative control. To test cell differentiation, the following set of genes was evaluated on SS and RS differentiated samples both at P3 and P7: bovine PPARγ for adipogenic differentiation, collagen type I (Coll I) and Osteopontin (OPN) for osteogenic differentiation and collagen type I and type II (Coll II) for chondrogenesis. In all the reaction 18S was used as house-keeping reference gene and the differentiated samples were normalized on the respective undifferentiated P3. The specific set of primers used is listed in Table 2.1.

Table 2.1: Sequence of primers used for qPCR analysis

Gene	Primer sequence FW and RV	Amplicon (bp)	(Reference)
b18S	FW: 5′-GAATAACGCCGCCGCATCG-3′	133	(Thelie et al. 2007)
	RV: 5′-CGGACCAGAGCGAAAGCATTTG-3′		
bOCT4	FW: 5′- TGCAGCAAATTAGCCACATC -3′	123	(Pant and Keefer 2009)
	RV: 5′- AATCCTCACGTTGGGAGTTG-3		
bNANOG	FW: 5′-GTCCCGGTCAAGAAACAAAA-3′	107	(Pant and Keefer 2009)
	RV: 5′-TGCATTTGCTGGAGACTGAG-3′		
bSOX2	FW: 5′-ACAGTTGCAAACGTGCAAAG-3′	114	(Pant and Keefer 2009)
	RV: 5′-AGACCACGGAGATGGTTTTG-3′		
bPPARγ	FW: 5′-CGCACTGGAATTAGATGACAGC-3	214	(Pant and Keefer 2009)
	RV: 5′-CACAATCTGTCTGAGGTCTGTC-3′ ′		
bOPN	FW: 5′-CCAATGAAAGCCCTGAG-3′	310	(Gao et al. 2014)
	RV: 5′-TCCTCCTCTGTGGCATC-3′		
bColl I	FW: 5′-AGAAGCATGTCTGGGTAGGAG-3′	358	(Gao et al. 2014)
	RV: 5′-AGGATAGGCAGGCGAGATR-3′		
bColl II	FW: 5′- ATCCATTGCAAACCCAAAGG -3′	147	(Sutradahar et al. 2012)
	RV: 5′- CCAGTTCAGGTCTCTTAGAG -3′		

For RNA extraction, cells were washed with PBS, dissociated using 0.25 % trypsin solution and the pellets were snap-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's instructions and it was reverse-transcribed using iScriptTM cDNA Synthesis Kit (Bio-Rad). Reactions were conducted for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. RT-products (cDNAs) were used

directly in PCR reactions. Real-time PCR reactions were performed in triplicate using iQ SYBR Green Supermix and a MyiQ Real-Time PCR Detection System. Data were analyzed with the iQ Optical System Software (BioRad) with the DDct method.

2.2.8 Statistical Analysis

CDs, DTs, cells yield, mean AF volume harvested, spheroids areas and percentages of migration are expressed as mean ± standard deviation. Statistical analyses were performed using IBM SPSS Statistics 21 (IBM Corporation, Milan, Italy). Data were analysed using one-way ANOVA or a Student's t-test (when comparing SS and RS populations only). For statistical analyses, the unique sample with the spindle-shaped morphology was cultured in 2 replicates. Significance has been assessed for $P<0.05$.

2.3 Results

2.3.1 Cell yield and morphology

It was possible to isolate cells in 25/39 samples (64 %). In 7/39 samples, harvested at the same time, it was not possible to isolate cells because of a bacterial contamination, probably due to the non-sterile conditions of the slaughterhouse. In other 7/39 samples, harvested from cows at the third trimester of gestation (4/7) or at the end of the second trimester (3/7), cells did not grow. Of the 25 samples where cells were isolated, 3 specimens were from the first trimester (0–93 days), 19 from the second one (94–187 days) and 3 were from the third one (188-term of pregnancy). Mean volume of amniotic fluid collected was 26.7 ± 20.8 ml in the first trimester, 41.1 ± 13.9 ml in the second one and

34.0±5.3 ml in the third one. Mean number of cell was 3533.3 ± 1747.4 cells/ml in the first trimester, 1035.0 ± 1260.9 cells/ml in the second trimester and 722.2 ± 502.3 cells/ ml in the third one. The cell yield was significantly higher in the first trimester compared to the second and the third ones ($P < 0.05$). At passage 0, it was possible to identify 4 different cell subpopulations in samples from all three trimesters: spindle-shaped cells, round-shaped cells, fibroblastoid cells and large and flat cells (Fig. 2.2). The last 2 types of cells (fi and LF) were lost after the first passage and they probably derived from foetal skin and urine. In 1/25 sample the spindle-shaped population predominated (SS sample) whereas in 7/25 samples (28 %) the round morphology prevailed (RS samples). In the rest of the samples (17/25, 68 %) both population were present, where the percentages of SS cells ranged from 10 to 40 % (mixed samples, MX). In these samples, spindle-shaped cells were only present until the third or fourth passage. This heterogeneity was found in samples from all three trimesters.

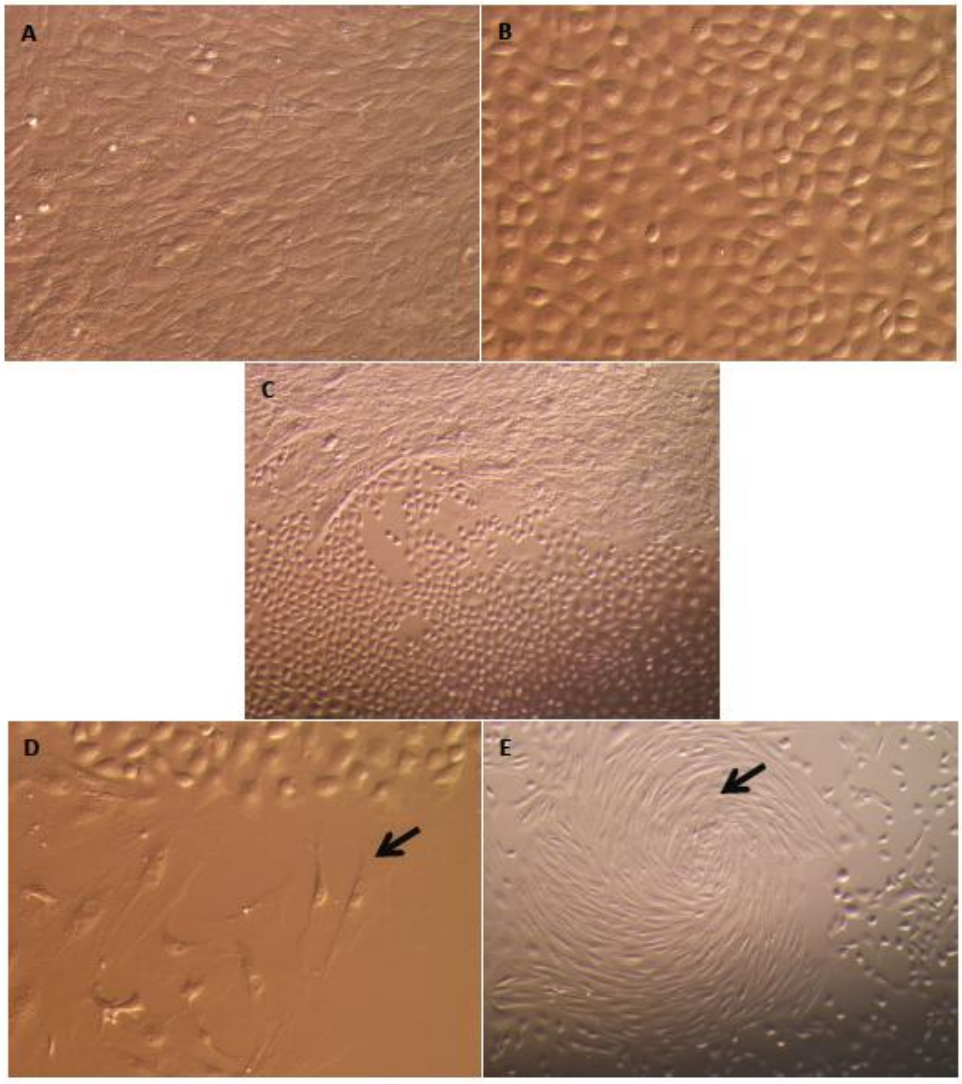


Fig. 2.2: Different types of adherent AF-cells isolated at P0: spindle-shaped (SS) cells (A), round-shaped (RS) cells (B), large and flat (LF) cells (D, black arrow) and fibroblastoid (fi) cells (E, green arrow). In most of the samples (17/25) a heterogeneous population composed of SS and RS cells, named mixed (MX) population, was found (C). Magnifications: A, B, D: 20X; C, E: 10X.

2.3.2 Cell culture and growth curve

Undifferentiated cells were cultured until the eighth passage and total cell doubling number (CDs) and population-doubling times (DTs) were calculated. At P8, total CDs were

statistically higher (P<0.05) in the SS sample (24.9 ± 0.8) than in the MX samples (18.6 ± 2.5) and in the RS ones (17.3 ± 4.8) (Fig. 2.3). Mean doubling time was statistically lower (P<0.05) for SS cells (38.4 ± 12.0 h) than MX samples (67.1 ± 25.0 h) and RS samples (82.9 ± 38.6 h).

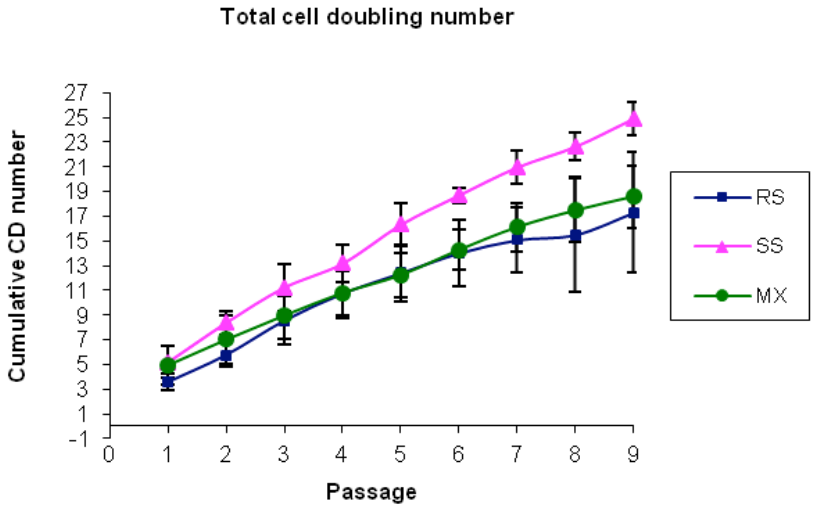


Fig 2.3: Cell doubling numbers of SS-, RS- and MX- populations. At P8, total CDs were statistically higher (*P<0.05*) in the SS sample than in the MX and in the RS ones**.**

2.3.3 Migration and adhesion assay

Both SS and RS cells formed spheroids when cultured in hanging drops. Average areas of the spheroids were 33138.1 ±1401.6 μm^2 for the SS and 45014.8 ± 1165.8 μm^2 for the RS populations respectively. Spheroids from SS cells were significantly smaller than from RS cells (P<0.05), indicating a higher adhesion capability. Average percentages of migration were significantly higher (P<0.05) for spheroids originated from SS cells (94.9 ± 3.0 %) than for the spheroids from RS samples (76.2 ± 4.5 %). The faster migration of the spindle-shaped population was also confirmed by the scratch test. Average percentage of migration

was significantly higher ($P<0.05$) in SS (96.9 ± 2.5 %) compared to RS (79.9 ± 2.2 %).

2.3.4 Molecular characterization

OCT4 expression was not detected both in SS and in RS (Fig. 2.4 C), there was instead a very weak expression of NANOG in all the samples (Fig. 2.4 B). Fibroblasts were negative both for *OCT4* and for *NANOG*. *SOX2* was not found in the RS population, while a weak expression was detected in the SS at both passages, but also in the bovine fibroblasts (Fig. 2.4 A).

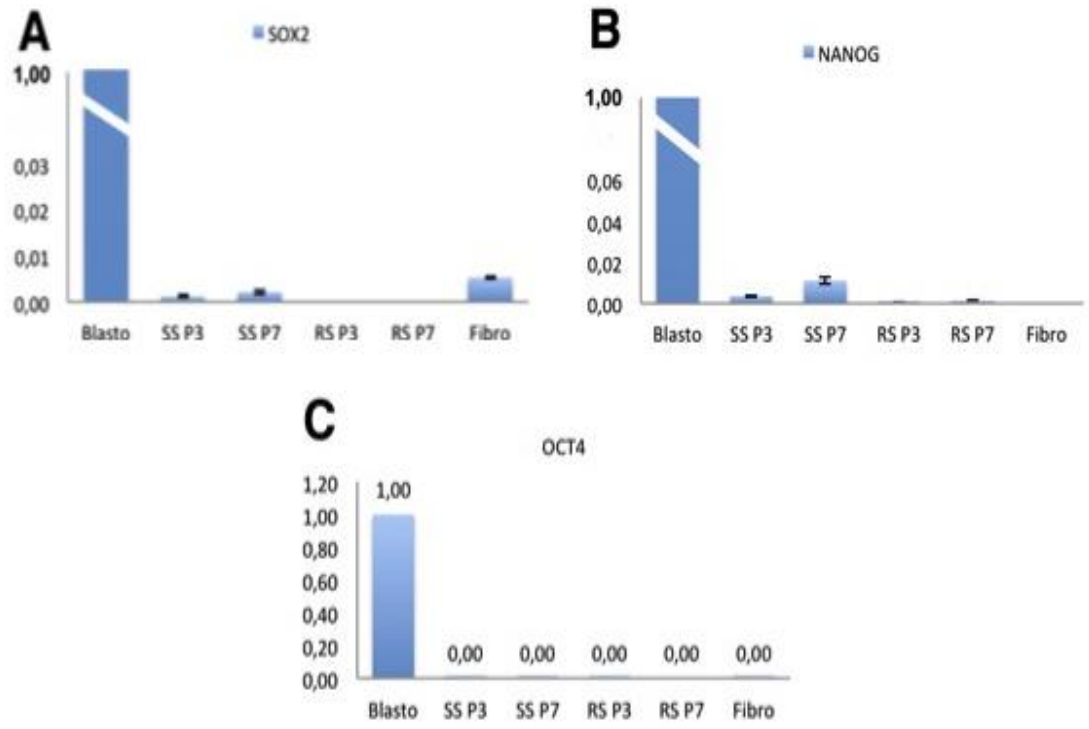


Fig. 2.4: qRT-PCR for the pluripotency genes OCT4, NANOG and SOX2. Evaluation of the expression in the two main cell type (SS- and RS-cells) at P3 and P7. Expression was normalized to bovine blastocysts, used as positive control.

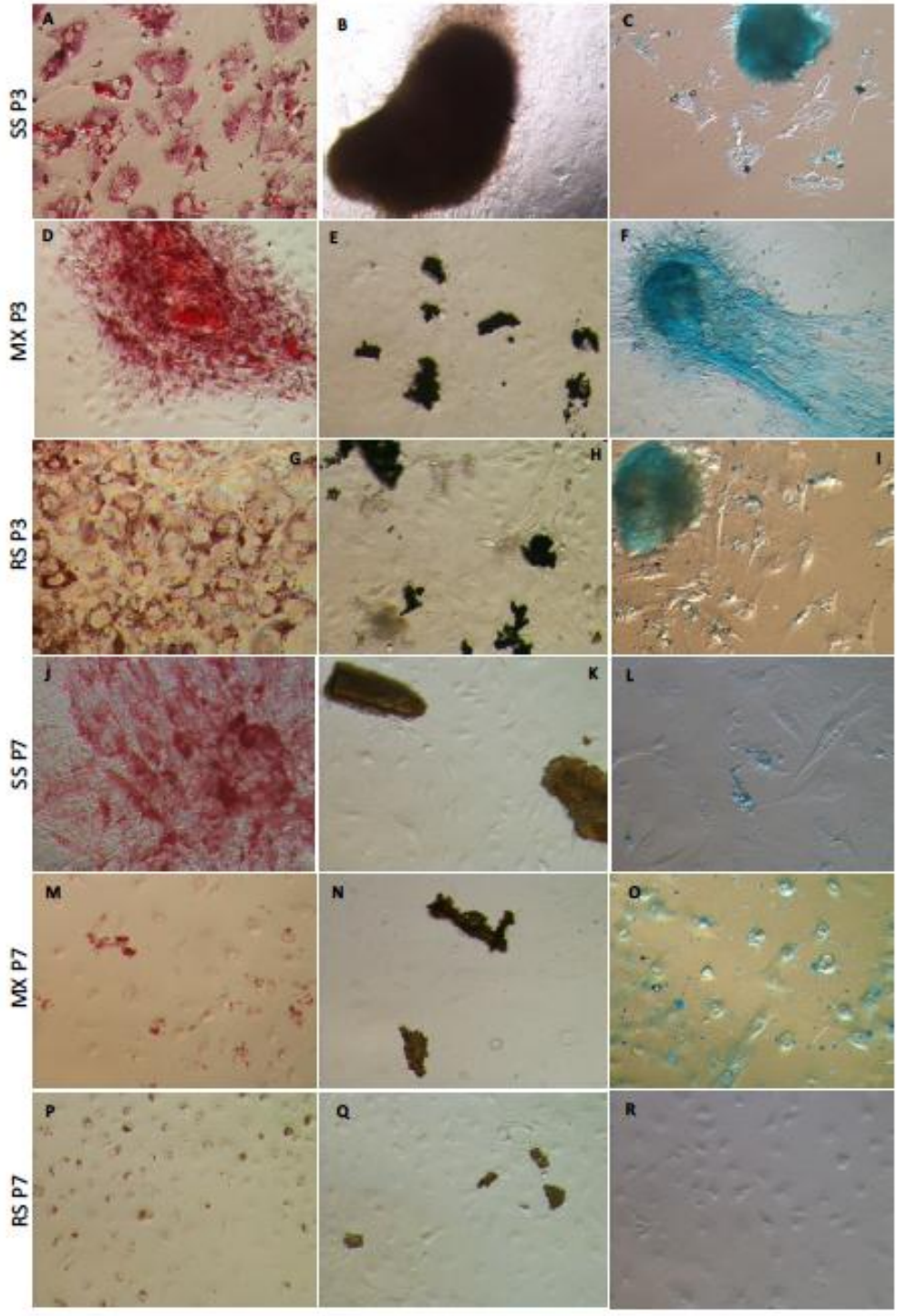
2.3.5 In vitro differentiation

All three types of cells (SS, RS and MX) were cultured in adipogenic, osteogenic and condrogenic condition. To better characterize the proliferating SS and RS subpopulations, real time PCR was performed both for P3 and P7 undifferentiated and differentiated. Generally, SS cells showed a higher differentiation potential compared to RS cells and in MX samples was evident how SS subpopulation had a higher adipogenic and chondrogenic differentiation potential (Fig. 2.5 D, F). In particular, in adipogenic induced cell cultures, after 1 week cells started to change morphology to a more rounded configuration. After 21 days, cells were round and showed evident lipid droplets within the cytoplasm, indicated by Oil Red O staining. At P3 (Fig. 2.5 A, D, G), samples of all 3 types (SS, MX and RS) showed an adipogenic differentiation displayed by an intense Oil Red O staining. At P7 (Fig. 2.5 M, J, P) SS cells showed a high accumulation of lipids in the cytoplasm, while MX and RS cells were still stained but with less intensity. Real Time PCR however indicated a significative ($P < 0.05$) upregulation of *PPARγ* mRNA in both passages of SS and RS cells (Fig. 2.6 A, B). All cell types grown in osteogenic medium both at P3 and P7 showed deposition of matrix-like substance that appeared positive for Von Kossa staining (Fig. 2.5 B, E, H). Gene expression analysis indicated an upregulation of collagen type I and osteopontin in both SS passages and in RS at P7, while no changes were observed in RS at P3 (Fig. 2.5 C, D). Cells cultured in chondrogenic medium showed, after 3 weeks, depositions of glycosaminoglicans, which were stained by Alcian Blue. As for adipogenic differentiation, also in chondrogenic one it was evident that all three populations showed a higher ability to differentiate under the specific culture conditions at P3 (Fig. 2.5 C, F, I),

while at P7 (Fig. 2.5 L, O, R) this potential was reduced in terms of glycosaminoglicans accumulation. Real-time PCR indicated a significative ($P < 0.05$) upregulation of collagen type I in SS cells at both passages and in RS cells only at P3 (Fig. 2.6 E, F). Also collagen II expression was evaluated but none of the samples showed a significant upregulation, even if an increasing trend was found in the differentiated ones. Negative controls for all the 3 differentiation lineages were cultured in regular culture medium and stained with the 3 different methods. No positive stained cells were found (data not shown). As additional control, differentiation into the three lineages described before was induced on bovine fibroblasts and no positive cells were found (data not shown).

Fig. 2.5: Bovine AF samples were cultured over 3 weeks both at the third. passage (from A to I) and at the seventh one (from J to R) under adipogenic (A, D, G, J, M, P), chondrogenic (C, F, I, L, O, R) and osteogenic (B, E, H, K, N, Q) medium. Adipogenic induction was verified staining intra-cellular lipid droplets with Oil Red O, extracellular calcium deposition produced by cells under osteogenic induction was stained by von Kossa and chondrogenic differentiation was proved staining glycosaminoglycans of the cartilage matrix by Alcian Blue. All samples (SS-, MX- and RS- ones) differentiated well at P3 into all three lineages. At P7 onlythe SS sample kept a good differentiation into adipogenic and osteogenic lineages. RS- and MX- samples differentiated less into these two lineages whereas they did not show chondrogenic differentiation. Magnifications: All figures are 20X, except for B (10X)

Fig. 2.5



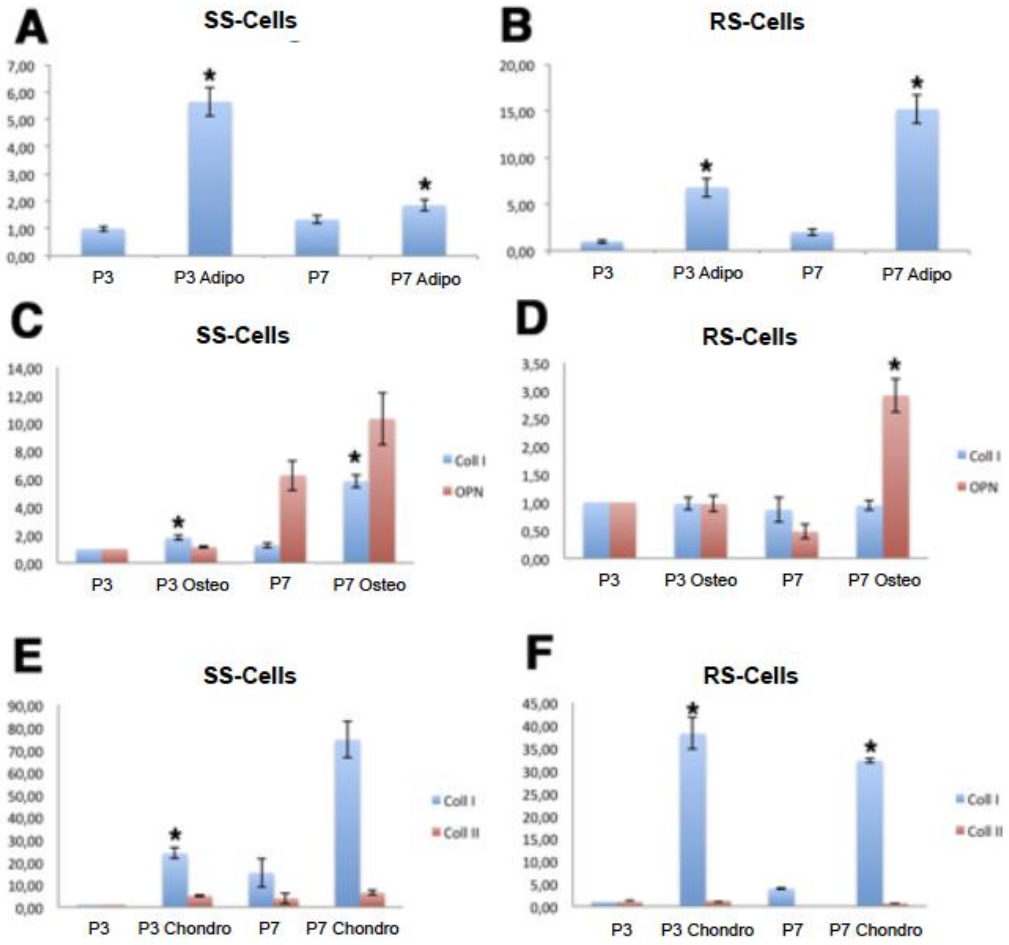


Fig. 2.6: Relative expression of markers related to differentiation lineages in both populations (RS and SS) at both passages (P3 and P7). In detail, *PPARγ* for the adipogenic differentiation (A, B), *Collagene type I* and *Osteopontin* for the osteogenic differentiation (C, D) and *Collagene type I* and *II* for the osteogenic one (E, F). The differentiated samples were normalized on the respective undifferentiated at P3.

2.3.6 Flow cytometry

All cell types were positive for CD44 and CD105, with an increasing of their expression at P7 for all cell populations except for SS one (Fig. 2.7). All cell types also showed cross-reaction with CD90 and the expression of this marker increased at P7 (Fig. 2.7). Overlay

histograms of these flow cytometric analyses are reported in Fig. 2.8. It was not possible to evaluate the expression of the markers CD45 and CD73 in the bAFSCs because these CDs were negative also for lymphocytes (data not shown). Surprisingly, all cell populations showed expression of CD34 both at P3 and at P7, and the percentages reached about 50 % in all samples at P7 (Fig. 2.7). It is important to underline that also bovine fibroblasts and MSCs from bovine adipose tissue at first passages showed CD34 expression at flow cytometry while bovine lymphocytes did not express this marker (data not shown).

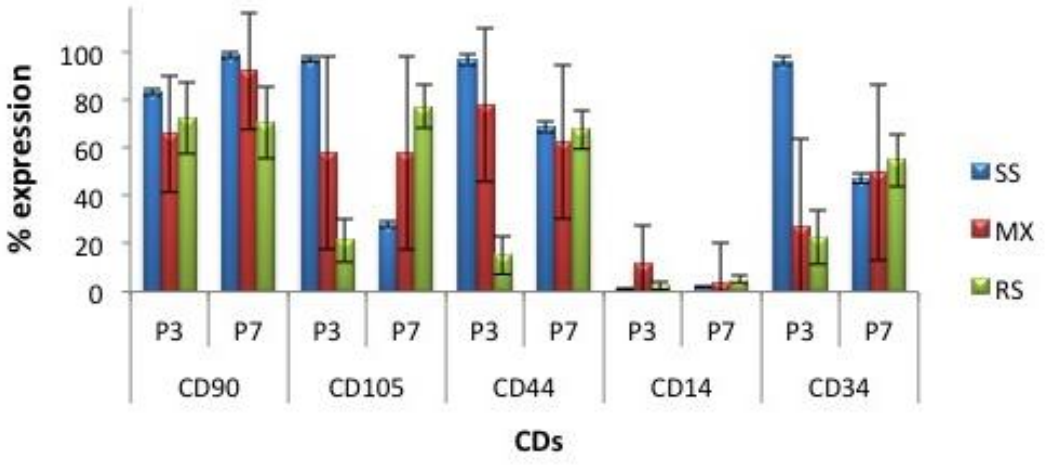


Fig. 2.7: SS-, MX- and RS samples analysed for different antigens expression (CD90, CD105, CD44, CD34, CD14) by FACS analysis both at P3 and at P7.

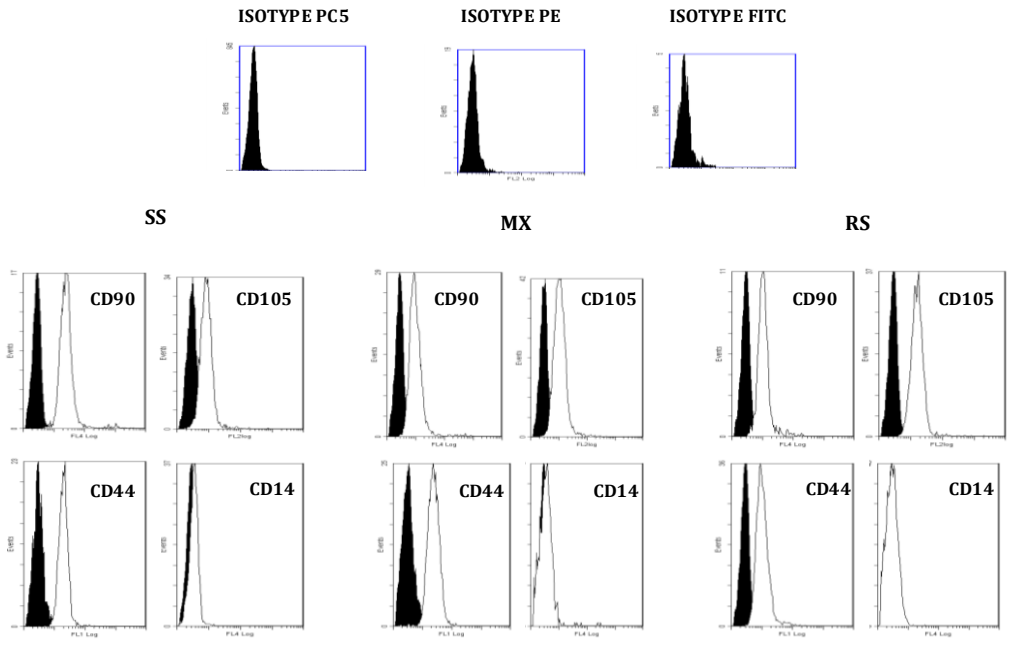


Fig. 2.8: Overlay histograms of cytometry analysis. In black isotypic controls are represented. Empty histograms represent the analysis with monoclonal antibodies on cell culture.

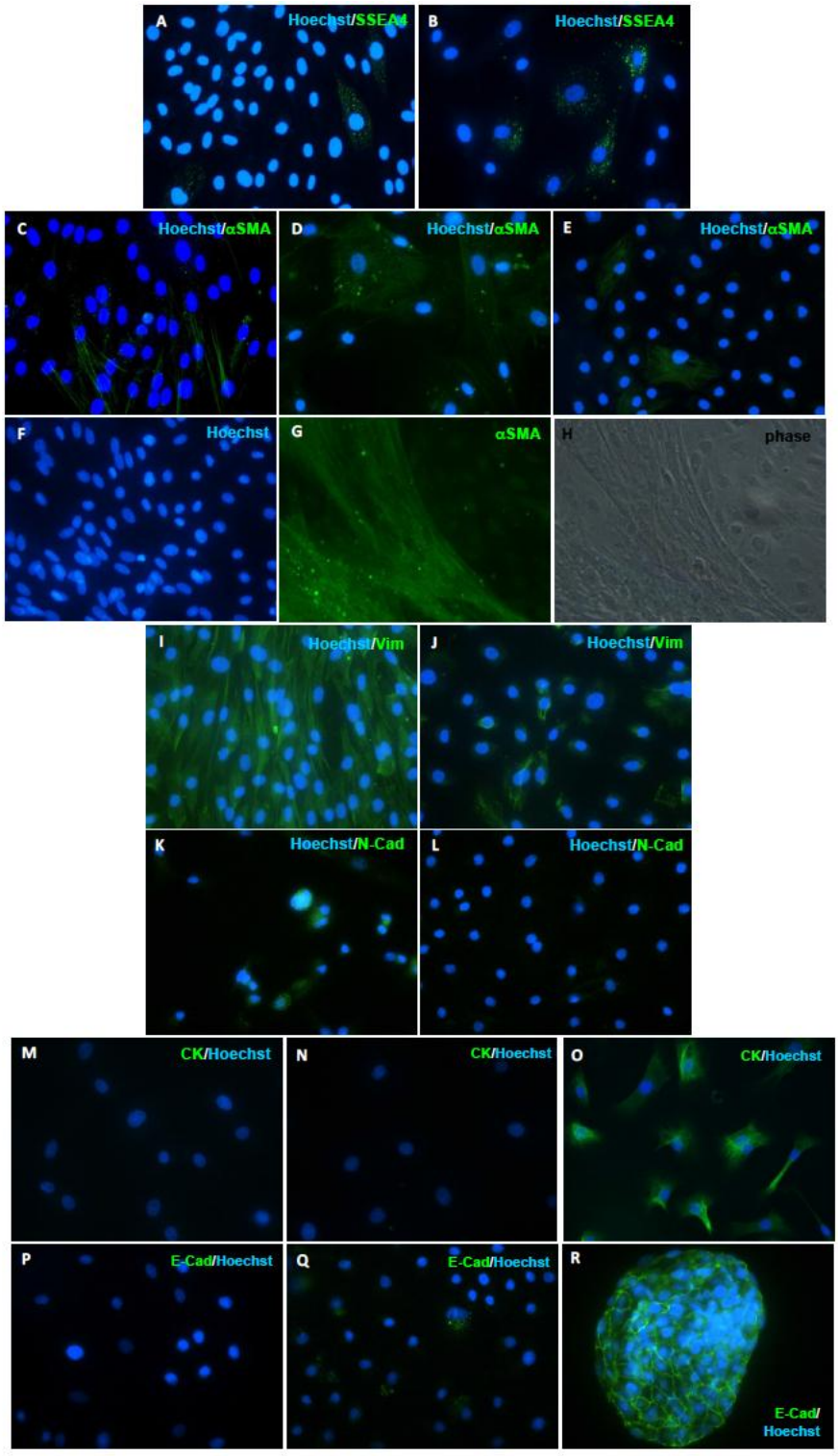
2.3.7 Immunocytochemistry

Immunocytochemical staining of bAFSCs showed heterogeneity in the expression of SSEA4, an antigen commonly used as a marker for undifferentiated cells, and in the expression of alpha-SMA, a mesodermal marker. All cell lines did not showed expression of Oct4. Only samples with spindle-shaped cells showed expression of SSEA4 both at P3 (8.0 %) and at P7 (22.0 %), with an increasing of the expression through passages (Fig. 2.9 A, B). Mixed populations (MX) and RS lines did not express this marker (data not shown). Alpha-SMA was expressed in all lines in different percentages at P3:9.4% in SS (Fig. 2.9 C), 0.9% in RS (Fig. 2.9 E) and 14.8% in MX samples (Fig. 2.9 G). In MX population is evident that percentages of positive cells are related to the presence of the spindle shaped cells (Fig. 2.9 F, G, H). At P7 only SS sample maintained and increased the expression of

this mesodermal marker, assessing it at 27.5 % (Fig. 2.9 D). Both RS and SS cells did not express cytokeratin (Fig. 2.9 M, N) and E-Cadherin was expressed in few RS cells (Fig. 2.9 Q) but not in SS cells (Fig. 2.9 P). Vimentin was expressed by SS cells (Fig. 2.9 I) while RS cells expressed this mesenchymal marker in a very low percentage, around 8 % (Fig. 2.9 J). N-Cadherin was clearly expressed in the spindle shaped population (Fig. 2.9 K) while in the round shaped one the expression was weaker and not present in all cells (Fig. 2.9 L).

Fig. 1.9: Expression of the embryonic stem cell marker SSEA4 in the SS sample at P3 (A) and at P7 (B). Expression of the mesenchymal marker alpha-SMA in the SS-sample (c), in a RS-sample (e) and in aMX-sample (F: Hoechst staining; G: FITC staining). It is clear how in the MX-samples cells positive to the FITC staining (G) are the spindle-shaped ones (H). F, G and H represent the same field. Expression of the mesenchymal marker Vimentin in the SS-population (I) and in the RS cells (J). Absence of expression of the epithelial marker Cytokeratin both for RS cells (N) and for SS ones (M). Bovine epithelial cells were used as positive control (O). Expression of the mesenchymal marker N-Cadherin in the SS-sample (K) and in a RS one (L). Absence of expression of E-Cadherin both in spindle shaped cells (P) and in round shaped ones (Q). All images, except for F, G and H, represent the merge of Hoechst and antibody stainings. Magnification: 40X.

Fig. 1.9


A
Hoechst/SSEA4
B
Hoechst/SSEA4
C
Hoechst/αSMA
D
Hoechst/αSMA
E
Hoechst/αSMA
F
Hoechst
G
αSMA
H
phase
I
Hoechst/Vim
J
Hoechst/Vim
K
Hoechst/N-Cad
L
Hoechst/N-Cad
M
CK/Hoechst
N
CK/Hoechst
O
CK/Hoechst
P
E-Cad/Hoechst
Q
E-Cad/Hoechst
R
E-Cad/
Hoechst

2.4 Discussion

Stem cells isolated from amniotic fluid represent an important source of cells with characteristics between pluripotent and adult stem cells (Kim et al. 2007; Klemmt et al. 2011). In the present study samples of AF have been easily collected at slaughterhouse from pregnant cows at different trimesters of pregnancy and nucleated plastic-adherent cells have been isolated, and subsequently cultured and characterized, from the most part of the samples processed. Difficulties in isolation were found only for AF samples harvested at the end of the second trimester (about 180 days of gestation) or at the third, probably due to the relative small volume of sampling compared to the increasing amount of AF at these stages of pregnancy (Brace and Wolf 1989). Furthermore, cells yield was almost threefold higher from samples collected in the first trimester than in the second and third, confirming that the ratio cells/ml decreases during pregnancy with the higher production of AF. On the other hand, differences about sub-populations isolated were not found among bAFSCs collected at different trimesters and changes in markers expression are to be associated with different cell populations. In fact, depending on the morphology, a heterogeneous population was found, as previously reported both for human amniotic fluid (Kaviani et al. 2003; Bossolasco et al. 2006; Bottai et al. 2011; Roubelakis et al. 2011) and in the veterinary field (Sartore et al. 2005). The two main populations isolated that persisted through passages were the spindle-shaped (SS) and the round-shaped (RS), even if at passage 0 there were also two other populations, as found by Bottai and colleagues (Bottai et al. 2011) in human AF samples, classified as large and flat cells and fibroblastoid cells, that were lost after the first-second passage. The best-represented subpopulation in our bovine-AF samples was the round-shaped. This is in contrast with results obtained by two

research groups (Corradetti et al. 2013, Gao et al. 2014) that found less heterogeneity among samples and a prevalence of the spindle-shaped subpopulation.

As shown by proliferation assay, SS cells showed a lower mean doubling time (1.6 ± 0.5 days) compared to RS ones (3.5 ±1.6 days). MX samples, which are composed by both populations until the third-fourth passage, had an intermediate average DT value (2.8 ± 1.0 days). This result suggests that the spindle-shaped subpopulation showed a rapid expansion in vitro, compared to the RS one. Moreover, SS subpopulation also showed a higher cell doubling number at P8 than the other 2 types of samples.

SS cells also migrated significantly faster and showed a statistically higher migration ability, compared to RS ones, as demonstrated by the migration assay. Migration ability is an important feature of MSCs because of its fundamental significance for systemic application (Burk et al. 2013). The expression of adhesion molecules and the homing to injured environments are also important characteristics of MSCs (Kavanagh et al. 2014). Migration assay showed a significant higher adhesion capability of the SS population than the round shaped one, as confirmed by higher expression at P3 of CD44, a glycoprotein which has a role in MSCs migration (Iacono et al. 2012a). At P7 all samples expressed this marker, highlighting an expression of CD44 through passages. In vitro differentiation assay supports results obtained with human AFCSs (Perin et al. 2008) and animal AFSCs (Lovati et al. 2011; Dev et al. 2012; Iacono et al. 2012a; Iacono et al. 2012b; Corradetti et al. 2013). The choice of the three differentiation protocols (adipogenic, osteogenic and chondrogenic ones) was established on the basis of the values listed by the International Society of Stem Cells (Dominici et al. 2006). When adipogenic and osteogenic differentiations were induced, SS population showed at both passages a higher differentiation potential compared

to RS capability in terms of accumulation of cytoplasmic lipids and deposition of glycosaminoglycans, respectively. At mRNA level, significant upregulation of specific differentiation markers was found for both populations, except for the expression of collagen type I in differentiated RS-cells at P3, indicating a selection through passages of cells able to differentiate toward the osteogenic lineage. Both cell types showed chondrogenic differentiation with production of cartilage matrix at P3 but not at P7. Observing differentiation potential of the mixed sample (MX) at P3, it is possible to see how SS cells differentiated better than the RS type, although there were not differences between the two cell types at mRNA level. Roubelakis and colleagues (Roubelakis et al. 2011) also found that human AF (hAF)-SS population showed a better chondrogenic and osteogenic differentiation potential compared to hAF-RS- population one. An examination of the immunophenotype was performed observing the expression of different surface MSCs markers. Since there are no bovine-specific antibodies for flow cytometry analyses, we used human ones, after checking for cross-reactions. Among our samples, all cell types had an increase in the expression of the MSC marker CD90 (Thy-1) from P3 to P7 and SS sample had a higher expression compared to RS and MX subpopulations at both passages. The other MSCs marker, CD105 (SH2 or endoglin or TGFβ1/3 receptor), increased its expression through passages, with the exception of the SS sample. All cell types were negative for the haematopoietic progenitor CD14. On the other hand, the lack of reactivity of bovine cells with the marker CD73 (NT5E, ecto-5′-nucleotidase) and bovine lymphocytes with CD45 (pan-leukocyte marker) maybe indicate that human antibodies did not cross-react with the corresponding bovine epitopes. It is important to underline that we found a percentage of positive cells for CD34 (marker for endothelial cells and

hematopoietic progenitors cells) in all samples. At P3 this marker was expressed in high percentages by SS sample while in low percentages in RS and MX populations. At P7 there were about 50 % of $CD34^+$ cells in all samples. Bovine MSCs isolated from the adipose tissue and bovine fibroblasts were also positive for this marker (unpublished data) whereas bovine lymphocytes revealed a CD34- profile, as expected. Since a human antibody anti-CD34 was used, we could not exclude an aspecific cross-reaction with bovine adult cells. Anyway, the CD34 antigen is expressed on hematopoietic progenitor cells of all lineages as well as the most pluripotential stem cells. CD34 antigen expression is highest on the most primitive stem cells and is gradually lost as lineage com-mitted progenitors differentiate (Civin et al. 1989). Moreover, Corradetti and co-workers (Corradetti et al. 2013) did not find this marker at RT-PCR analyses, so further investigation is necessary.

It was proved that there is an amniotic fluid subpopulation that exhibit gene expression associated with pluripotency (Siegel et al. 2008; Pappa and Anagnou 2009; Antonucci et al. 2011; Klemmt et al. 2011) so investigation for markers such as OCT4, NANOG, SOX2 and SSEA4 was performed. By ICC it was discovered that SS population increased the expression of SSEA4 through passages, so it is probably due to a positive selection of a $SSEA4^+$ population. Molecular analysis showed that only SS cells weakly expressed NANOG whereas OCT4 and SOX2 were not expressed by both subpopulations. The lack of expression of the embryonic marker OCT4 was also supported by ICC analyses. This is in contrast with previous studies performed on bAFSCs (Corradetti et al. 2013; Gao et al. 2014). This result is also in contrast with phenotypic characterization of AF-MSCs made by numerous groups in human medicine (Prusa et al. 2003; Tsai et al. 2004; Kim et al. 2007;

Siegel et al. 2008; You et al. 2008; You et al. 2009) and in the veterinary field (Lovati et al. 2011; Chen et al. 2011; Filioli Uranio et al. 2011; Dev et al. 2012) but recent studies on human AFSCs did also not find a population positive for Oct4 (Zia et al. 2013).

ICC investigations also showed different expression of alpha-SMA, a protein expressed by cells towards the mesodermal lineage. At P3 this marker was expressed by SS sample and by MX samples, where it was clear that this marker was expressed only by cells with a spindle-shaped morphology. At P7 only the SS sample showed an alpha-SMA^{+} profile, MX specimens did not express the protein and this is probably related to the absence of spindle-shaped cells over the fourth passage. The spindle-shaped population also expressed other typical mesenchymal markers such as Vimentin and N-Cadherin, as found by other researchers (Kim et al. 2007; Siegel et al. 2008; Bottai et al. 2011; Zagoura et al. 2013), while RS-cells expressed these markers in a very low percentages. Furthermore, SS cells did not express epithelial markers like Cytokeratin and E-Cadherin, and RS-cells expressed only E-Cadherin in a very low percentage, revealing that they were not epithelial cells.

2.5 Conclusions

In conclusion, both subpopulations isolated at early passages, SS and RS, showed expression of mesenchymal markers (CD90, CD105, CD44), a multilineage differentiation into mesenchymal lineages (adipogenic, osteogenic and chondorgenic ones) and average doubling times (DTs) comparable to human ones (Bottai et al. 2011) and to DTs of AFSCs from other species (Filioli Uranio et al. 2011; Iacono et al. 2012a; Iacono et al. 2012b). SS cells also showed a low expression of stemness markers such as Nanog and SSEA4, which

are expressed over P3. Thus it is likely that SS cells are the presumptive AF mesenchymal subpopulation because they showed stem cells characteristics in addition to mesodermal markers, as for other species (Sartore et al. 2005) and for humans (Kim et al. 2007). Although RS cells showed some mesenchymal surface markers and had a good differentiation potential at early passages, the SS population is the ascribable to the mesenchymal one. The lack (Cytokeratin) or the very low expression (E-Cadherin) of epithelial markers in the RS population allows to exclude that these rounded cells are epithelial ones, and the low expression of mesenchymal markers (N-Cadherin, Vimentin), whose expression increases during the Epithelial to Mesenchymal transition, or EMT (Eastham et al. 2007), collocate these cells in an intermediate stage. The isolation of these two different populations does not depend on the trimesters of pregnancy and differences are only ascribable to different cell types.

These results were published in:

- Rossi B, Merlo B, Iacono E, Tazzari PL, Ricci F, Galli C. Isolation and characterization of mesenchymal stem cells from bovine amniotic fluid at different gestational ages. Proceedings of the ISSCR 11th Annual Meeting, Boston, Massachusetts, June 12-15 2013: W3066.

- Rossi B, Merlo B, Iacono E, Pagliaro PP, Tazzari PL, Ricci F, Galli C. Bovine amniotic fluid mesenchymal stem cells characterization after culture in vitro. *Reproduction, Fertility and Development*, 2014, 26(1): 207-8.

- Rossi B, Merlo B, Colleoni S, Iacono E, Tazzari PL, Ricci F, Lazzari G, Galli C. Isolation and in vitro characterization of bovine amniotic fluid derived stem cells at different trimesters. *Stem Cell Reviews and Reports* 2014, 10(5): 712-24. doi: 10.1007/s12015-014-9525-0.

3. Molecular and phenotypical characterization of pluripotency marker expression in equine mesenchymal stem cells isolated from amniotic fluid, Wharton's jelly and umbilical cord blood

3.1 Introduction

It is reported in literature that human foetal adnexa such as amniotic fluid (AF), Wharton's jelly (WJ) and umbilical cord blood (UCB), contain a mesenchymal stem cell population that express a panel of pluripotency markers, such as OCT4, NANOG, SOX2 and SSEA4 (Pappa and Anagnou 2009). In the veterinary field, results are conflicting (Iacono et al. 2015), expecially for equine foetal MSCs (De Schauwer et al. 2011). Pluripotency markers, generally analyzed are the transcription factors OCT4, also known as POU5F1, NANOG and SOX2. These three transcription factors are the master regulators of pluripotency and they act in a complex network where the balance of their activation and inhibition maintains ESCs in the pluripotent state (Pan and Thomson 2007; Shi and Jin 2010). Several groups were able to identify equine OCT4+ MSCs at the protein level (Hoynowski et al. 2007; Reed and Johnson 2008; Corradetti et al. 2011) while Guest and colleagues (2008) found no expression. The absence of specific antibodies for equine cells represents a big deal for the research in the field. Gene expression analysis at the mRNA level is a valuable alternative when no cross-reacting antibodies are available, although these analyses must be interpreted with care (De Schauwer et al. 2011).

Considering the conflicting and confusing results reported in literature, in addition to the species-specificity of stemness marker expression by MSCs, the aim of this study was to evaluate the expression of pluripotency markers in mesenchymal stem cells isolated from equine amniotic fluid (eAF-MSCs), Wharton's jelly (eWJ-MSCs) and umbilical cord blood

(eUCB-MSCs), as a continuation of the characterization of these cells previously performed by our research group (Iacono et al. 2012a).

3.2 Materials and Methods

3.2.1 Sample harvesting, cells isolation and culture

AF, WJ and UCB samples were harvested as previously described (Iacono et al. 2012a). In summary: AF samples were taken soon after foal or placenta membranes passed through vulva. Each sample was diluted 1:1 with Dulbecco's Phosphate Buffer Solution (DPBS) and centrifuged for 15 min at 1500 rpm. The pellet was resuspended in 5 ml of culture medium containing DMEM and TCM199 (1:1), 10% (v/v) FBS (Gibco, Life Technologies), 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were isolated by carefully loading sample on 5 ml of 70% Percoll solution in a 50 ml polypropylene tube, centrifuging for 30 min at 25° C at 2100 rpm. The interphase was collected after aspirating and discarding the supernatant and it was washed 3 times in culture medium. Cells were then resuspended in 1 ml of culture medium and counted by haemocytometer.

UCB was collected immediately after foaling and before the umbilical cord (UC) breaks spontaneously or was broken according to management protocol. Venipuncture of the umbilical vein was performed with a 21 guage hypodermic needle attached to a 60 ml sterile syringe (IMI), containing 1 ml of heparin (Eparina Vister 5000 IU/ml; Marvecs Pharma) as anti-coagulant. Each sample was diluted 1:1 with DPBS containing 100 IU/ml penicillin and was centrifuged for 15 min at 1500 rpm. Supernatant was removed and the pellet was re-suspended in 5 ml of culture medium. The mononuclear cell fraction was isolated by carefully loading sample on 5 ml of 70% Percoll solution, centrifuging for 30

min at 2100 rpm at 25 °C. The interphase was then collected after aspirating and discarding the supernatant washed with 20 ml of culture medium by centrifuging at 470 g for 10 min at 25 °C. The supernatant was aspirated, cells were washed twice with culture medium and then resuspended in 1 ml to count them by haemocytometer.

WJ is a gelatinous substance that could be present or not at birth, because of reduction of the water and substances content towards the end of pregnancy (Fig. 3.1).

Fig. 3.1: Wharton's jelly is clearly visible in the equine umbilical cord. In this figures WJ is highlighted by black arrows.

It was harvested immediately after breaking the UC from the part closest to the colt. Umbilical cord was rinsed by repeated immersion in DPBS and WJ was isolated, weighed and minced finely (0.5 cm) by sterile scissors. Minced WJ was digested by 0.1% (w/v) collagenase type I (Gibco, Life Technologies) dissolved in DPBS solution and sterilely filtered. The tissue and digestion solution were mixed thoroughly, incubated in a 37 °C water bath for 30 min, and mixed every 10 min. After incubation, collagenase was inactivated by diluting 1:1 with DPBS plus 10% (v/v) FBS. The solution obtained was

filtered and undigested tissue was discarded. Nucleated cells were pelleted at 1500 rpm for 10 min, then the supernatant was discarded and the pellet was washed in culture mediun three times. After the last wash, cell pellet was resuspended in 1 ml of culture medium and cells were counted at haemocytometer.

Cells isolated from 3 AF, 3 WJ and 3 UCB samples (total of 9 samples) were cultured in DMEM/M199 in a humidified atmosphere with 5 % CO_2 at 38.5 °C. When cultures reached about 80–90 % of confluence, cells were dissociated with 0.25 % (w/v) trypsin solution, counted and re-seeded to the subsequent passage. Immunophenotyping and RT-PCR were carried out at P3-4 of culture.

3.2.2 Immunocytochemistry

Cultured cells were washed with DPBS, fixed with 4 % PFA for 20 min at RT and then washed in phosphate buffer (PB). Cells were blocked in goat serum 10 % (Sigma) for 1 h and incubated overnight with primary antibodies against Oct4 (Abcam, ab18976, 1:50) and SSEA4 (Hybridoma Bank, MC-813-70, 1:50). Cells were washed in PB2 and incubated with anti-mouse- or anti-rabbit- FITC conjugated secondary antibodies for 1 h. After that, nuclei were labelled using Hoechst 33342 and the excess of secondary antibody and Hoechst was removed by 3 washes with PB2. Equine fibroblasts were used as negative control. Percentages of positive cells were obtained doing ratio between FITC+ cells and nuclei.

3.2.3 RT-PCR

Gene expression profile was performed by RT-PCR to search for *OCT4*, *NANOG* and *SOX2* mRNA and *GAPDH* was employed as reference gene. Equine blastocysts and equine fibroblasts were used respectively as positive and negative controls. RNA extraction was performed as described in Paragraph 2.2.7 for bovine AFSCs.

Reactions were conducted for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. RT-products (cDNAs) were used directly in PCR reactions. The 25 µL reaction mixtures contained 10X *Taq* buffer, 1U HotStarTaq® Plus (Qiagen), forward and reverse gene-specific primers (final concentration: 0.25 µM). Amplification was carried out for 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec. PCR products were separated on a 2% agarose gel, results of the electrophoresis were captured with a Gel Logic 100 Kodak Imaging System and images were analysed with a Kodak Molecular Imaging Software. Primers used are listed in Table 3.1

Table 3.1: Sequence of primers used for RT-PCR analysis

Gene	Primer sequence FW and RV	Amplicon (bp)	Ref.
eGAPDH	FW: 5′- GTCCATGCCATCACTGCCAC-3′ RV: 5′- CCTGCTTCACCACCTTCTTG-3′	262	(Desmarais et al. 2011)
eOCT4	FW: 5′-CCCAGGACATCAAAGCTCTGCAGA-3′ RV: 5′ - TCAGTTTGAATGCATGGGAGAAGCCCAGA -3	679	
eNANOG	FW: 5′- GACAGCCCCGATTCATCCACCAG-3′ RV: 5′- GCACCAGGTCTGACTGTTCCAGG-3′	492	
eSOX2	FW: 5′- GGCGGCAACCAGAAGAACAG-3′ RV: 5′- AGAAGAGGTAACCACGGGGG-3	663	

3.3 Results

3.3.1 Cell culture

It was possible to culture and expand spindle-shaped plastic-adherent cells from all samples of AF, WJ and UCB (Fig. 3.2). Cells were cultured until P3-4 before characterization.

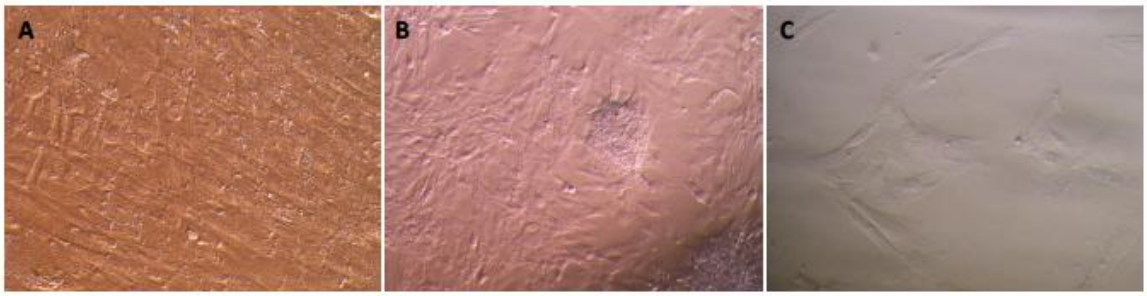


Fig. 3.2: Equine MSCs isolated from AF (A), WJ (B) and UCB (C) at P4. Magnifications: A, B: 20X; B: 10X.

3.3.2 Immunophenotyping by immunocytochemistry

ICC analysis showed a weak expression of the embryonic stem cells antigen SSEA4 in eAF-MSCs (14.8 ± 9.5 %), eWJ-MSCs (8.9 ± 2.6 %) and eUCB-MSCs (7.1 ± 4.8 %). In Fig. 3.2 results for one sample of each tissue are reported.

All samples showed no nuclear expression of OCT4 (Fig. 3.3 B, D, F) but also the positive control did not show a strong nuclear expression (Fig. 3.3 H), so the antibody did not cross-react with equine cells and it is clear that there is an aspecific cytoplasmic binding, very strong in the negative control (Fig. 3.3 J). For this reason, we further investigated mRNA profile.

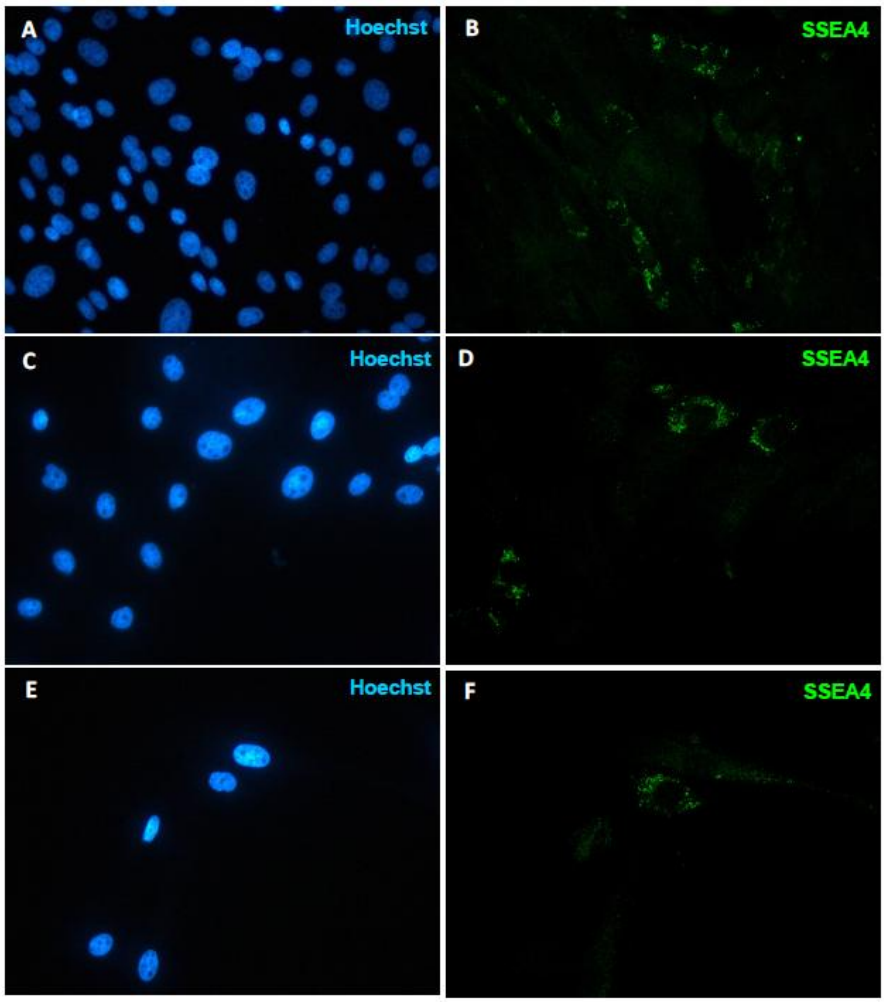


Fig. 3.3: Expression of the embryonic stem cell marker SSEA4 at P3-4 in AF (A-B), WJ (C, D) and UCB (E, F). The first coloumn (A, C, E) represents Hoechst staining of the same fields of the pictures on the right (B, D, F). Here are reported, as example, pictures from 1/3 sample for each MSCs source. Magnification: 40X.

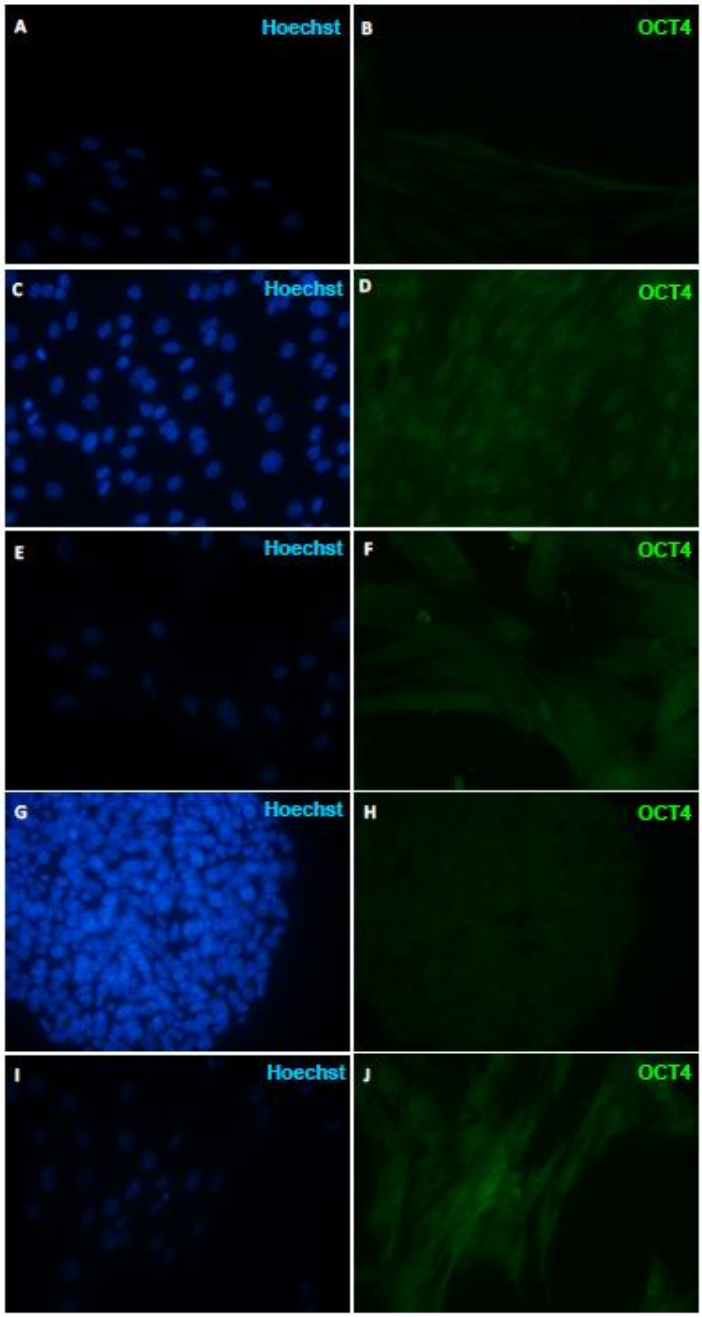


Fig. 3.4: Expression of the key pluripotency regulator OCT4 was evaluated at P3-4 in AF (A-B), WJ (C, D) and UCB (E, F). The first coloumn (A, C, E, G, I) represents Hoechst staining of the same fields of the pictures on the right (B,D, F,H, J). Here are reported, as example, pictures from 1/3 sample for each MSCs source. An equine blastocyst was used as positive control to test the antibody (Hoechst: G; FITC: H) while equine fibroblasts were used as negative control (Hoechst: I; FITC: J). Images with the FITC filter were taken with the same time exposure. Magnification: 40X.

3.3.3 Molecular characterization by RT-PCR

RT-PCR demonstrated no expression of *NANOG* and *SOX2* in all samples and some samples demonstrated a weak expression of *OCT4*, also weakly present in fibroblasts (Fig. 3.5).

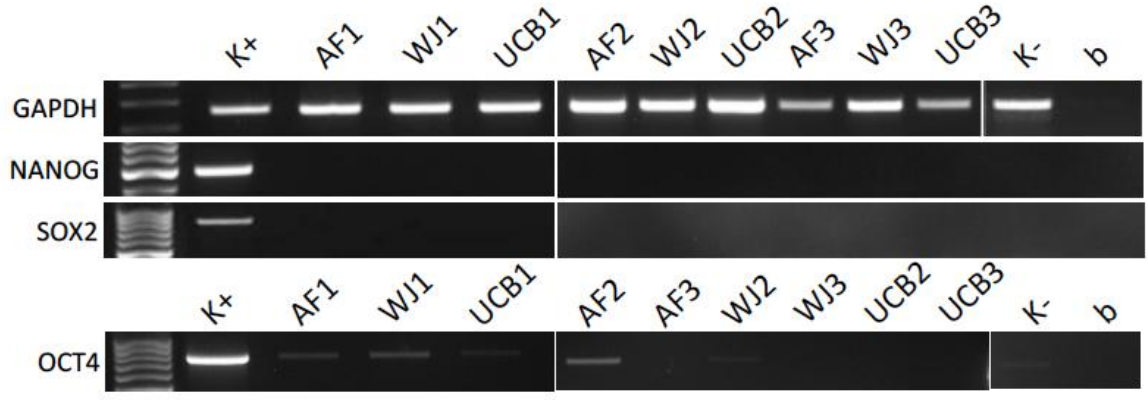


Fig. 3.5: Gene expression profile of the stemness markers *OCT4*, *NANOG* and *SOX2* in all three samples from each source of equine MSCs analyzed. Equine balstocysts were used as positive control (K+) and equine fibroblasts as negative (K-). *GAPDH* was used as internal control.

3.4 Discussion

Isolated cells from equine foetal adnexa showed a subpopulation positive for the pluripotency markers SSEA4. *NANOG* and *SOX2* markers were not detected, unlike human AF-MSCs (Klemmt et al. 2011) and human WJ-MSCs (Fong et al. 2007).

Weak expression of *OCT4* found by RT-PCR could be related to the expression in a low percentage of cells or to a low expression in all cells. On the other hand, at least six *OCT4* pseudogenes are described to date and this implies that the expression of this marker at the gene level should be performed using variant specific primers (Zuk 2009). Further characterization at a protein level with an appropriate antibody will be also necessary.

3.5 Conclusion

Equine MSCs isolated from foetal sources, previously characterized by our research group (Iacono et al. 2012a) through flow-cytometry analysis for MSCs markers expression (CD44+ CD90+, CD105+) and lack of expression of hematopoietic markers (CD14-, CD34-) and for their multipotent mesodermal potential (adipogenic, osteogenic, chondrogenic), were here studied to understand their stemness features. From our results, we cannot assert that equine MSCs are a source of cells expressing the key regulators of pluripotency.

These results were published in:

Rossi B, Merlo B, Iacono E, Colleoni S, Galli C. Stemness features in mesenchymal stem cells from equine amniotic fluid, Wharton's jelly and umbilical cord blood. Atti del 26° Convegno Annuale dell'Associazione Italiana di Colture Cellulari (ONLUS-AICC) - 4th International Satellite Symposium AICC–GISM, Brescia, Italy, 20-22 novembre 2013: 50.

4. Production of induced pluripotent stem cells from bovine adult fibroblasts (biPSCs)

4.1 Introduction

Since the pioneering work of Takahashi and Yamanaka in 2006, the generation of pluripotent stem cells from livestock animals has started to be attempted hardly because of their importance in world food supply, as meat and dairy products, and because they represent also a good model for biomedical and clinical research. The initially generation of iPSCs in mice and humans was then extended to the production of iPSCs from agriculturally important species, including cow, sheep, and pig (Paragraph 1.1.3). Among these, the bovine has a pivotal economic importance in the livestock industry and a few research groups were able to generate bovine iPSCs lines until now. The generation of robust bovine pluripotent stem cell lines may allow complex genetic manipulations, including gene knockin and knockout technology (Sumer et al. 2011). Bovine iPSCs have been generated from fetal fibroblasts and skin fibroblasts by retroviral transduction, lentiviral transduction and virus-free polypromoter vector. Transcription factors cloned from cow, human or the combination of human and pig have all been used (Paragraph 1.1.3, Table 1.1). In all these works biPSCs were culture on MEFs. iPSCs generated by OKSM (bovine or human) were not stable and could only be passaged 6 times (Han et al. 2011).

The aim of this project was to induce pluripotency in bovine adult fibroblasts following the protocol used and standardized for human iPSCs at Istituto Clinico Humanitas (Rozzano, Milano, Italy), where the most part of this work was carried out.

4.2 Materials and Methods

4.2.1 Bovine fibroblasts reprogramming and culture

Bovine adult fibroblasts were cultured in DMEM/M199 supplemented with 10 % FBS in a humidified atmosphere with 5 % CO_2 at 38.5 °C. They were transfected between P1 and P3. After viral transduction, fibroblasts were cultured in Nutristem (Life Technologies) with the addition of 100 ng/ml bFGF for approximatively 3 weeks on matrigel-coated dishes in a feeder-free condition in a humidified atmosphere with 5 % CO_2 at 37°C. After the first picking, colonies were propagated in Essential 8 medium (Life Technologies).

Lentivirus was produced by transfecting HEK293 cells with a human STEMCCA Cre-excisable constitutive polycistronic (OCT4, KLF4, SOX2 and C-MYC) vector and with the lentiviral packaging vectors (tat, rev, gag/pol, Vsv-g). Supernatant was collected by ultracentrifugation (20200 rpm at 4°C) and bovine fibroblasts were infected overnight. Experimental outline of transduction and culture is shown in Figure 4.1 and a timeline of biPSCs production attempt is reported in Figure 4.2.

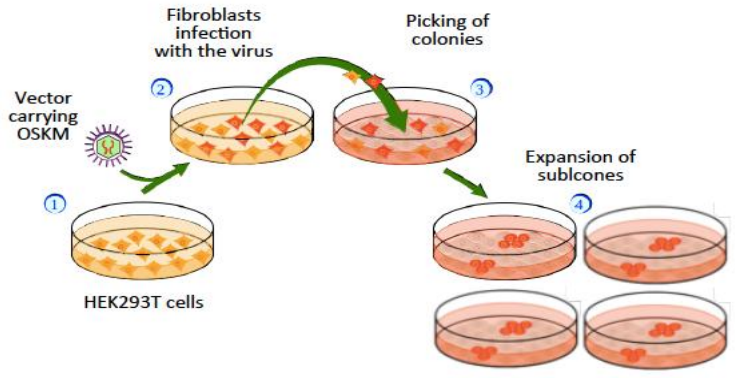


Fig. 4.1: (1) HEK293T cells were transfected with a 48h of incubation with a human STEMCCA Cre-excisable constitutive polycistronic (OSKM) vector and with the lentiviral packaging vectors. 2) Virus were used to infect bovine fibroblasts overnight and (3) when colonies with an ESCs-like (morphology started to appear, they were picked and expanded (4) in order to create subclones (modified from http://en.wikipedia.org/wiki/Induced_pluripotent_stem_cell).

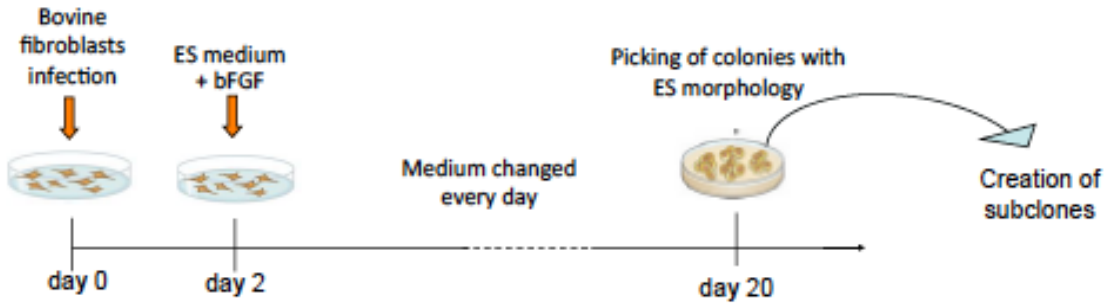


Fig. 4.2: Schematic diagram of transduction protocol for the generation of biPSCs.

4.2.2 Karyotyping

Bovine iPSCs were prepared for karyotype analysis by incubation in medium containing 0.1 mg/ml KaryoMAX® Colcemid™ (Gibco, Life Technologies) for 5 hours. Cells were trypsinized, resuspended in 0.075 M KCl, incubated at 37°C for 30 min, and fixed in 3:1 methanol:acetic acid at room temperature for 5 min. Correct chromosome arrangement of bovine fibroblasts used for reprogramming was evaluated by using CytoVision® software.

4.3 Results

4.3.1 Cell culture and fibroblasts karyotyping

Karyotype analysis of bovine fibroblasts before transduction (Fig. 4.3) showed that they had a normal karyotype: 60, XY (Fig. 4.4).

4.3.2 Reprogramming

Putative biPS colonies, observed between 14 to 21 days post-transduction, were manually

picked and re-plated onto matrigel-coated dishes.

Bovine adult fibroblasts were transduced with retrovirus encoding for human OCT4, SOX2, KLF4, and c-MYC, four transcription factors that were sufficient to reprogram human (Takahashi et al. 2007), monkey (Liu et al. 2008), rat (Liao et al., 2009), and porcine cells (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009; West et al, 2010; Hall et al 2012; Rodriguez et al. 2012). During the first transduction, fibroblasts were not able to survive so it was not possible to see the formation of colonies. After the second and the third transduction, despite the identification of putative iPSCs colonies 2-3 weeks after transduction, these colonies could not be expanded beyond the first passage because they were not able to proliferate.

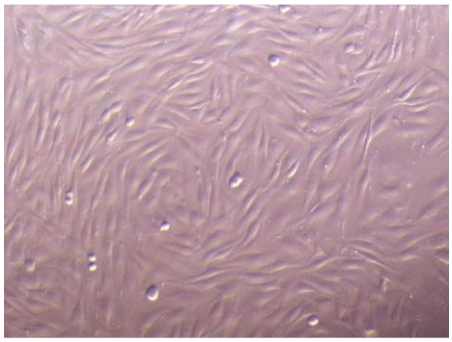


Fig. 4.3: Bovine adult fibroblasts at P2 of culture.

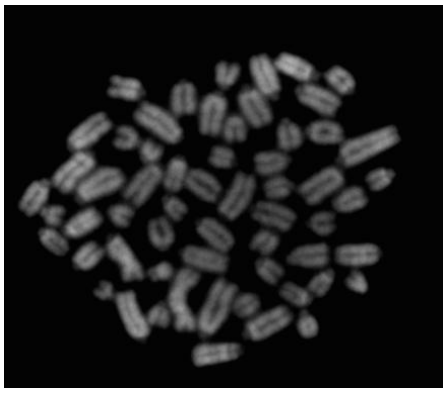


Fig. 4.4: Bovine adult fibroblasts were karyotyped and they showed normal male karyotype of 60 chromosomes.

4.4 Discussion

In this work the generation of biPSCs in a feeder-free condition by using a lentiviral polycistronic vector carrying OSKM factors was attempted but this protocol failed to yield biPSCs clones that could be expanded beyond the first passage. It is possible that the feeder-free culture conditions were not suitable for biPSCs, so it is worth to try to culture them on feeders for the first passages (around 10), and then switch to the culture on matrigel-coated dishes. Moreover, there could be problems related to the factors transfected or to the reprogramming type. The successful generation of biPSCs reported in literature were performed by retroviral transduction of the OSKM factors plus NANOG (Sumer et al. 2011) and by transduction of six bovine transcription factors (Han et al. 2011). It was also reported that only by using human or bovine OKSM, reprogramming seems to be incomplete and none of the colonies could be expanded beyond passage six (Cao et al. 2011). But in our case, the problem was related to the very first steps, because we were not able to go beyond the first passage, so it is more ascribable to some culture condition. First, the adaption of iPSCs to a culture without MEFs was only achieved for human iPSCs and porcine iPSCs by only one research group (Montserrat et al. 2011; Montserrat et al. 2012). It is possible that these cells need to be cultured on feeder-layers for the first passages (until P10, for example), and then adapted to a feeder-free culture. Second, the use of commercial media for human iPSCs could not be the best choice for bovine cells. Third, based on the apparent inefficiency of OSKM cocktail to induce pluripotency in the bovine, it is possible to assert that species-specific requirements exist regarding the transcription factors necessary for a complete reprogramming in bovine somatic cells.

4.5 Conclusions

Undoubtedly, one of the major problems regarding production, isolation, and characterization of pluripotent cells from ungulates is the lack of understanding of the species-specificities in the development process during the very early stages post-conception (Malaver-Ortega et al. 2012). Our aim was to generate a line of biPSCs by using the standardized protocol (commercial media, feeder-free culture) routinely used at Istituto Clinico Humanitas, where biPSCs were generated, but it did not seem to work. Direct extrapolation from the knowledge accumulated from human and mouse work could be misleading for new advances in the field for livestock (Malaver-Ortega et al. 2012), so it is necessary to adapt and develop techniques and criteria to the particularities of bovine species.

5. Stimulus Triggered Acquired Pluripotency (STAP) in porcine embryonic fibroblasts: Could pH variation really induce pluripotency?

5.1 Introduction

After the discover of iPSCs in 2006 (Takahashi and Yamanaka), represented by the possibility to induce pluripotency by transduction of pluripotency genes, the current trend is to simplify the generation of iPSCs minimizing genetic manipulations (Masuda et al. 2013), for example incorporating the use of small chemical molecules (Paragraph 1.1.3). Induction of pluripotency by reprogramming techniques presents drawbacks, like low reprogramming efficency (Liao et al. 2009), time consuming protocols and high costs. In this contest, at the beginning of 2014, a group of japanese scientists at RIKEN institute asserted that, simply bathing somatic cells in a mild acid solution, they could be reprogrammed to become pluripotent. Murine CD45+ splenocytes were chemically stressed in acidified HBSS (pH 5.7) and then cultured in DMEM-F12 for 25 min supplemented with B27 and LIF up to 7 days, observing that the procedure activated the OCT4 promoter 2 days after the treatment. The authors named this procedure "Stimulus Triggered Acquired Pluripotency (STAP)" (Obokata et al. 2014a) (Fig. 5.1).

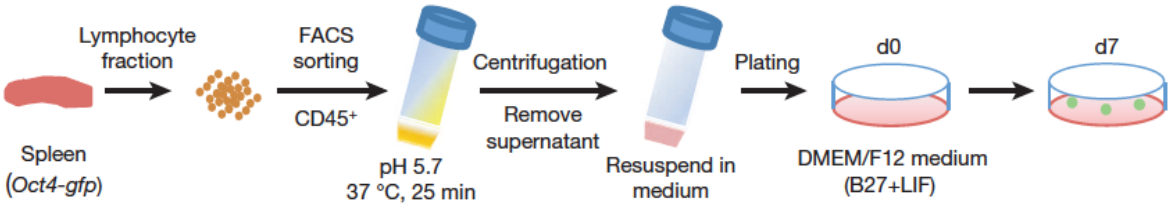


Fig. 5.1: Schematic summary of low-pH pluripotency induction (from Obokata et al. 2014a)

In a second pubblication, STAP cells were demonstrated to be able to form all organs and

tissues in chimeric embryos, and also they asserted that STAP cells could contribute to placenta, highlightening not only their pluripotency, but also totipotency (Obokata et al. 2014b) (Fig. 5.2).

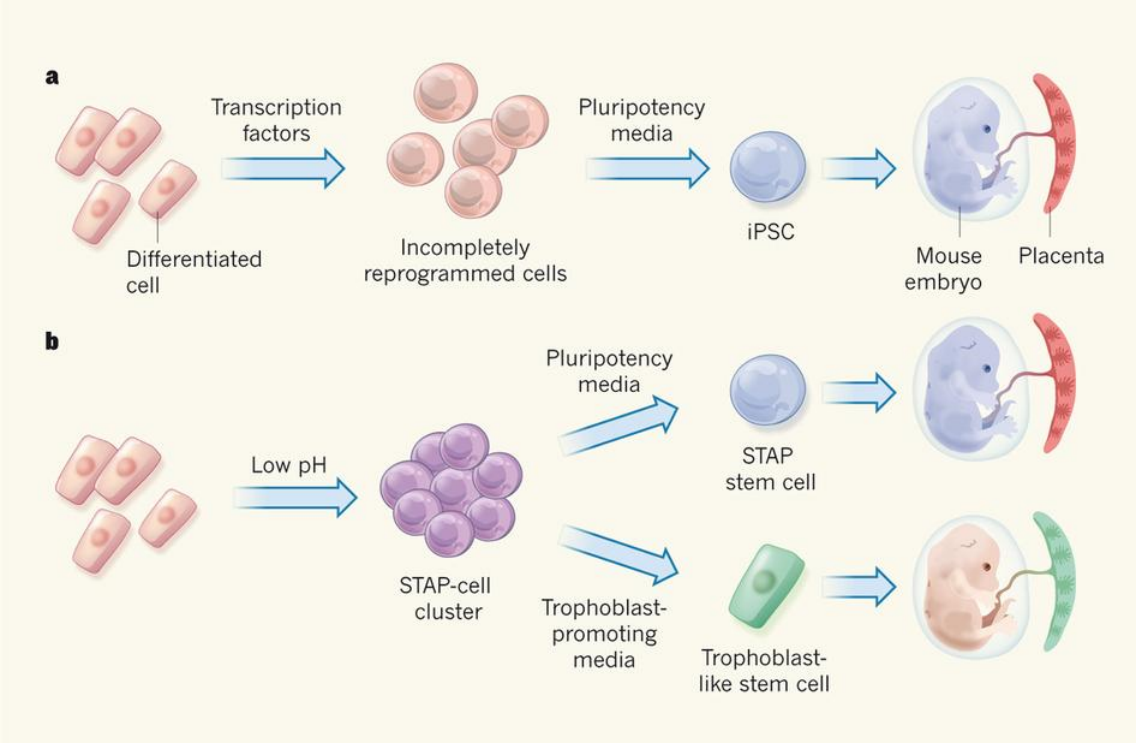


Fig 5.2: Unlike iPSCs, that are able to self-renew and contribute to all the cell types in a developing embryo but not the placenta, STAP stem cells, when cultured in a medium that promotes the growth of trophoblast stem cells (a placenta-generating cell type), STAP cells acquire trophoblast-like characteristics, so they can contribute to the placenta (Smith 2014).

These astonishing findings led us to try the chemical induction of pluripotency in porcine somatic cells. If the production of STAP stem cells could be reproduced in other species, human and large animal models, in the easy and efficient way as reported, it would dramatically increase the production, and consequently the avaiability, of patient-derived pluripotent cells for use in research for both biomedical and agricultural purposes. The pig

is a particularly desirable species to create pluripotent cell lines not only for the livestock industry, but also because it has a huge value as biomedical model in transplantation, considering its longer lifespan compared to laboratory mice, and organ size and physiology more similar to human. (Brevini et al. 2007; Telugu et al. 2010). The pig has already yielded iPSCs by several research groups, even though through the use of the less desirable viral approach, so the application of a virus-free method in this species could be of big interest. The ability to provide iPSC from animals with valuable traits would provide a permanent source of cells for clonal propagation that would likely avoid the inefficiencies and the problems arising from somatic cell nuclear transfer (SCNT), where many of the cloned offspring die or are developmentally abnormal even if they survive to term (Ezashi et al. 2009).

The aim of this study was to attempt to replicate the production of STAP stem cells following their most updated protocol (Obokata et al. 2014c) starting from porcine embryonic fibroblasts (PEFs) instead of splenocytes, in order to study a new way to induce pluripotency in the pig model without any molecular modification.

5.2 Materials and Methods

5.2.1 *Cell culture*

Embryonic fibroblasts isolated from male pig embryos were treated with low-pH medium. Before the acid treatment, PEFs were cultured and expanded in DMEM (Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies). To collect them, PEFs were trypsinized (0,25% trypsin, Gibco, Life Technologies), centrifuged at 1000 rpm for 5 min and counted at haemocytometer.

5.2.2 *Acid treatment*

Around 2,4 - 3 millions of PEFs were treated with mild-acidic HBSS medium as described by Obokata et al. (2014c), titrating pH to 5.7 with HCl. pH was measured by a calibrated pH meter and titrated when cells have already been in the acid-medium, in order to reveal the real pH of the suspension. The acid bath was performed for 25 min at 39 °C. As negative control, 1 million of PEFs was kept in HBSS medium at 39 °C for 25 min, without modifying pH. The acid-treated cells and the related control were centrifuged at 1000 rpm for 5 min. Since these treated cells were not able to grow up, the experiment was repeated other two times with some modifications about the time of acidic-exposure (15 min instead of 25 min). Like for the first sample, 1 million of PEFs were treated in the same way, except for the acid-bath, as negative control (Fig. 5.3). Pellets were resuspended in DMEM-F12 medium supplemented with 1,000 U LIF (Sigma) and 2% B27 (Gibco, Life Technologies) and plated onto non-adhesive culture plates (Nunc).

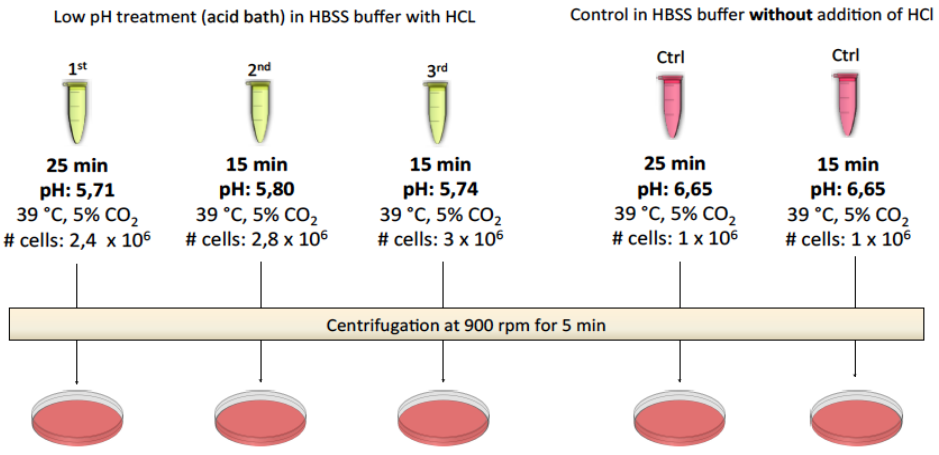


Fig 5.3: The low-pH treatment was carried out three times. Firstly following the time of exposure reported by Obokata and colleagues (25 min), then other two times decreasing the acid exposure to 15 min. Two negative controls were performed using the same medium, except for the addition of HCl, and pH was evaluated.

5.2.3 *STAP stem cells conversion and culture*

Obokata and colleagues reported that STAP cells do not proliferate, but subsequent treatment with pluripotency-promoting media produces STAP stem cells, which have the same properties as iPSCs. To establish STAP stem cell lines, STAP cell clusters were transferred to adrenocorticotropic hormone (ACTH)-containing medium. ACTH (1-24) is available from American Peptide and other companies. The composition of the medium was GMEM, 15% Knockout Serum Replacement (KSR, Gibco, Life Technologies), 1% non-essential amino acids (NEAA), 1% Sodium Pyruvate, 0.1 mM β-mercaptoethanol, 1000 U/ml LIF, and 10 μM ACTH (American Peptide). Several clusters (more than a dozen clusters per well of 96-well plates) were seeded on mouse embryonic fibroblasts (MEFs). After 7 days of culture, cells were subjected to a classic trypsinization method (trypsin 0,05%) and plated in ESC maintenance medium containing 20% KSR. Alkaline phosphatase (AP) activity was analyzed with the AP kit (Sigma) following the manufacturer's instructions during the ACTH-medium culture (day 2) and before the passage in ESC maintenance medium. The protocol is summarized in Fig. 5.4.

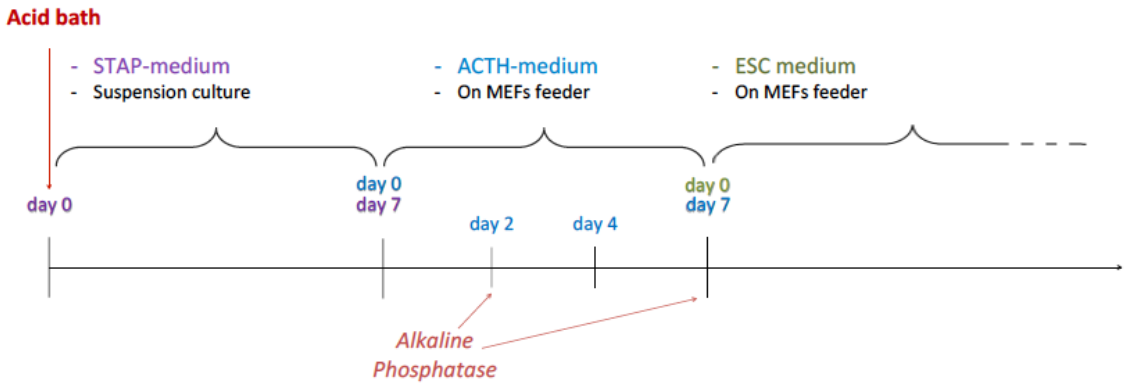


Fig 5.4: Schematic protocol for porcine STAP and STAP stem cells production.

5.3 Results

5.3.1 *Low pH exposure and cell culture*

In the first experiment (25 min treatment), after the hydrocloridic acid treatment, cells were centrifuged and it was possible to see a lot of dead cells in the supernatant. Also Obokata and colleagues noticed high cell death and they asserted that this was due to the fact that the HCl-treatment stresses cells to the point of death. During the suspension culture in DMEM-F12, supplemented with B27 and LIF, it was possible to observe the formation of clusters, both in the treated sample and in the control (Fig. 4-5). The clusters showed a little growth during the week of suspension culture but there were not differences between treated and not-treated samples. Although Obokata and colleagues noticed cell death either, we tried to decrease the time of exposure to the acid bath (15 min) and we repeated the experiment 2 times. It was still possible to see a lot of dead cells in the supernatant (low-pH solution), but we plated precipitated cells anyway. Both in the samples and in the control, it was possible to see clusters as we saw during the first experiment, without any difference (Fig. 5.5).

5.3.2 *STAP stem cells conversion and culture*

Porcine STAP cells were induced into a proliferative state by culturing them in a medium containing (ACTH) on MEFs feeder layer. During this second step of the protocol, cells were not able to show growth (Fig. 5.6) and, moreover, they were negative to the AP assay both on day 2 and on day 7 (data not shown).

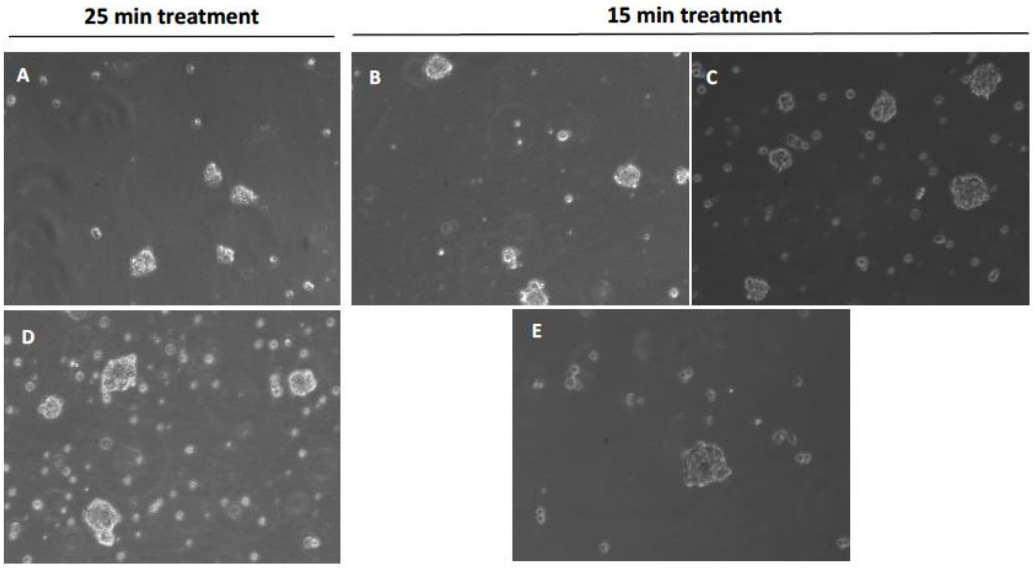


Fig 5.5: During the suspension culture in the STAP medium, it was possible observe clusters both during in the samples treated for 25 min (A), and in the samples HCl-stressed only 15 min (B, C). There were no differences among samples, as well as no differences were observed with both controls (D, E).

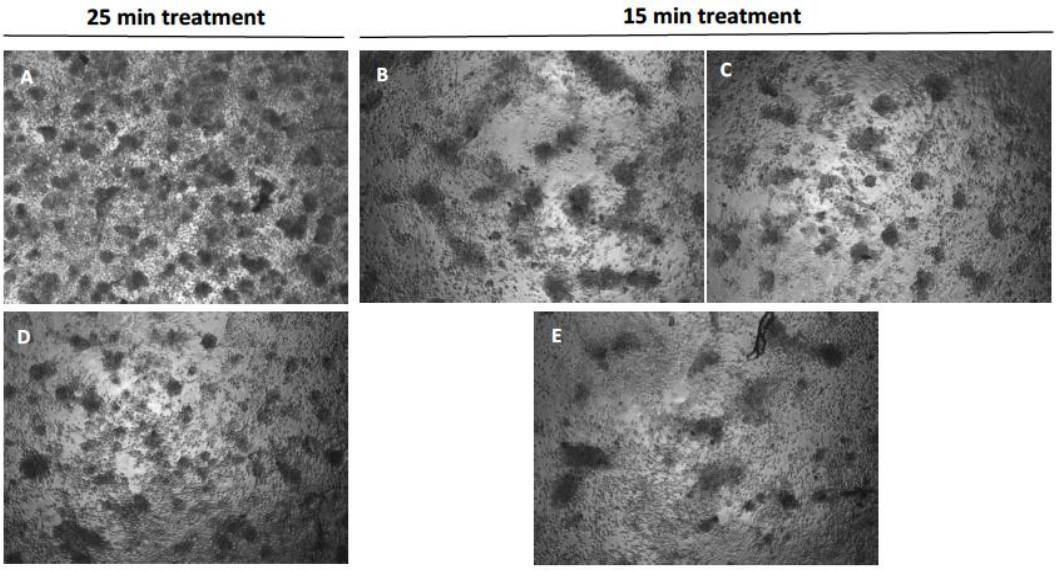


Fig. 5.6: In both the acid treatments (A, 25 min; B, C, 15 min) it was not possible to see cell growth during the seven days in ACTH-medium, so it underlines that the conversion to STAP-stem cell did not work. Moreover, neither control samples (D, E) were able to grow under this medium.

We kept cells in ACTH-medium 7 days and then they were passaged in ESC maintenance medium, although it was not possible to see them grow and they were AP negative.

For each treated sample it was possible to see a very few colonies (around 1-2 per sample) but they were not able to proliferate and cells seemed to die (Fig. 5.7).

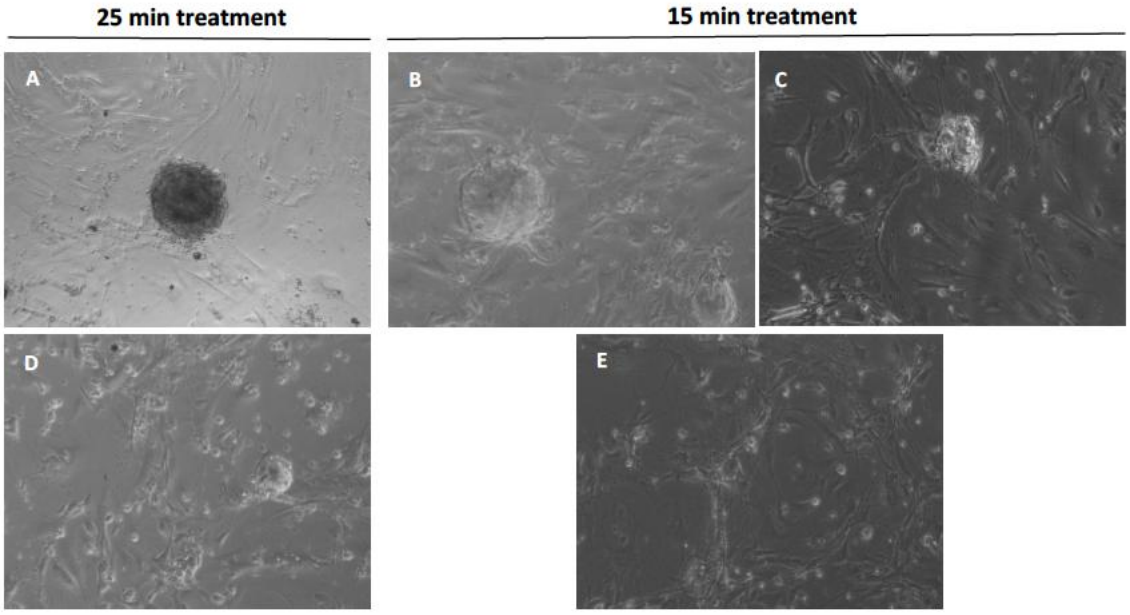


Fig. 5.7: Also in ESC maintenance medium cells were not able to grow and proliferate and the few putative colonies attended in each sample (A, B and C) did not increase in diametre and they seemed to die. Controls cells (D, E) were not able to survive too.

5.4 Discussion

In 2013 it has been reported the generation of mouse iPSCs cells using a cocktail of seven chemical molecules without any genetic manipulation (Hou et al. 2013). These intersting findings have been strengthened by astonishing results of two papers published on Nature at the beginning of 2014 by Obokata and colleagues (2014a, b). As described before, this research group asserted that they were able to activate the Oct4-GFP transgene in CD45+

murine splenocytes, isolated from Oct4-GFP neonates, by a chemical stress made by a treatment with hydrocloric acid only. They called these cells "STAP cells" and they asserted that these cells, when injected into host blastocysts, were able to partecipate to the development of all tissues and organs, placenta included, giving to these cells not only features of pluripotency, but also totipotency. These astonishing findings lead us to try to replicate immediately STAP protocol on porcine somatic cells. The possibility to induce pluripotency in somatic cells without any genetic modification and in a very cheap way made us interested on this protocol. Simultaneously to our study, many research groups have started to debate about the truthfulness of Obokata's papers because several laboratories failed when they tried to replicate the protocols, and one group reported that they were not able to produce STAP stem cells from murine neonatal splenocytes or lung fibroblasts using the acid-based treatment (Tang et al. 2014).

5.5 Conclusions

According to our results, low pH treatment is not an efficient external stimulus for inducing pluripotency in porcine foetal fibroblasts. The clear thing is that the method to produce STAP cells is not as simple and reproducible as it has been reported, and this is underlined by the fact that Obokata was later accused of misconduct and the paper was retracted. This fact is used as an example to highlight the importance of trust and repeatability of the protocols in biological science (Lancaster 2015). Despite these events, easily obtainable pluripotent stem cells are highly sought after and the study of transgene-free protocols to induce pluripotency in somatic cells represents the future of this field.

6. Production of porcine induced pluripotent stem cells (piPSCs) doxycycline-dependent from porcine embryonic fibroblasts

6.1 Introduction

Since the acid-bath treatment turned out to be an insufficient method to induce pluripotency in porcine fibroblasts, we focused again on the classic strategy, namely ectopic expression of reprogramming genes in somatic cells by the use of lentiviral vectors. As previously explained in Chapter 5, the pig has been widely used as a model for preclinical studies because of its similarities in size and physiology to humans and for reasons due to its agricultural and economic interest.

Here we induced pluripotency by a doxycycline-inducible lentiviral vector previously used to generate piPSCs (Rodriguez et al. 2012). The use of tetracycline- or doxycycline-regulated expression of transgenes is one approach considered for silencing the transgenes effectively that has already been applied in the pig (Wu et al. 2009; Hall et al. 2012; Rodriguez et al. 2012), but on the other hand the withdrawal of doxycycline results not only in the expected loss of exogenous gene expression, but also in the loss of the ability to proliferate and maintain pluripotency *in vitro*, giving an incomplete and unstable reprogramming (Hall et al. 2012). Since our final aim was represented by the differentiation of piPSCs into the myogenic lineage (Chapter 7), we decided to use a doxycycline-inducible vector to ease the differentiation program by the withdrawal of doxycycline.

6.2 Materials and Methods

6.*2.1 Porcine embryonic fibroblasts reprogramming and culture*

Porcine iPSCs

Porcine embryonic fibroblasts (PEFs) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and containing also 1% glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids and 0.1 mM β-mercaptoethanol. Induced pluripotent stem cells were generated from PEFs at P3. They were plated onto gelatinized dishes (0.1% porcine skin gelatin, Sigma) at a density of 0.1 million cells per 9.6 cm^2 and they were infected with virus containing-medium overnight. Lentiviruses were produced by transfecting HEK293 cells with doxycycline inducible FUW-tetO vectors (Addgene), encoding the human cDNA sequences of the four transcription factors OCT4, KLF4, SOX2 and C-MYC plus FUW-M2rtTA (Addgene), as previuosly described (Rodriguez et al. 2012). The media containing the 4 factors and FUW-M2rtTA viruses were collected 48h after transfection and were pooled in equal volume, filtered through a 0.45 mm filter and supplemented with 4 mg/mL polybrene (Sigma). PEFs were passaged 48 h after transduction and cultured in KO-DMEM containing 20% Knock-out serum replacement (KSR) supplemented with 20 ng/ml bFGF (Peprotech) and 1 μg/mL doxycycline (Dox, Sigma) (ESC medium). Between 11 and 19 days after Dox induction, colonies were mechanically picked by a 25 gauge 1 inch needle and plated onto feeder layers of MEFs mitotically inactivated by mitomycin C treatment. We selected colonies with a well defined edge and with a translucent light-refractive appearance, composed by cells showing a high ratio nucleus/cytoplasm, criteria used for identifying iPSCs (Wakao et al. 2012). piPSCs cells were passaged every 4-5 days and the timeline is summarized in Fig. 6.1

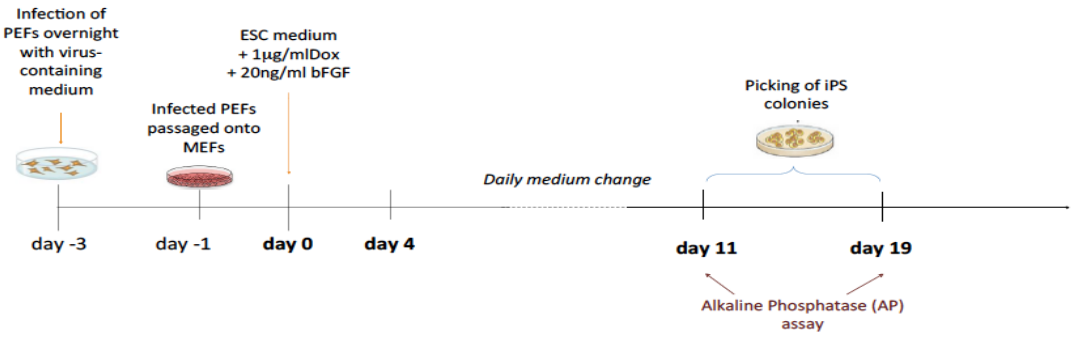


Fig. 6.1: Overview of the reprogramming protocol aimed to the production of piPSCs.

6.2.2 Alkaline Phosphatase assay

Alkaline phosphatase (AP) activity was analyzed with the AP kit (Sigma) following the manufacturer's instructions. AP activity was checked both during the picking period, in order to understand which morphology was equivalent to AP positive colonies, and through passages to evaluate if cells were able to maintain AP activity.

6.2.3 Embryoid Bodies assay

We attempted to assess the ability of piPSCs to differentiate *in vitro* by producing embryoid bodies (EBs). EBs were prepared from piPSCs cells using the *hanging drop* method. Briefly, piPSCs cells were collected by StemPro® Accutase® (Gibco, Life Technologies) and counted at haemocytometer before preparing 30 µl hanging drops at a density of 250 cells/EB. EBs were subsequently allowed to adhere and grown out under *in vitro* differentiation conditions. The differentiation medium consisted of DMEM supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids and 0.1 mM β-mercaptoethanol. Aggregated EBs were transferred to non-adherent dishes after 4 days. After 10 days, EBs were plated into matrigel-coated plates and

cultured for up to 22 days, when cells were collected for further molecular analysis.

6.2.4 Immunocytochemistry

Cells were fixed in 2,5% PFA, permeabilized with 0.1% Triton X-100 in PBS and blocked in 5% BSA in PBS. Pig iPSCs cells incubated with SSEA1-4 and TRA1-60 antibodies were not permeabilized. The following primary antibodies were incubated overnight at 4°C: NANOG (1:300, Peprotech), OCT4 (1:100, Santa Cruz Biotechnology), SOX2 (1:100, Santa Cruz Biotechnology, SSEA1 (1:50; Hybridoma bank), SSEA4 (1:50; Hybridoma bank), TRA1-60 (1:50; Hybridoma bank), all in 5% BSA in PBS. After three washes, cells were transferred to the appropriate secondary antibody and incubated for 1 h at RT, followed by two washes in PBS + 5% BSA. Cells were mounted in Vectashield with DAPI (49-6-diamidino-2-phenylindole, Vector Laboratories).

6.2.5 RT-PCR

RNA extraction was carried out using NucleoSpin® RNA kit (MACHEREY-NAGEL) following the manufacturer's instructions. RNA reverse transcription was performed using Omniscript synthesis kit (Qiagen) and PCR was performed with ReadyMix (Sigma-Aldrich) and 0.4 uM of each primer, amplyfing 10 ng of cDNA. Primers used are listed in Table 6.1. RT-PCR thermal profile included an initial step of 95°C (5 min), followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C and an extension step of 30 sec at 72°C. PCR products were run in 1.5% agarose gel to determine the amplicon size.

Table 6.1: List of the primers used.

Gene	Primer sequences FW and RV	Amplicon (bp)	Accession number or (Reference)
GAPDH	FW: 5'- GGGCATGAACCATGAGAAGT – 3' RV: 5'- GTCTTCTGGGTGGCAGTGAT – 3'	162	AF017079.1
OCT4	FW: 5'- GCAAACGATCAAGCAGTGA – 3' RV: 5'- GGTGACAGACACCGAGGGAA – 3'	201	NM_001113060
NANOG	FW: 5'- TTCCTTCCTCCATGGATCTG – 3' FW: 5'- ATCTGCTGGAGGCTGAGGTA – 3'	214	DQ447201
SOX2	FW: 5'- AAGAGAACCCCAAGATGCACAACT – 3' RV: 5'- GCTTGGCCTCGTCGATGAAC – 3'	219	TC208722
KLF4	FW: 5'- CCATGGGCCAAACTACCCAC - 3' RV: 5'- TGGGGTCAACACCATTCCGT - 3'	154	EU669075.2
STELLA	FW: 5'- CTGAGTAGGTTGAGCCCACA – 3' RV: 5'- CCAAAAGAGGCAAAACCTGA – 3'	281	AJ656181.1
FGF5	FW: 5'- GGAGCAGAGCAGCTTTCAGT – 3' RV: 5'- GGAGCAGAGCAGCTTTCAGT – 3'	170	ENSSSCG00000009253
CARDIAC ACTIN	FW: 5'- CAGGTATTGCTGATCGCATGCA – 3' RV: 5'- ATTTGCGGTGGACGATGGA – 3'	201	TC270296
TYROSINE HYDROX.	FW: 5'- GTCTCGGACGAGGAAATTGA – 3' RV: 5'- CGAAGTAGACGGGCTGGTAG – 3'	226	TC298649
NESTIN	FW: 5'- TACCTGGAAGCGGAAGAGAA – 3' RV: 5'- CTGATCCAGGTCTGCCTTGT – 3'	201	TC295480
GATA4	FW: 5'- AGATTCCTGCATGGACTTGG – 3' RV: 5'- CTCAACTGGAAAGGCCTGAG – 3'	234	TC338844
FUW- hOCT4	FW: 5'- CCCCTGTCTCTGTCACCACT – 3' RV: 5'- CCACATAGCGTAAAAGGAGCA – 3'	148	(Rodriguez et al. 2012)
FUW- hSOX2	FW: 5'- ACACTGCCCCTCTCACACAT – 3' RV: 5'- CATAGCGTAAAAGGAGCAACA – 3'	122	(Rodriguez et al. 2012)
FUW- hKLF4	FW: 5'- GACCACCTCGCCTTACACAT – 3' RV: 5'- CCACATAGCGTAAAAGGAGCA – 3'	137	(Rodriguez et al. 2012)
FUW- hCMYC	FW: 5'- CAGCTACGGAACTCTTGTGC – 3' RV: 5'- CCACATAGCGTAAAAGGAGCA – 3'	125	(Rodriguez et al. 2012)

6.3 Results

6.3.1 Generation of a Doxycycline dependent porcine iPSCs line

ESC-like colonies started to appear only 4 days following transduction (Fig. 6.2 A, B). Twenty colonies were isolated from days 11 to 19 and cultured independently in order to generate different cell lines. During these days it was possible to identify colonies with a thick center and a well defined edge with cells showing a high ratio nucleus/cytoplasm (Fig. 6.2 C, D).

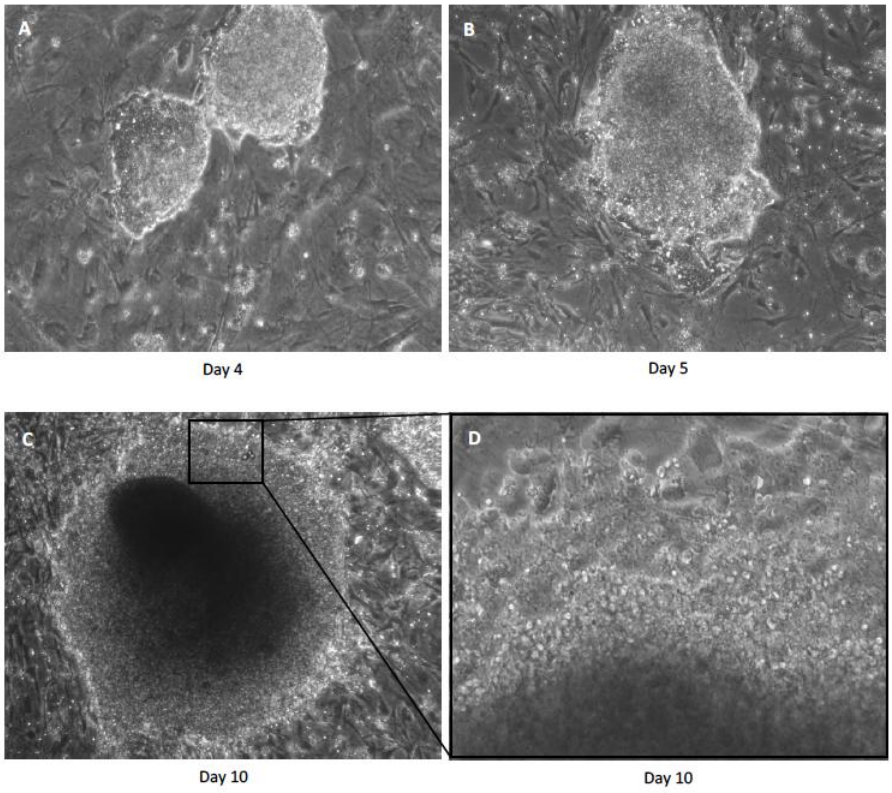


Fig. 6.2: Four-five days after transduction it was possible to observe small colonies with an ESCs-like morphology (A, B: 10X). Colonies grew very fast showing a very thick center (C, day 10: 10X) and cells at the edge with a ratio nuclei/cytoplasms around 1 (D, day 10, 20X).

Colonies were strongly positive to AP at day 11 (Fig. 6.3 A-D) while it was almost totaly

lost at day 19, when colonies started to lose defined edge and cells started to die (Fig. 6.3 E-F).

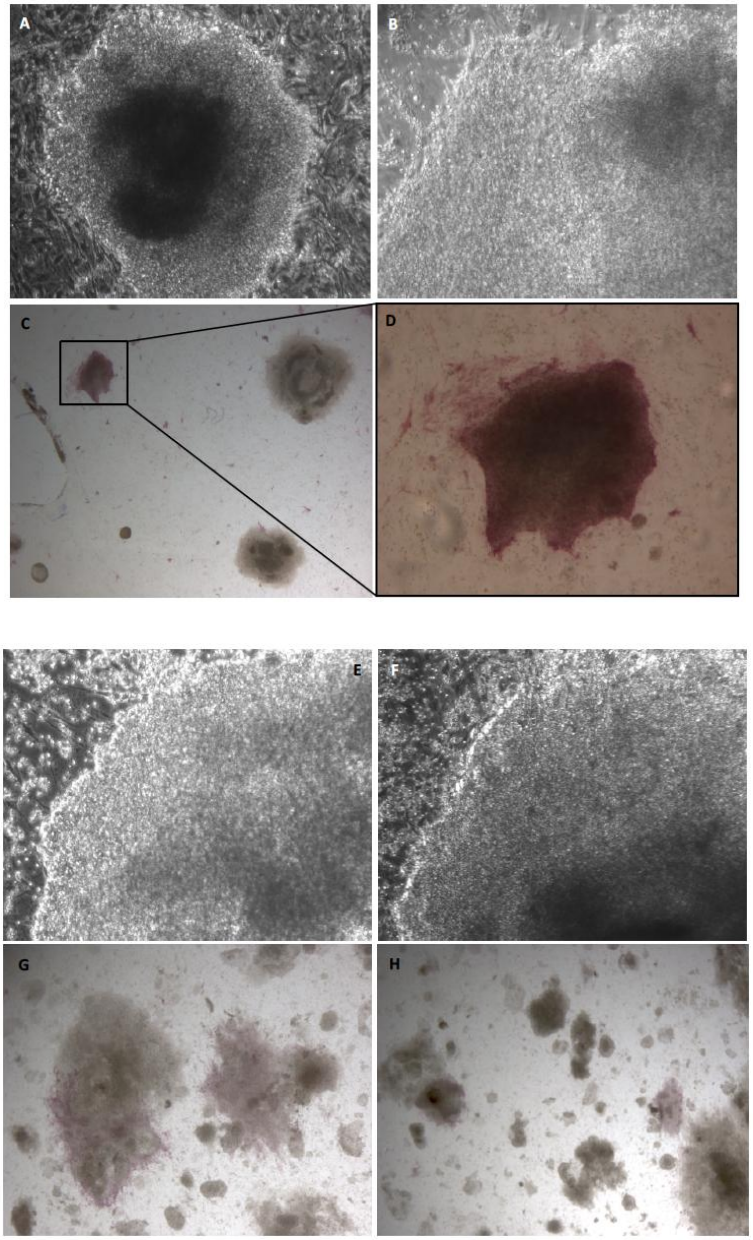


Fig. 6.3: Colonies were passaged between day 11 (A: 10X; B: 20X) and day 19 (E, F: 10X), picking only the homogeneous external part of the colonies and avoiding the thick center. In parallel expression of AP was evaluated to understand which kind of colonies were AP^{+}: strong positive colonies were found on day 11 (A: 1.6X; B: 5X) while no positive colonies were found on day 19 (G: 1.6X; H: 0.71X), so no colonies were picked after this day.

In order to expand subclones, 20 colonies were picked and one line passaged on day 12 showed homogeneous colonies with a very defined border, a high nuclei-cytoplasm ratio and a phase-bright flat aspect (Fig. 6.5), so it was selected for further culture and in-depth analysis (piPSCs#1). Also other two lines showed homogeneous colonies but with a less defined edge, whereas the other 17 colonies picked were too thick and they did not proliferate, until death (Fig. 6.4).

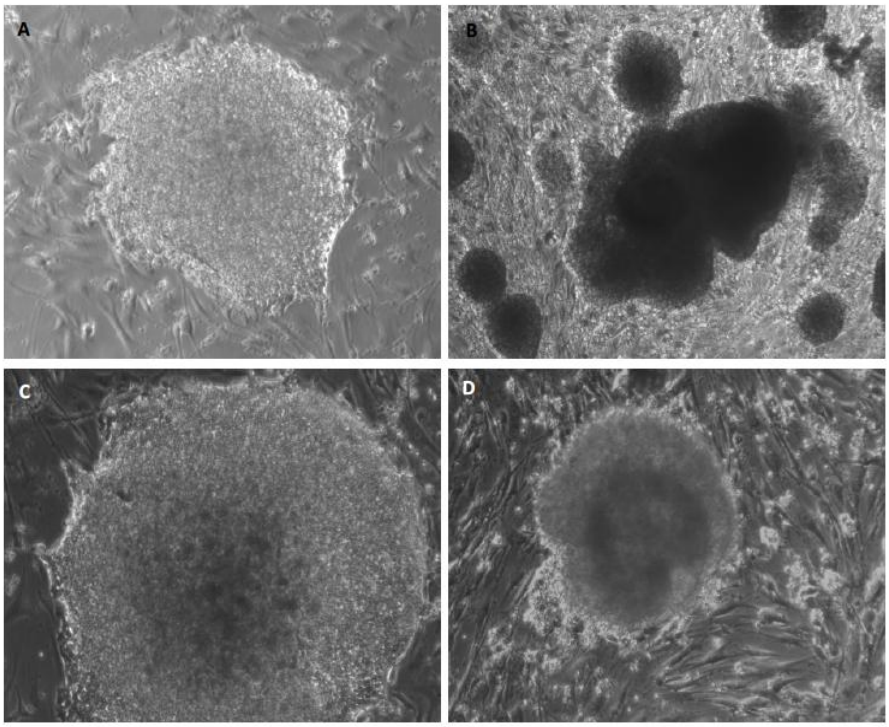


Fig. 6.4: Among 20 colonies picked to expand subclones, two lines showed colonies with a defined edge composed by cells with a high ratio nuclei/cytoplasm (A: 10X; C: 20X) while 17 subclones appeared very thick and they were not able to grow up (B, 10X; D; 20X).

Porcine iPSCs#1 were grown until passage P12 without any changes in colony morphology, proliferation rate and AP positivity (Fig. 6.5). After that, they were frozen for

further use.

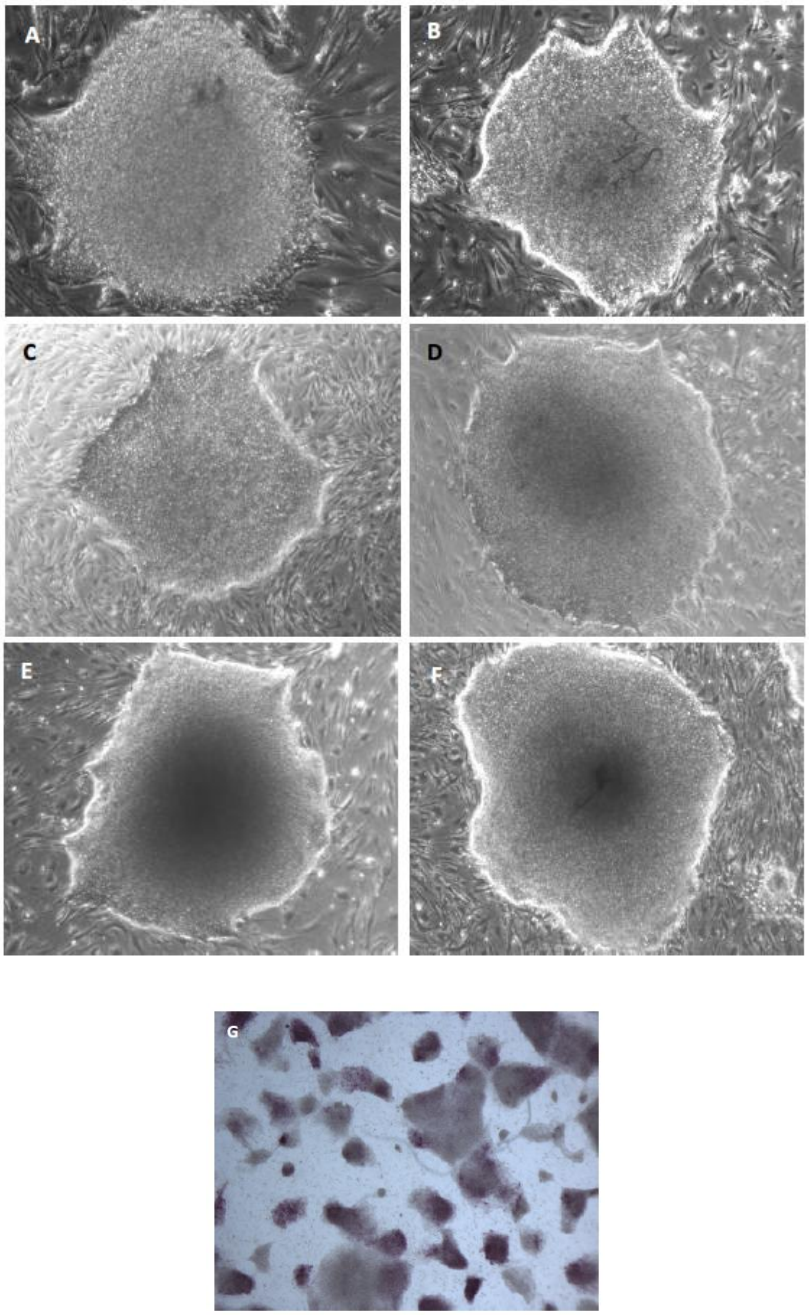


Fig. 6.5: Among 20 colonies picked to expand subclones, one line picked on day 12 (piPSCs#1) showed very homogeneous colonies with a well defined border, with a high nuclei-cytoplasm ratio and a phase-bright flat aspect. These features were kept through culture passages (A, B: P0; C: P1; D: P7; E: P8; F: P12). At P4 AP activity was evaluated and piPSCs#1 were strongly AP^+ (G). Magnifications: A, 10X; B-G: 5X.

At P5 doxycycline was withdrew to evaluate if piPSCs#1 were able to proliferate without the exogenous genes activated. It was possible to passage them for 2 more passages without drastic morphology changes but, after that, colonies started to be more flattened and they stopped proliferating (Fig. 6.6).

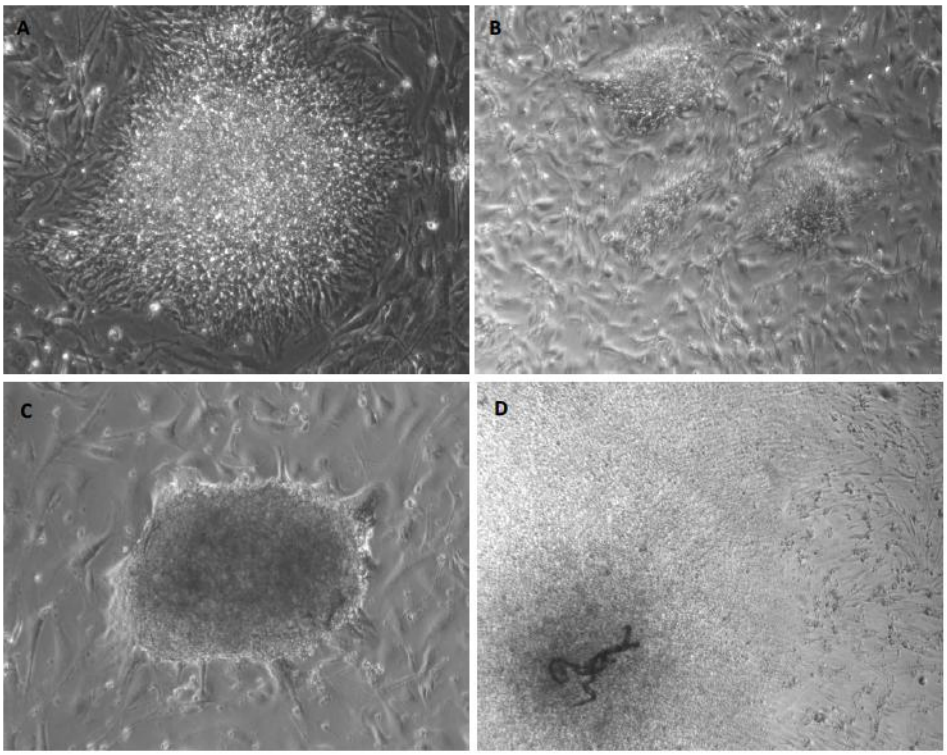


Fig. 6.6: When Dox was withdrew, after only 2-3 days it was possible to see many differentiated cells at the edge (A, 10X). It was tried to passage not differentiated cells and, although they seemed to survive after one (B, 5X) or two (C, 10X) passages, after the third one colonies started to be flatten, with a differentiated edge and cells were very slow in proliferation (D, 10X).

6.3.2 Embryoid Bodies assay

EBs of 250 cells each, after 48 h of hanging drop culture and 3 days in suspension culture, were plated on matrigel-coated plates where they started to spread as monolayer after 3-4 days (Fig. 6.7)

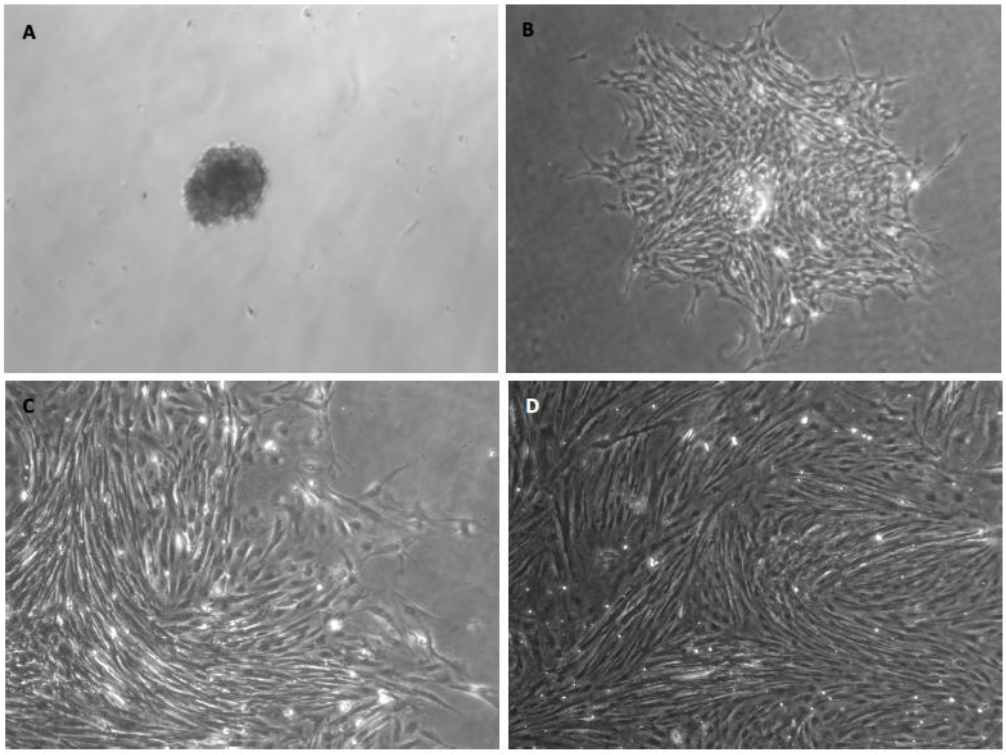


Fig. 6.7: 250 cells EBs appeared compact after 2 days in hanging drop culture (A, 5X) and, when plated on matrigel-coated dishes, after only 6 days cells were already spread as monolayer (B, 5X). Monolayer cells kept growing for 3 weeks changing medium every three days and on day 22 (D, 10X) they were harvested for gene-expression profile study. C: day 11, 10X.

After 22 days of culture as monolayer, expression of ectoderm (*NESTIN*, *THYROSINE HYDROXILASE*), mesoderm (*CARDIAC ACTIN*), and endoderm markers (*GATA4*) was revealed by RT-PCR (Fig. 6.8).

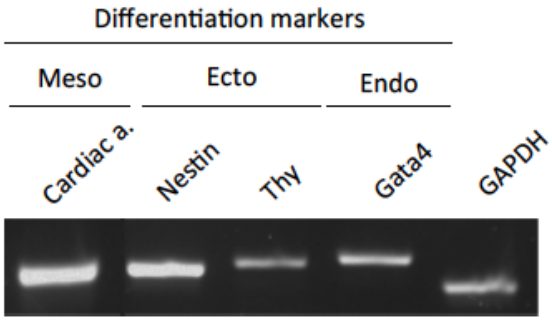


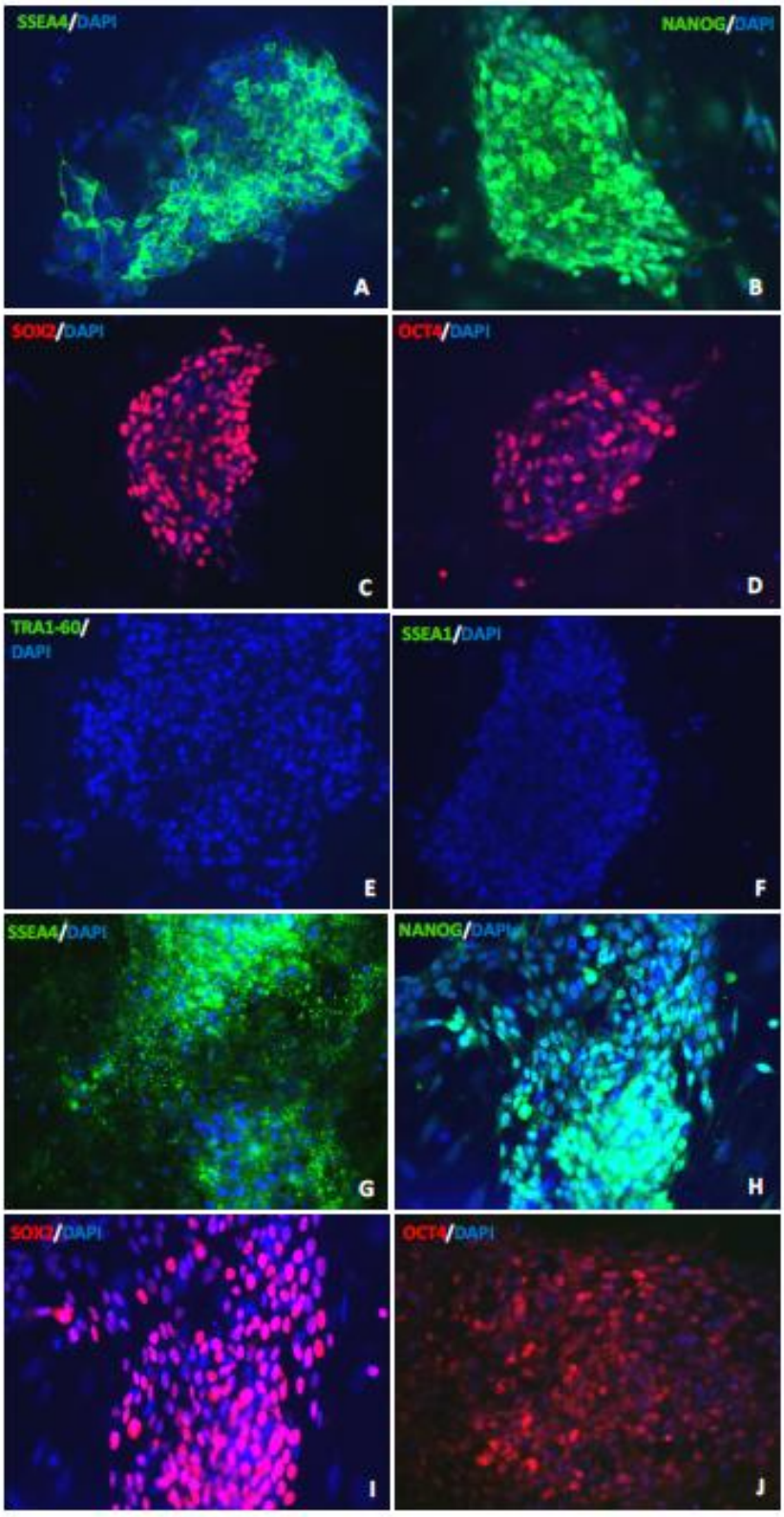
Fig. 6.8: On day 22 cells grew from EBs resulted positive for markers of all the three germ layers: mesoderm (*CARDIAC ACTIN*), ectoderm (*NESTIN* and *THYROSINE HYDROXILASE*) and endoderm (*GATA4*). *GAPDH* was used as internal control.

6.3.3 Immunocytochemistry

Immunocytochemistry was performed at P4 and P11 on piPSCs#1 for the pluripotent markers OCT4, NANOG and SOX2, and the surface markers, SSEA1, SSEA4 and TRA-1-60. OCT4 and SOX2 expression was detected (Fig. 6.9 C, D) but it was not possible to understand if they were proteins transduced by exogenous or endogenous genes. More important, cells were positive through passages to the surely endogenous markers NANOG and SSEA4 (Fig. 6.9 A, B). They did not express the cell-surface markers SSEA1 and TRA-1-60 at P4, so these markers were not studied further in culture.

Fig. 6.9: Immunofluorescence images of iPSCs#1 colonies stained for SSEA4, NANOG, SOX2, OCT4 at P4 (A-D) and P11 (G-J). SSEA1 and TRA1-60 expression was evaluated only at P4. All images represent the merge of DAPI staining (nuclei, blue) and staining for the indicated protein (FITC, green or TRITC, red). Magnification: 40X.

Fig. 6.9


SSEA4/DAPI
A
NANOG/DAPI
B
SOX2/DAPI
C
OCT4/DAPI
D
TRA1-60/
DAPI
E
SSEA1/DAPI
F
SSEA4/DAPI
G
NANOG/DAPI
H
SOX2/DAPI
I
OCT4/DAPI
J

6.3.5 Gene expression profile of piPSCs#1

RT-PCR analyses revealed that all the four exogenous factors were expressed in piPSCs#1 through passages, due to the presence of Dox in culture that induced their expression. Gene expression profile was studied using porcine specific primers both at P2 and at P10, revealing that piPSCs#1 expressed endogenous *OCT4*, *SOX2*, *NANOG*, *KLF4* and *FGF5* while *STELLA* was weakly expressed (Fig. 6.10).

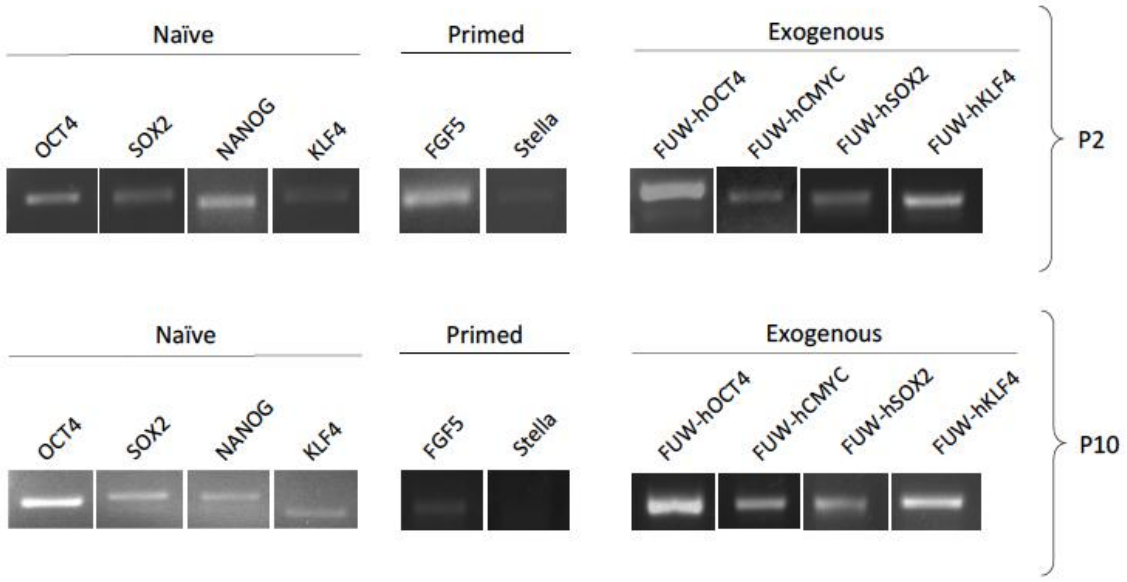


Fig. 6.10: Porcine iPSCs#1 expressed naïve pluripotency endogenous markers through passages. Also *FGF5* and a weak *STELLA* expression (primed pluripotency markers) was kept from P2 to P10. Exogenous factor were strongly expressed at both passages because of the maintenance of Dox in culture. *GAPDH* was used as internal control (not shown).

6.4 Discussion

In this study a doxycycline-regulated and bFGF-dependent porcine iPSCs line was generated with genomic human OSKM integration in porcine embryonic fibroblasts. This line was stable in culture beyond passage 10, cells were AP positive and

immunocytochemistry analysis showed that they expressed other markers similar to those expressed in ESCs, such as SSEA4, NANOG, OCT4, and SOX2. While we cannot be sure if OCT4 or SOX2 represented the protein synthesized from endogenous or exogenous genes, the strong expression of SSEA4 and NANOG was endogenous for sure. Differently form Park et al. (2013), we were not able to find TRA1-60 positive cells. RT-PCR showed expression of all 4 exogenous factors (*hOCT4*, *hSOX2*, *hKLF4* and *hCMYC*) as well as the expression of their endogenous counterparts (pig *OCT4*, pig *SOX2* and pig *KLF4*), in contrast with Hall et al. (2012). Cells showed expression not only of naïve pluripotency markers, but also primed ones, showing how piPSCs cultured with bFGF displayed features of primed and naïve pluripotency, as found for porcine iPSCs LIF-dependent (Rodriguez et al. 2012). Cells were able to form EBs and differentiate *in vitro* into all the three germ layers. This differentiation was evaluated only by studying the gene-expression profile, so the effective protein expression needs to be further studied. Our piPSCs#1 line could not be sustained in the absence of doxycycline, as reported by Hall and colleagues (2012), underlining the inability of cells to proliferate in the absence of transgene expression.

6.5 Conclusions

Since our main aim was the differentiation of this line into the musculo-skeletal lineage (Chapter 7), we used Dox-dependence feature to induce differentiation by the simple withdrawal of Dox in culture, but appropriate culture conditions to sustain piPSCs only on the basis of their endogenous pluripotency machinery needs to be studied. It could be worth trying to remove Dox from the very beginning, in order to select not-dependent colonies. Moreover, considering the strong positivity of our piPSCs#1 line for SSEA4 marker, it

could be considered a live SSEA4 staining to select these cells and evaluate if this surface antigen protein may be a good markers for selection in the pig, as done for human iPSCs by using other selection markers (Chan et al. 2009).

7. Myogenic differentiation by a 4-hydroxytamoxifen-inducible lentiviral vector encoding human *PAX7*

7.1 Introduction

After the generation of a stable porcine iPSCs line, the project was carried out by studying piPSCs differentiation into a specific lineage, in detail the musculo-skeletal differentiation. The myogenic differentiation in a pig model is of interest both for agricultural and for clinical purposes. First, we are committed to a future in which a greater percentage of the world's population will have easy access to food. For this reason, the creation of a porcine line of myoblastic precursors meets agronomic interest aimed to the improvement of the muscle mass in the pig. Second, therapeutic strategies that focus on the replacement of diseased muscle tissues with stem cells that can give rise to healthy myofibers are very attractive in human medicine (Darabi et al. 2012). In fact, considering the features of the pig as a good model for human medicine, the generation of a line of myoblastic precursors starting from piPSCs could be of interest for the possibility of generating patient-specific iPSCs for autologous therapies and a critical prerequisite for a potential therapeutic application is the generation of abundant engraftable tissue-specific cell preparations (Darabi et al. 2012).

The mature skeletal muscle fiber is a complex multinucleated cell that is specialized for contraction (Sinowatz 2010). In mature skeletal muscle, most satellite cells are quiescent and are activated in response to injury, so their activation mediates muscle regeneration (Meregalli et al. 2011). After division, satellite cell progeny, termed myoblasts, undergoes terminal differentiation and becomes incorporated into muscle fibers (Bischoff and Heintz 1994). Myogenesis is orchestrated by key transcription factors that regulate the progression

from quiescence, activation and proliferation of satellite cells to their self-renewal or differentiation (Yin et al. 2013). Quiescent satellite cells, as well as proliferating myoblasts, are characterized by the expression of the paired box transcription factor *PAX7*. Adult myoblasts express Myogenic factor 5 (*MYF5*) and Myogenic determination factor (*MYOD*) and they begin differentiation by downregulating *PAX7* (Yin et al. 2013) (Fig. 7.1).

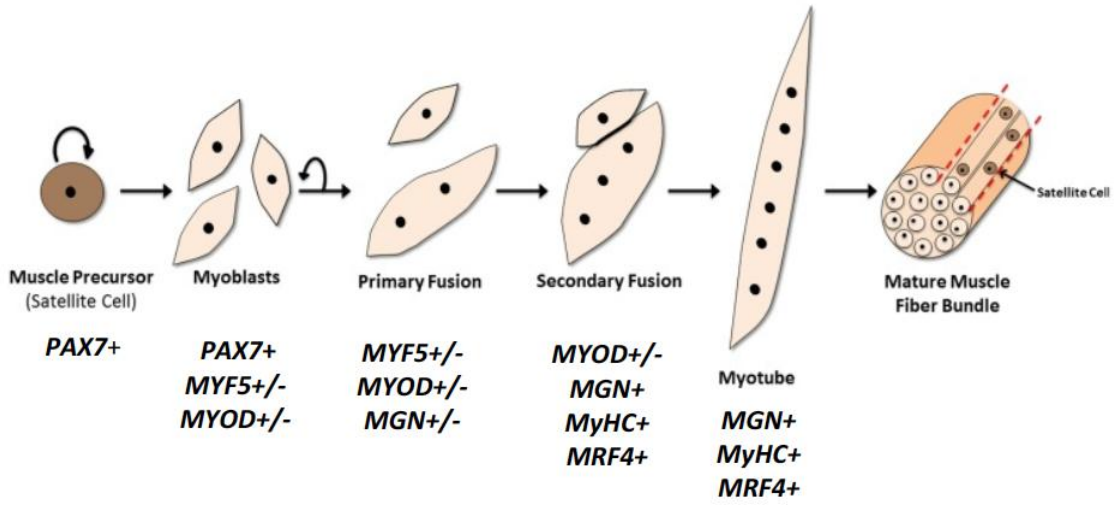


Fig 7.1: Molecular control of myogenesis (modified from Enwere et al. 2014).

Satellite stem cells would enter different developmental programs depending on whether MYF5 or MYOD expression predominates. Predominance of MYOD would drive the program toward early differentiation, analogous to the behavior of $MYF5^{-}$ myoblasts, whereas predominance of MYF5 would drive the program toward enhanced proliferation and delayed differentiation (Rudnicki et al. 2008). Myoblasts coexpressing MYF5 and MYOD would exhibit an intermediate growth and differentiation program (Rudnicki et al. 2008) (Fig. 7.2).

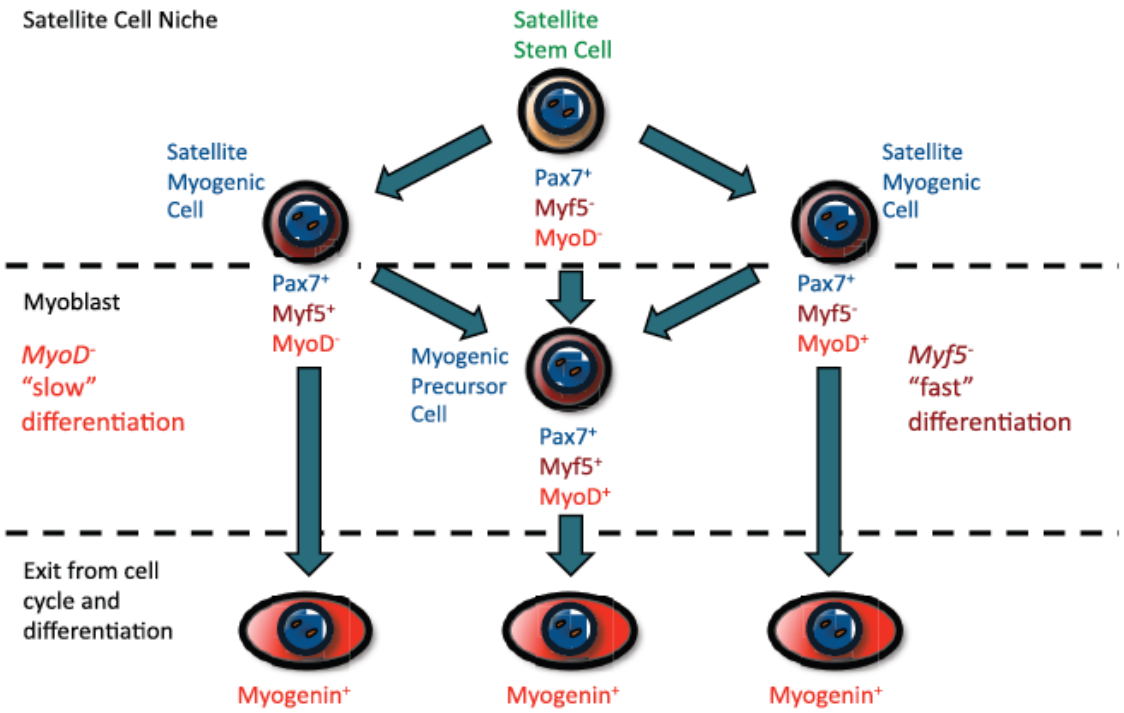


Fig 7.2: Depending on whether MYF5 or MYOD expression predominates, satellite stem cells enter different developmental programs. $MYF5^{+}/MYOD^{-}$ myoblasts are driven to a "slow" differentiation program while $MYF5^{-}/MYOD^{+}$ myoblasts enter a "fast" differentiation. $MYF5^{+}/MYOD^{+}$ myoblasts show an intermediate differentiation (from Rudnicki et al. 2008).

The initiation of terminal differentiation and fusion begins with the expression of Myogenin (MGN), that in concert with MYOD will activate muscle specific structural and contractil genes (Yin et al. 2013) (Fig. 7.1). Finally, differentiated mononuclear myocytes positive for MGN and Myosin Heavy Chain (MyHC) fuse together to form multinucleated myotubes or nascent myofibers (Fig. 7.1) (Kuang and Rudnicki 2008).

The aim of this study was to derive a proliferating population of porcine skeletal myogenic progenitors from porcine iPSCs (piPSCs#1 line) previously generated (Chapter 6) and to verify their ability to fuse into myotubes, characteristic units of mature skeletal muscle.

7.2 Materials and Methods

7.2.1 Lentivirus preparation

Fresh lentiviral soup was generated by transfecting subconfluent HEK293T cells with a pSIN vector encoding the human *PAX7* (h*PAX7*). The vector was designed with a HA-tag downstream of the h*PAX7* gene and an estrogenic receptor (ER) domain upstream of h*PAX7*. The vector contained also a puromycin resistance marker to select transduced cells. Viral supernatant was collected 48h after transfection and it was filtered through a 0.45 mm filter and supplemented with 4 mg/mL polybrene (Sigma). It was then used to infect porcine iPSCs (piPSCs#1) previously generated (Chapter 6).

7.*2.2 Generation and culture of porcine inducible* PAX7-*iPSCs line (*PAX7-*piPSCs)*

Porcine iPSCs#1 at P6 of culture were infected for 6h plus an overnight with a dilution 1:2 of the virus. In order to verify the transduction, cells were tested with a 24h induction of 4-hydroxytamoxifen (4OH-Tam, Sigma), that is a selective *ER* modulator (Miyazaki et al. 2012), and expression of HA protein was evaluated by immunocytochemistry (Fig. 7.2.6). Cells were cultured in ESC medium (Paragraph 6.2.1) on mitomycin C-inactivated MEFs. Selection of h*PAX7* transgenic iPSCs (*PAX7*-piPSCs) was conducted 2 passages after transduction by adding puromycin 0.5 μg/ml for five days, in order to kill most untransduced cells. The optimal concentration of puromycin was evaluated by testing different concentrations of the antibiotic on piPSCs#1 (data not shown). Porcine iPSCs#1 transduction is summarized in Fig. 7.3.

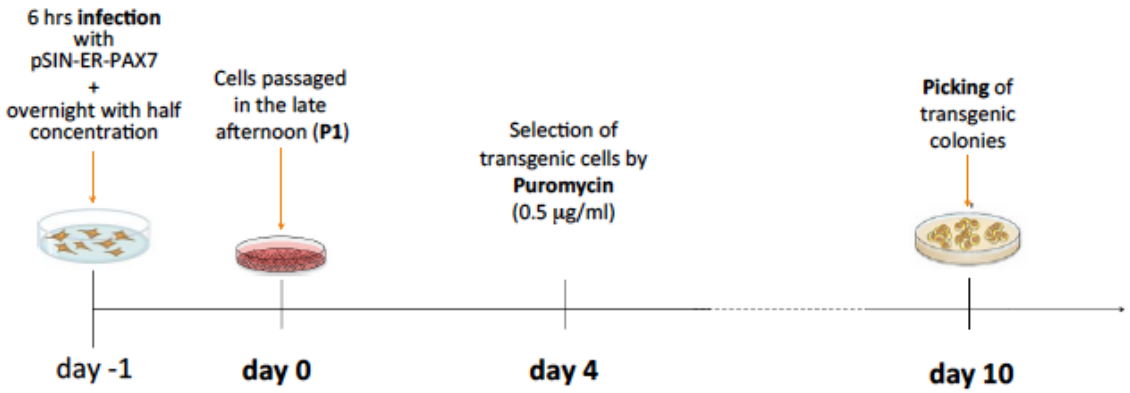


Fig. 7.3: Timeline of lentivirus preparation and transduction of *PAX7*-piPSCs.

7.2.3 Differentiation by Embryoid Bodies (EBs) formation

The generated line *PAX7*-piPSCs was firstly differentiated as EBs. Five passages after selection, puromycin-selected cells were collected by StemPro® Accutase® (GIBCO), counted at haemocytometer and cultured for 48h in 30 µl hanging drops (250 cells each drop) of ESC medium. After that, EBs were transferred in 3.5 cm low attachment Petri dishes and cultured in suspension in myogenic induction medium (Darabi et al. 2012). It was composed by IMDM (GIBCO), 15% fetal bovine serum (GIBCO), 10% horse serum (Sigma), 1 % chick embryo extract (US Biological), 50 µg/ml ascorbic acid (Sigma), 4.5 mM monothioglycerol (Sigma) and 10 µM ROCK inhibitor (Tocris - for only 24 h). After 5 days, EBs were plated in matrigel-covered dishes and cultured 3 additional days in myogenic induction medium. Then medium was switched to a terminal differentiation medium composed by DMEM + 2% horse serum (HS medium). During culture in myogenic medium, 2 µM 4OH-Tam was added every 2 days in order to induce h*PAX7*. At the same time, one group was cultured without 4OH-Tam as negative control. A summary of the protocol is reported in Fig. 7.4.

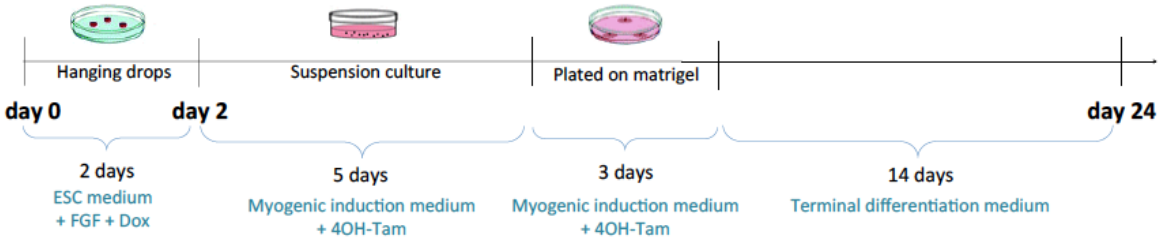


Fig. 7.4: Timeline of EBs protocol aimed to the differentiation into the myogenic lineage of *PAX7*-piPSCs.

7.2.4 Direct differentiation into myotubes

The *PAX7*-piPSCs line was secondly differentiated following a protocol of direct differentiation of colonies cultured on MEFs, without EBs stage. Since myogenic induction medium did not work well with EBs and cells seemed to die (Paragraph 7.3.2), we cultured cells in DMEM + 10% FBS. *PAX7*-piPSCs colonies were firstly left 2 days after the picking in ESC medium to let them grow up. Medium was then switched to DMEM + 10% FBS supplemented with 2 μM 4OH-Tam and changed every 2 days. After 6 days, medium was switched to a terminal differentiation medium composed by DMEM + 2% horse serum (HS medium) and it was changed every three days for 2 weeks. Cells were characterized by immunocytochemistry after 6 days of differentiation (before the switching to HS medium), after 1 week in HS medium (13 days of differentiation) and at the end of the terminal differentiation (21 days). In this last step the gene expression profile was also studied. Differentiation protocol and characterization steps are summarized in Fig. 7.5.

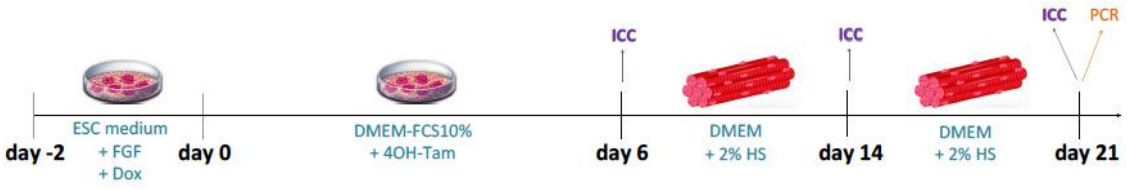


Fig. 7.5: Direct differentiation protocol of *PAX7*-piPSCs cultured on MEFs.

7.2.5 Generation and culture of porcine myoblastic cells line

In order to generate a line of myogenic precursors, *PAX7*-piPSCs were plated into matrigel-coated 3.5 cm Petri dishes, expanded in DMEM + 10% FBS containing 10 ng/mL bFGF and 2 μM 4OH-Tam (myoblasts medium), and passaged by trypsinization (trypsin 0.05%) every 3 days. It was avoided the reaching of confluency to prevent cells fusion, so they were passaged at 60-70% of confluency. Cells were plated in 12-wells (60000 cells each well) and counted by haemocytometer every passage. Medium was changed every day after until P6 and 10 ng/ml bFGF was added every day.

Cell-doublings (CD) and cell-doubling time (DT) were calculated through the following formulae (Rainaldi et al. 1991):

1) $CD = \ln(N_f / N_i)/\ln(2)$

where N_f is the final number of cells and N_i the initial number of cells;

2) $DT = CT/CD$

where CT is the cell culture time.

We further wanted to evaluate if these cells were able to fuse into myotubes. Since we observed differentiation into myotubes of *PAX7*-piPSCs when differentiated on MEFs (Paragraph 7.3.2), we plated myoblasts at high density (around 400000 cells in a well of a 12-well) on MEFs in DMEM + 10% FBS supplemented with 10 ng/ml bFGF and 2 μM 4OH-Tam. The day after, medium was switch to HS medium

7.2.6 Immunocytochemistry

Cells were rinsed with PBS and fixed with 2,5% PFA for 20 min at RT. Subsequently, cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with PBS + 5% BSA for 1h at RT. Cells were incubated overnight at 4°C with the following primary antibodies: PAX7 (1:50; Hybridoma bank), Myogenin (1:100, Abcam), Myosin Heavy Chain (1:100, Abcam), HA (1:200, Millipore). After three washes in PBS + 5% BSA, cells were incubated with the appropriate secondary antibody for 1h at RT and then rinsed 2 times in PBS + 5% BSA. Cells were mounted in Vectashield with DAPI (49-6-diamidino-2-phenylindole; Vector Laboratories).

7.2.7 RT-PCR

RNA was isolated using Nucleospin® RNA kit (MACHEREY-NAGEL) and cDNA was synthesized using Omniscript synthesis kit (Qiagen), following the manufacturers' instructions. Then, PCR was performed with ReadyMix (Sigma-Aldrich) and 0.4 μM of each primer (primers used are listed in Table 7.1) following a thermal profile including an initial step of 95°C (5 min), 40 cycles of 30 sec at 95°C, 30 sec at 60°C, and an extension step of 30 sec at 72°C. PCR products were run in 1.5% agarose gel to determine the amplicon size. For what concern myotubes, in order to select only them and not the some leftover pieces of the iPSCs colonies, myotubes were harvested by a cell-scraper.

Table 7.1. Sequences of primers used for RT-PCR analysis.

Gene	Primer sequences FW and RV	Amplicon (bp)	Accession number or (Reference)
p*GAPDH*	FW 5'- GGGCATGAACCATGAGAAGT -3' RV 5'- GTCTTCTGGGTGGCAGTGAT -3'	162	(Rodriguez et al. 2012)
h*PAX7*	FW 5'- CCACTGCGGGCTCTACTTC -3' RV 5'- GACCCCTCCCAGCTGATTGAC -3'	227	Designed by Dr. Li
MYOD	FW 5'- GATAGAGCAGGGTGGTGGAC -3' RV 5'- TGCAAAGTTGCAGAGAGAGC -3'	222	U12574.1
PAX7	FW 5'- GGTGGGGTTTTCATCAATGG -3' RV 5'- GTCTCTTGGTAGCGGCAGAG -3'	155	(Wilschut et al. 2008)

7.3 Results

7.3.1 Generation of PAX7*-piPSCs*

The piPSCs#1 line generated by a 4OH-Tam inducible lentiviral vector encoding h*PAX7* and selected by puromycin showed good colonies with a very defined edge and a thick center (Fig. 7.6). Except for the middle thick part, genetic modification did not alter morphology of piPSCs. When manually passaged, it was picked only the external part avoiding to passage cells from the center.

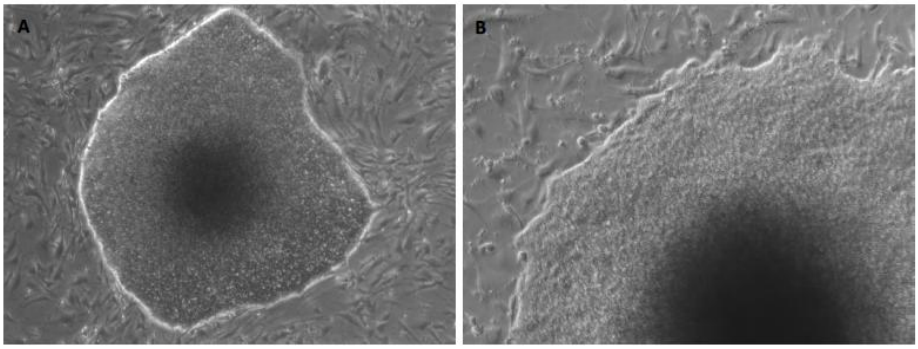


Fig. 7.6: *PAX7*-piPSCs colonies at the fifth passage after selection by puromycin. Colonies did not modify their morphology after transduction. Magnifications: A, 10X; B, 20X.

Immunofluorence analysis showed expression of the HA-tag upon 24h of 4OH-Tam induction while in the not-induced negative control there were only a few positive cells, probably due to a leaky expression (Fig. 7.7).

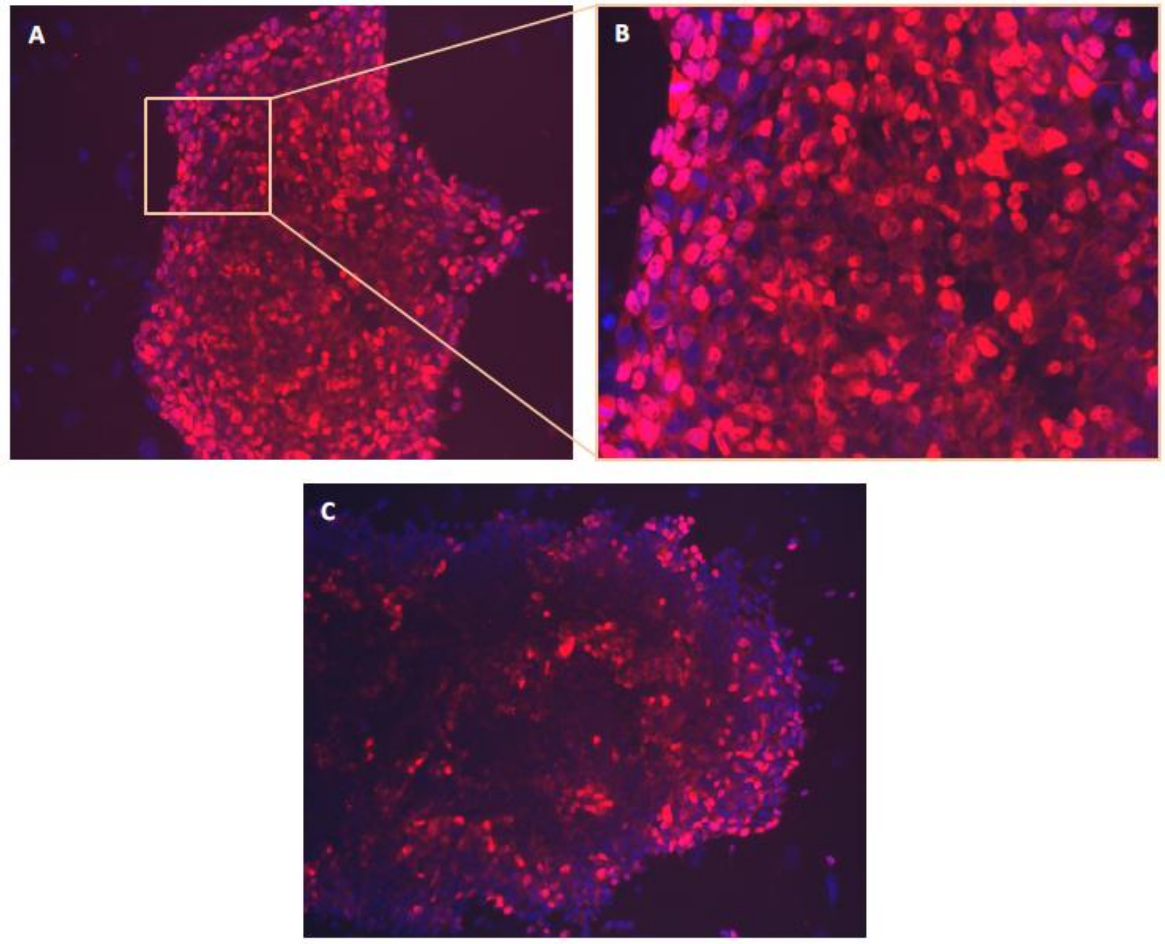


Fig. 7.7: HA was strongly expressed after 24h 4OH-Tam treatment in *PAX7*-piPSCs colonies (A, 20X; B, 40X) and expressed by only a few cells in the not-treated sample (C, 20X).

7.3.2 PAX7*-piPSCs were not able to differentiate as EBs*

When *PAX7*-piPSCs were plated with the hanging drop method, after 24h they were able to form EBs, so genetic modification did not alter this ability. After one week of suspension culture in myogenic induction medium, cells were plated on matrigel-coated dishes where

they started to grow up as monolayer. Unfortunately after 2-3 days cells started to die, underling how either the medium or the EBs protocol were not the suitable way to induce the myogenic program in the modified piPSCs line. For this reason, we proceeded by inducing *PAX7*-piPSCs colonies directly, only culturing them under differentiation medium.

7.3.3 Differentiation of PAX7-*iPS into myotubes*

When plated on feeders under myogenic medium, transgenic iPSCs differentiated directly into myotubes. It was possible to start observing a morphology clearly ascribable to myotubes after only 3 days of culture (Fig. 7.8). When the medium was switched to the HS medium, after 2-3 days myotubes started making spontaneous contractions.

Fig. 7.8

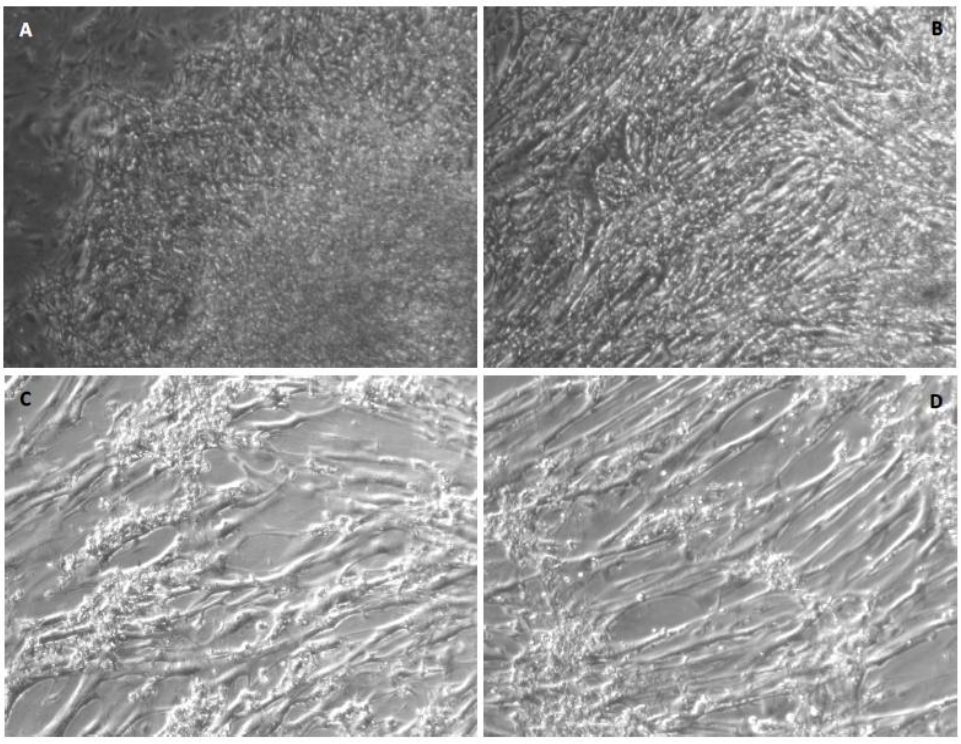


Fig. 7.8: During direct differentiation, after only 3 days in DMEM-10% FBS supplemented with 4OH-Tam it was possible to notice cells at the edge of the colonies that started to assume a morphology ascribable to myotubes (A, 5X). Number of myotubes increased during 4OH-Tam induction and after 6 days of induction (B, 5X) the culture was switched to HS medium. During these culture conditions, myotubes started making contractions in the plate (C: day 12, 10X; D: day 19, 10X).

Characterization by immunocytochemistry was performed for the late myogenic markers MGN and MyHC at different steps. At day 6, before the switch to HS medium, it was clear how MGN^{+} cells from h*PAX7*-iPSCs colonies were going to organize themselves to undergo fusion and form myotubes (Fig. 7.9). The already formed myotubes were positive both for MGN (Fig. 7.9) and MyHC (Fig. 7.10).

Fig. 7.9

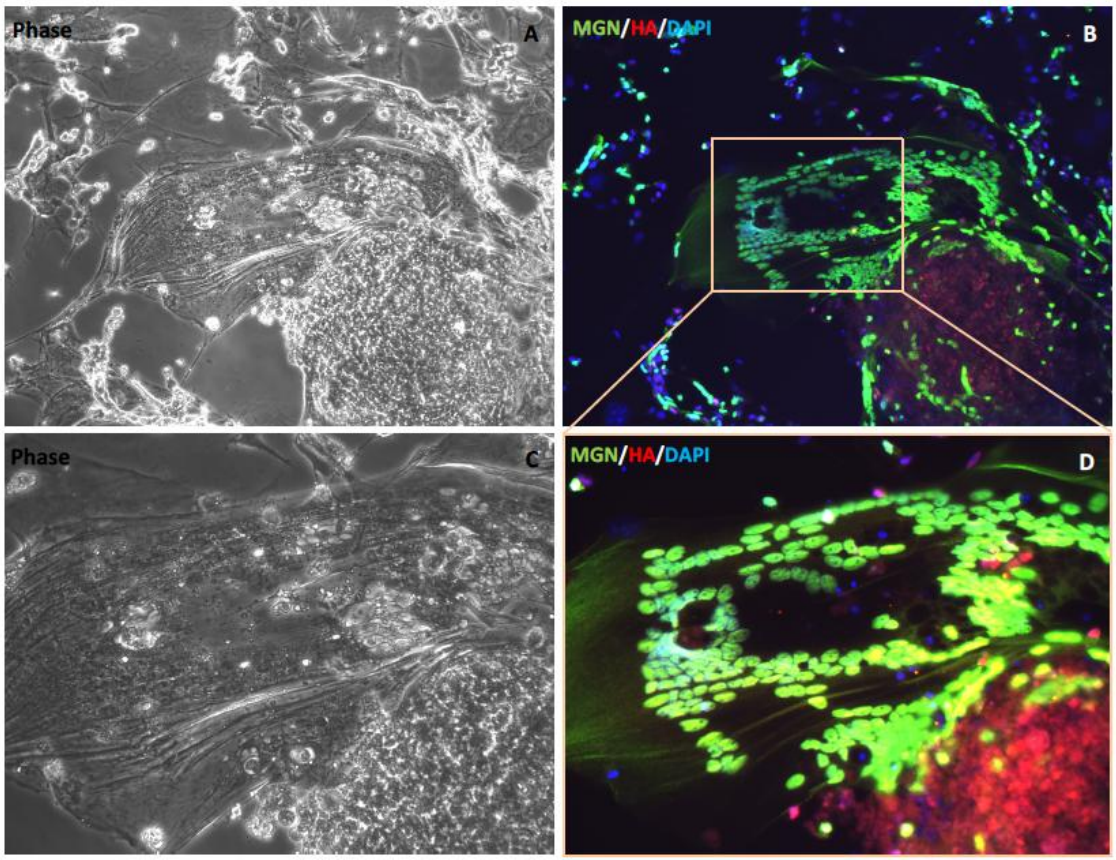


Fig. 7.9: After 6 days of 4OH-Tam induction, it was possible to see already formed myotubes that resulted positive for MGN (B: 20X; D, 40X). Looking at the nuclei, it was clear how cells from the colonies were organizing themselves to alineate and fuse to form myotubes. Cells were double-stained with MGN (FITC, green) and HA (TRITC, red) and it was possible to observe a pool of 4OH-Tam-induced HA^+ cells in the center of the colonies ready to go to form myotubes. A and C (magnification: 40X) represents the phase contrast images of the same fields of B and D pictures, respectively.

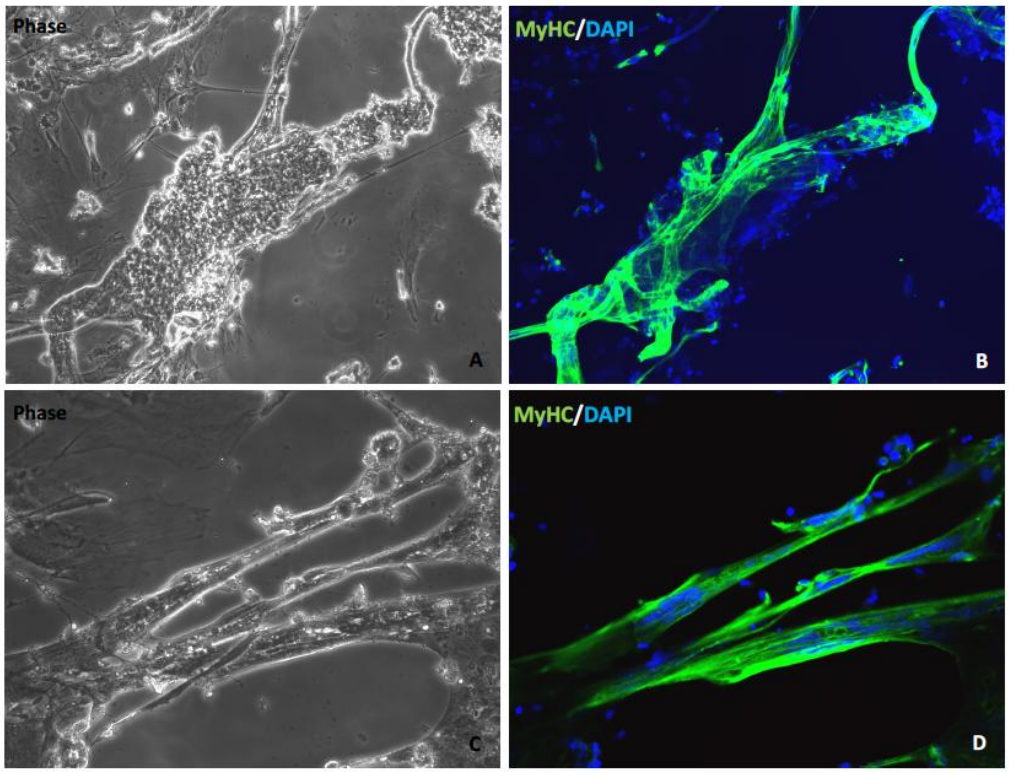


Fig. 7.10: After 6 days of 4OH-Tam induction, cells stained positive also for the late differentiation marker MyHC (B, D: 40X). A and C (magnification: 40X) represents the phase contrast images of the same fields of B and D pictures, respectively.

Myotubes maintained a profile $MGN^+/MyHC^+$ also during the culture under HS medium and contracting myotubes showed a strong expression of MyHC with the classic banding both after 7 days in HS medium (Fig. 7.11) and after 13 days (Fig. 7.13). Moreover, MGN+

nuclei were clearly organizing themselves to go aligned in order to let cells fusion and myotubes formation (Fig. 7.12).

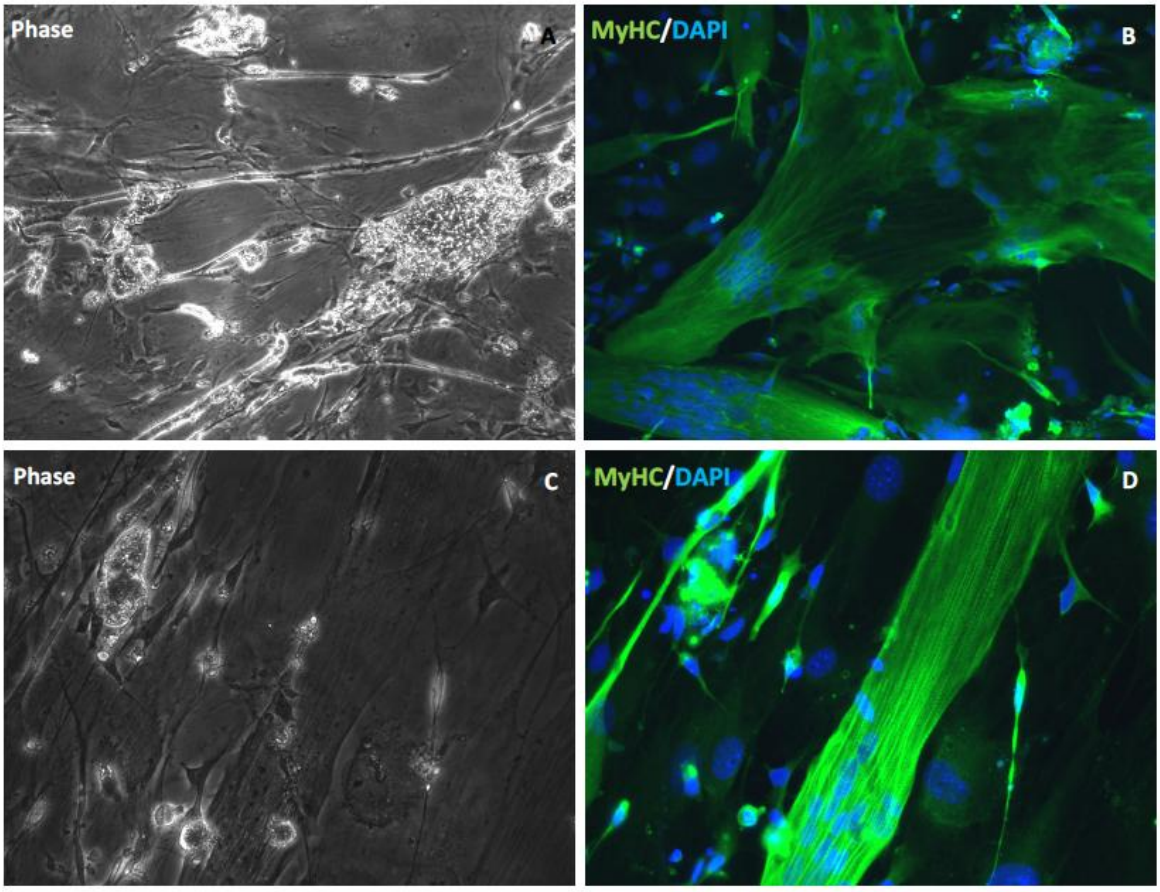


Fig. 7.11: When cultured in HS medium, after 7 days (13 total days of differentiation culture) MyHC was clearly expressed by myotubes (C, D: 40X). A and C (magnification: 40X) represents the phase contrast images of the same fields of B and D pictures, respectively.

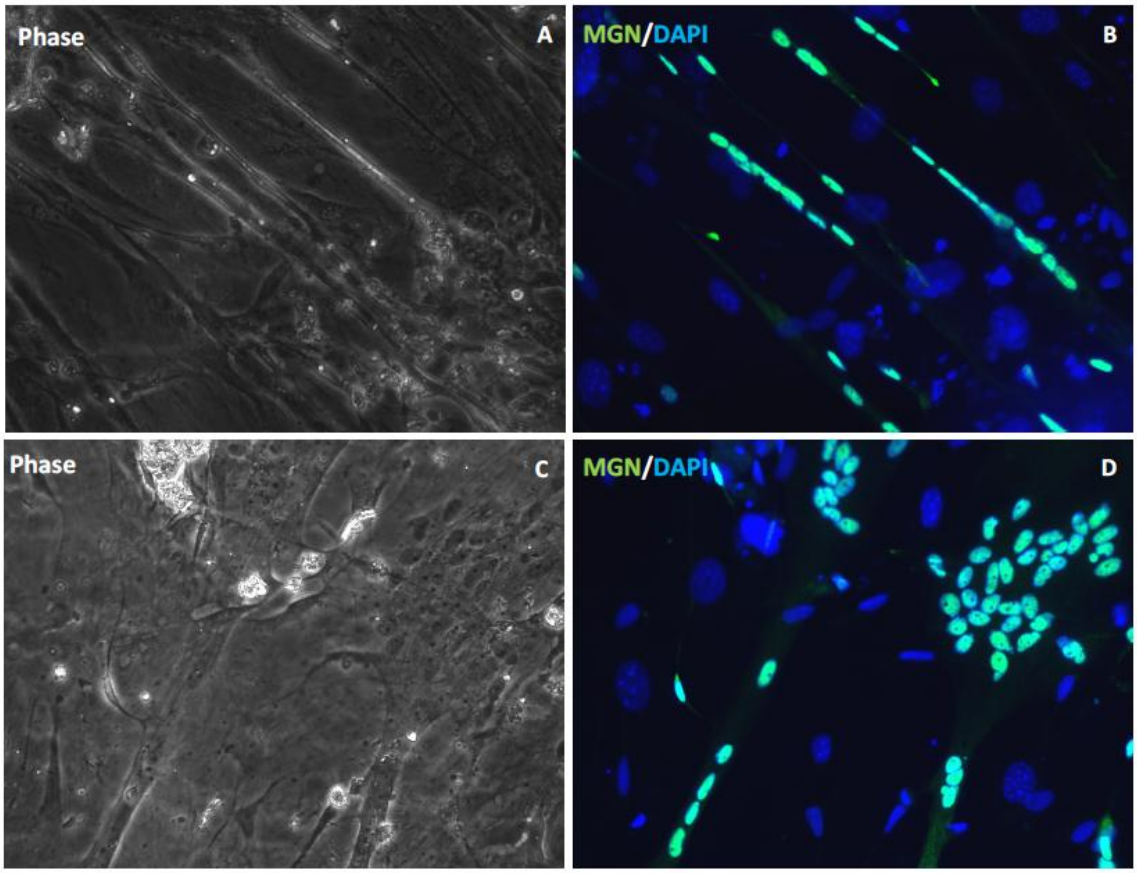


Fig. 7.12: During culture under HS medium (7h day) MGN+ nuclei showed how cells were going to fuse together to form multinucleated myotubes (B, D: 40X). A and C (magnification: 40X) represent the phase contrast images of the same fields of B and D pictures, respectively.

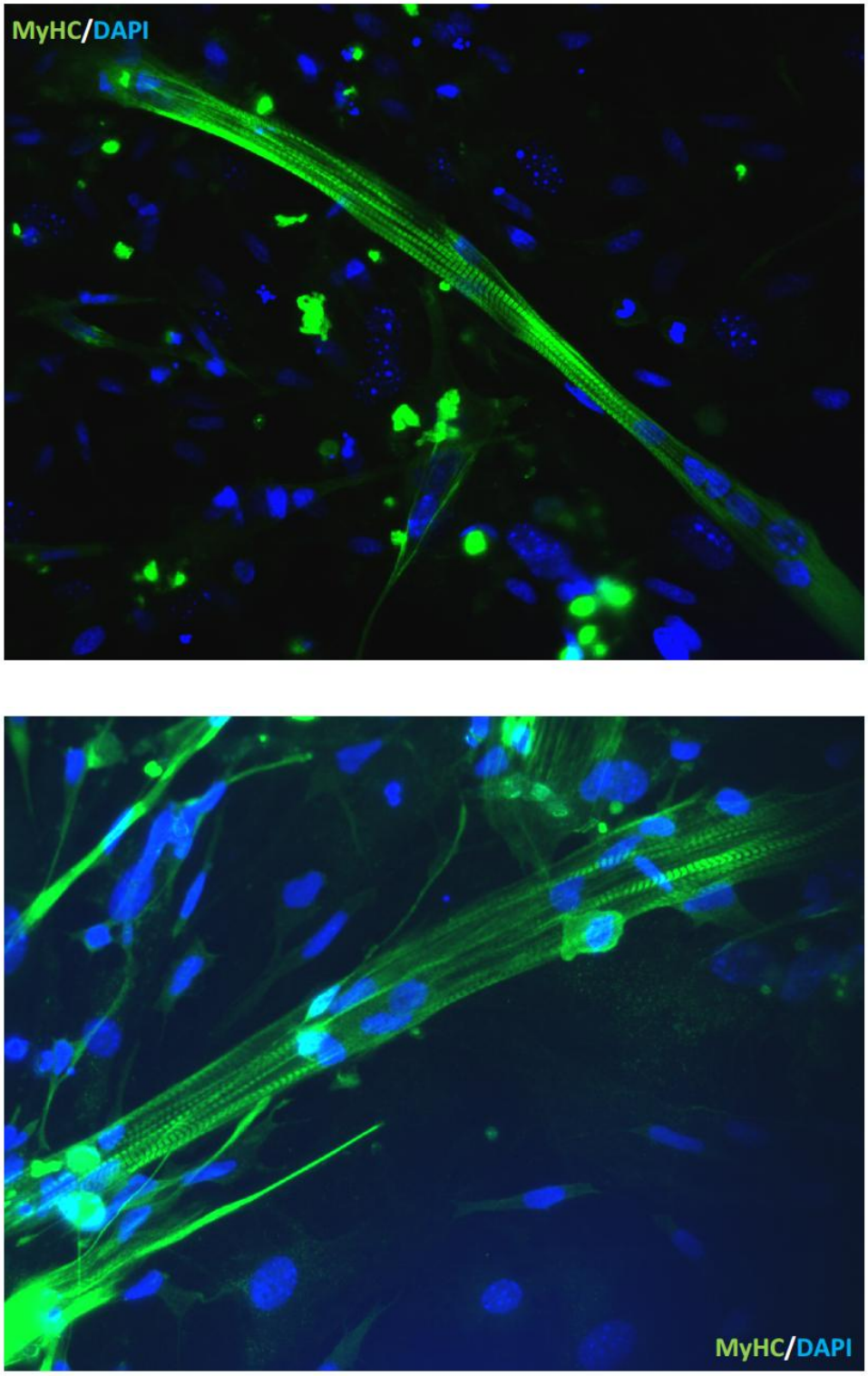


Fig. 7.13: Contracting myotubes on day 22 of differentiation showed a strong expression of the late marker Myosin Heavy Chain with the classic banding. Magnifications: 40X.

7.3.4 Direct differentiation into myoblasts

PAX7-piPSCs were plated into matrigel-coated wells, expanded in ESC medium and then induced under myoblasts medium. Four days after induction it was possible to observe at the edge of the colonies cells with a morphology ascribable to myoblasts, kept through passages (Fig. 7.14).

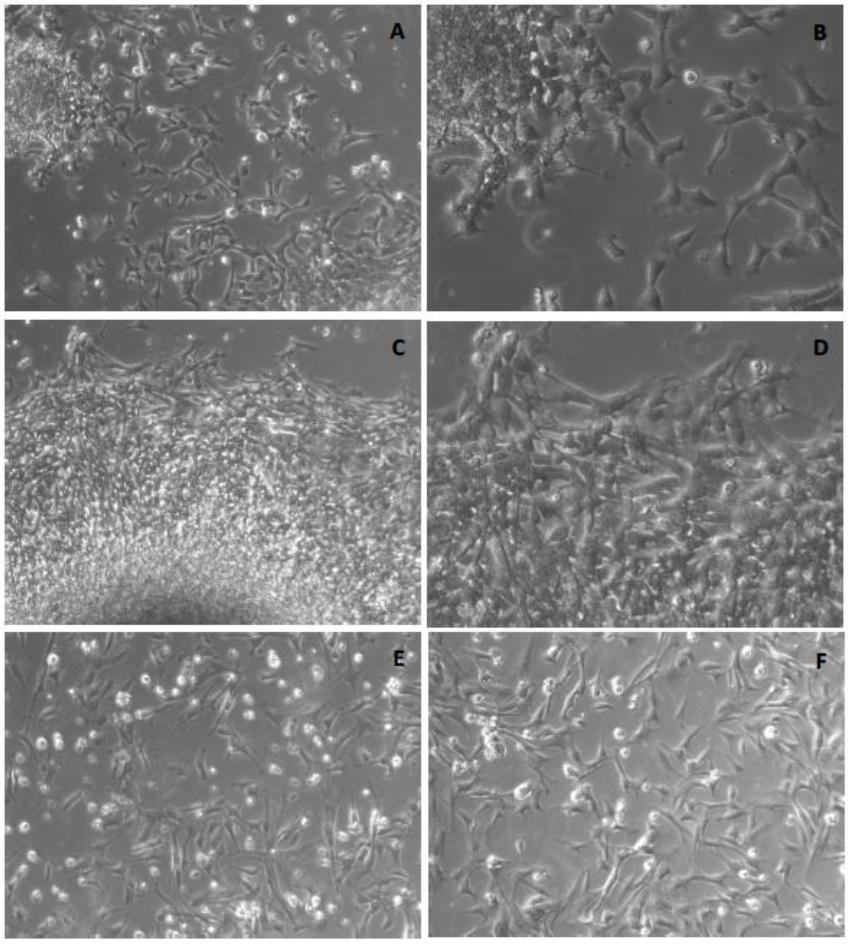


Fig. 7.14: Differentiation of PAX7-piPSCs into myoblasts. Colonies were plated on matrigel-coated dishes (P0) and induced in myoblasts medium. Four days after 4OH-Tam induction, it was possible to observe a morphology ascribable to myoblasts of the cells at the border of the colonies (A, C: 10X; B, D: 20X). The line was purified by passaging and morphology was kept through passages (E: P2, 10X; F: P5, 10X).

PAX7-piPSCs showed a good expansion potential, averaging a total of 6.4 doublings during 2 weeks of expansion, as found by Darabi and colleagues (2012) for PAX7-human iPSCs. Under these proliferation conditions, cells from P1 to P5 decreased the expression of Myogenin, a marker of terminal muscle differentiation, and increased the expression of the earlier marker PAX7, as demonstrated by immunocytochemical analysis (Fig. 7.15).

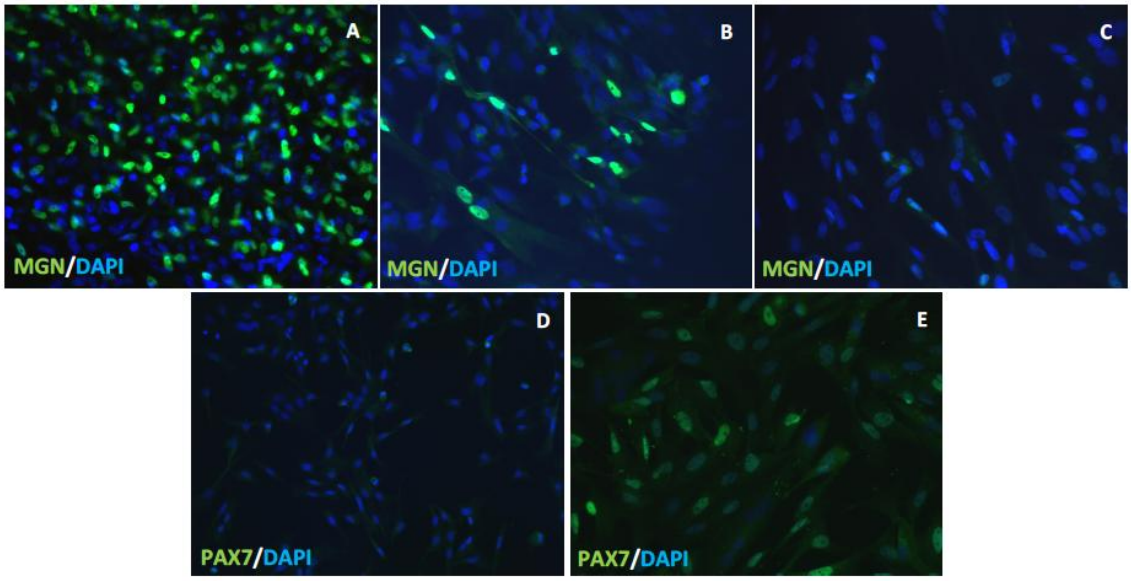


Fig. 7.15: Immunophenotype of proliferating myoblasts through passages. The intermediate marker MGN was highly expressed a few days after induction (A: P0, day 5 after 4OH-Tam induction, 20X), and decreased its expression going forward with passaging (B: P2, 40X; C: P5, 40X). On the other hand, PAX7 expression was not detected at P2 (D, 20X) but there was expression at P5 (E, 40X).

When myoblasts were further plated at high density on MEFs, after 4-5 days they underwent fusion forming multinucleated myotubes (Fig. 7.16).

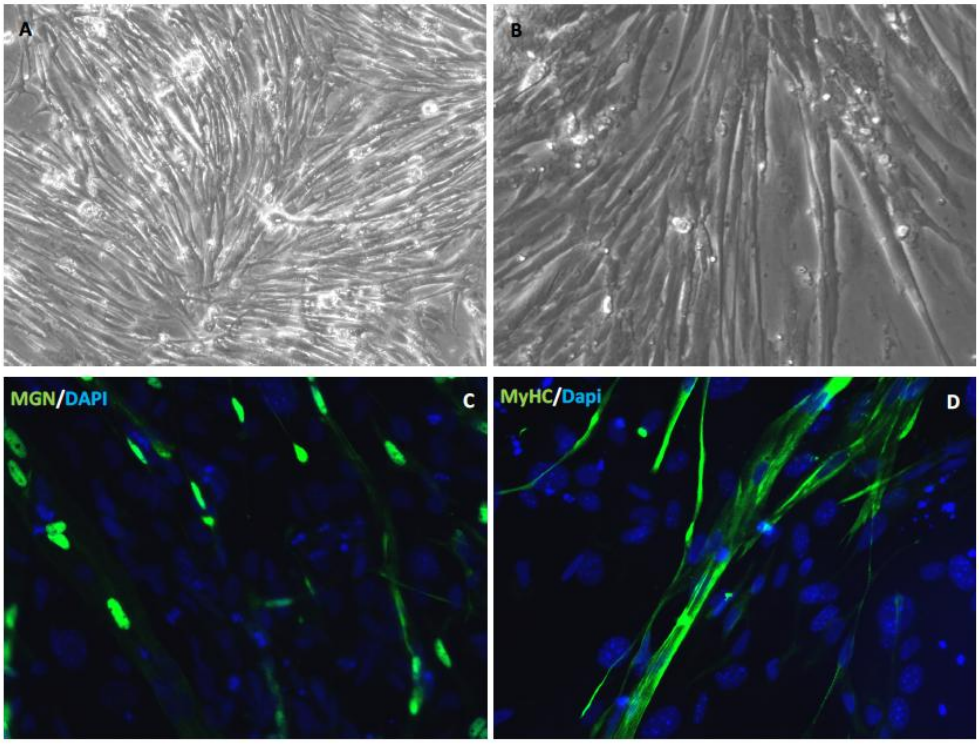


Fig. 7.16: Myoblasts plated in MEFs at high concentrations underwent fusion forming myotubes (A, 10X; B, 20X) that were positive both for MGN (C, 40X) and for MyHC (D, 40X).

7.3.5 RT-PCR

Gene expression profile was evaluated both in contractin myotubes and in myoblasts at P1 of culture. RT-PCR showed expression of the endogenous early markers *PAX7* (weak expression in myotubes) and *MYOD*, both in myotubes and in myoblasts. All samples expressed the exogenous human PAX7 and no *MYF5* (Fig. 7.17).

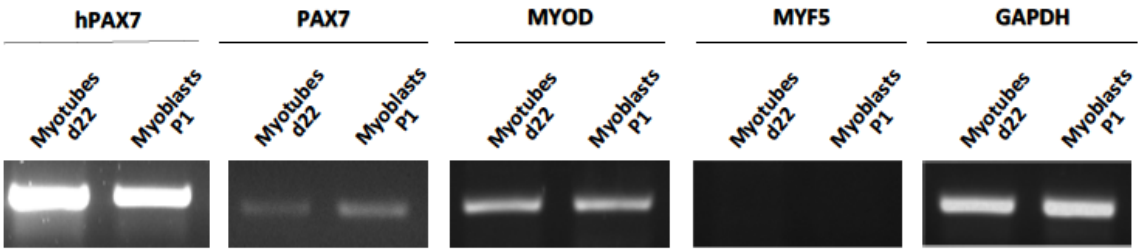


Fig. 7.17: Gene-expression profile of a sample of contracting myotubes at the end of the differentiation (day 22) and a sample of myoblasts at P1 of culture. Both samples were positive for *MYOD* and negative for *MYF5*. Endogenous *PAX7* was weakly expressed by myoblasts and very weakly expressed by myotubes. Exogenous *PAX7* was present in both samples, also in myotubes that were not under 4OH-Tam induction anymore. *GAPDH* was used as internal control.

7.4 Discussion

The growth and development of skeletal muscle has long been of interest to animal scientists not only for human health implications, but also because a better understanding of this process leads to improved strategies aimed to increase the efficency of lean tissue deposition in domestic animals (Reecy et al. 2003). Here it was demonstrated how the overexpression of h*PAX7* by a 4OH-Tam inducile vector was an efficient system to induce the myogenic program in porcine iPSCs and how piPSCs#1 Dox-regulated previously generated (Chapter 6) were amenable to genetic modifications oriented to musculo-skeletal differentiation. It was possible to generate myotubes able to make contractions *in vitro*, but more important it was possible to generate a purified line of myoblasts able to proliferate and differentiate in proper conditions. When cultured on MEFs feeder layers, *PAX7*-iPSCs were able to differentiate very fast into myotubes, and it was the same when myoblasts were plated on MEFs. Considering the $MYOD^{+}/MYF5^{-}$ profile of myoblasts, it is possible that cells were driven to a "fast differentiation" into *MGN+/MyHC+* myotubes able to do

contractions when cultured in the terminal differentiation medium (HS medium). It is also possible that some key molecules were produced by MEFs and this needs to be further studied. Both myotubes and myoblasts showed expression of the exogenous h*PAX7*. While myoblasts were expected to express it, considering that they were continuously under 4OH-Tam induction, on the other side we did not expect to see its upregulation in myotubes, as well as for endogenous *PAX7* and *MYOD*. It could be ascribable to the presence of estrogenic substances in FBS that are able to bind the ER domain, and activate h*PAX7* as a consequence. Despite the role of these unknown molecules, we were able to control the early (myoblasts) or terminal (myotubes) differentiation of the modified *PAX7*-iPSCs line in the pig model.

There are studies that have shown myogenic differentiation of murine iPSCs (Darabi et al. 2011) or human iPSCs (Darabi et al. 2012; Tanaka et al. 2013), but to our knowledge this is the first time that the generation of a myoblastic line in the pig has been achieved.

7.5 Conclusions

In the system described in this study, porcine myogenic progenitors were derived by forced expression of h*PAX7* and expanded *in vitro*. It was verified their ability to fuse into myotubes, the musculoskeletal subunit. *In vivo* functional regeneration needs to be evaluated, as well as the development of a safer viral- and transgenic- free method of *in vitro* differentiation, as it has been studied and achieved for human iPSCs (Borchin et al. 2013, Shelton et al. 2014).

CHAPTER 8: FINAL DISCUSSION AND CONCLUSIONS

Stem cells represent a booming area of research, because of their huge potential for clinical applications in current incurable diseases. However, the word "stem cell" is sometimes used without the demonstration of stem cell markers or the confirmation of stemness through gene expression analysis or differentiation (De Schauwer et al. 2011). In the case of mesenchymal stem/stromal cells, the ISCT postulated that the term mesenchymal *stem* cell, in contrast to the more general term mesenchymal *stromal* cell, should be reserved for those cells that show long-term survival *in vivo*, have self-renewal capacities and possess the ability for tissue repopulation with multilineage differentiation (Horwitz et al. 2005). MSCs isolated from bovine and equine foetal sources studied in this thesis did not show a significant expression of pluripotency markers nor a long life-span that would allow to use them as an alternative source for gene targeting experiments in genetic engineering. In any case, their immunomodulatory properties, well reported in literature, and their multi-differentiation potential make them an interesting source of cells in the field of the regenerative medicine, even if they are not the best candidate to elucidate pluripotency networks.

Therefore our attention was later focused on the generation of iPSC line in domestic animals. Considering the unsuccessful 30 years efforts to generate *bona fide* ESCs line in these species, the scientific community switched to the field of iPSCs. However the current focus in this field for domestic animals is still represented by the definition of a standard protocol to generate iPSCs with an endogenous pluripotency machinery able to sustain themselves. In our experiments we started trying to induce pluripotency in bovine fibroblasts, as continuation of the study performed on bovine AF-MSCs, and because there

were very few studies reported on iPSCs from this species. Unfortunately, the protocol used did not work and this could be due to the reprogramming factors used or to the culture conditions. In fact, the culture on feeders could be crucial for these cells, as for the murine iPSCs lines, while in this work the culture of biPSCs was done on matrigel only. It is necessary to start from the beginning both by changing the factors transfected and optimizing the culture conditions. Later, studies were finalized to the production of a porcine iPSC line with the objective to use them to create a line of myoblastic progenitors. Since the chemical induction of pluripotency is not working yet, the classic Yamanaka's technology was used. The reprogramming protocol used in porcine embryonic fibroblasts was successful and the line generated showed a consistent pluripotent profile, but issues related to exogenous factors silencing have to be solved.

The ability of iPSCs to differentiate into cells belonging to all the three germ layers has been well demonstrated by the scientific community, both *in vitro* by EBs formation and differentiation, and *in vivo*, by teratoma formation after inoculation into immuno-deficient mice. The real challenge is represented by the driven differentiation into a specific lineage. Porcine iPSCs demonstrated a full differentiation potential into the myogenic lineage by forced expression of *PAX7* expression vector, demonstrating that they could be induced into a desired lineage by genetic modification and appropriated culture conditions. Only a few cell types have been differentiated from domestic animal iPSCs to date, so the development of a reliable directed-differentiation protocol represents a very important result. Further studies are required for the production of iPSCs with a viral- free system in order to create a source of undifferentiated cells, with high proliferative capacity, useful both for genetic manipulation and for clinical applications.

REFERENCES

Aasen T., Raya A., Barrero M. J., Garreta E., Consiglio A., Gonzalez F., Vassena R., Bilic J., Pekarik V., Tiscornia G., Edel M., Boue S., Izpisua Belmonte J. C. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol*, 26 (11): 1276-84. doi: 10.1038/nbt.1503.

Antonucci I., Stuppia L., Kaneko Y., Yu S., Tajiri N., Bae E. C., Chheda S. H., Weinbren N. L., Borlongan C. V. (2011). Amniotic fluid as a rich source of mesenchymal stromal cells for transplantation therapy. *Cell Transplant*, 20 (6): 789-95. doi: 10.3727/096368910x539074.

Ashwood E.R., Knight G.J. (2006). Chapter 54: Clinical chemistry of pregnancy. In Burtis C.A., Ashwood E.R., Bruns D.E. (Eds.), Teitz textbook of clinical chemistry and molecular diagnostics 4th ed. (pp. 2153-2206). St. Louis, MO: Elsevier Saunders.

Bao L., He L., Chen J., Wu Z., Liao J., Rao L., Ren J., Li H., Zhu H., Qian L., Gu Y., Dai H., Xu X., Zhou J., Wang W., Cui C., Xiao L. (2011). Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors. *Cell Res* 21 (4): 600-8. doi: 10.1038/cr.2011.6.

Bauer G., Dao M. A., Case S. S., Meyerrose T., Wirthlin L., Zhou P., Wang X., Herrbrich P., Arevalo J., Csik S., Skelton D. C., Walker J., Pepper K., Kohn D. B., Nolta J. A. (2008). In vivo biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Mol Ther*, 16 (7): 1308-15. doi: 10.1038/mt.2008.93.

Bischoff R., Heintz C. (1994). Enhancement of skeletal muscle regeneration. *Dev Dyn*, 201 (1): 41-54. doi: 10.1002/aja.1002010105.

Borchin B., Chen J., Barberi T. (2013). Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. *Stem Cell Reports* 1 (6): 620-31. doi: 10.1016/j.stemcr.2013.10.007.

Bossolasco P., Montemurro T., Cova L., Zangrossi S., Calzarossa C., Buiatiotis S., Soligo D., Bosari S., Silani V., Deliliers G. L., Rebulla P., Lazzari L. (2006). Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential. *Cell Res* 16 (4): 329-36. doi: 10.1038/sj.cr.7310043.

Bottai D., D. Cigognini, E. Nicora, M. Moro, M. G. Grimoldi, R. Adami, S. Abrignani, A. M. Marconi, A. M. Di Giulio, and A. Gorio. (2011). Third trimester amniotic fluid cells with the capacity to develop neural phenotypes and with heterogeneity among sub-populations. *Restor Neurol Neurosci*. doi: 10.3233/rnn-2011-620.

Brace R. A., Wolf E. J. (1989). "Normal amniotic fluid volume changes throughout pregnancy." *Am J Obstet Gynecol* 161 (2):382-8.

Brevini T. A., Antonini S., Cillo F., Crestan M., Gandolfi F. (2007). Porcine embryonic stem cells: Facts, challenges and hopes. *Theriogenology* 68 Suppl 1:S206-13. doi: 10.1016/j.theriogenology.2007.05.043.

Burk J., Ribitsch I., Gittel C., Juelke H., Kasper C., Staszyk C., Brehm W. (2013). Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources. *Vet J* 195 (1):98-106. doi: 10.1016/j.tvjl.2012.06.004.

Cao H., Yang P., Pu Y., Sun X., Yin H., Zhang Y., Li Y., Liu Y., Fang F., Zhang Z., Tao Y., Zhang X.. (2012). Characterization of bovine induced pluripotent stem cells by

lentiviral transduction of reprogramming factor fusion proteins. *Int J Biol Sci* 8 (4): 498-511. doi: 10.7150/ijbs.3723.

Chan E. M., Ratanasirintrawoot S., Park I. H., Manos P. D., Loh Y. H., Huo H., Miller J. D., Hartung O., Rho J., Ince T. A., Daley G. Q., Schlaeger T. M.. (2009). Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. *Nat Biotechnol* 27 (11):1033-7. doi: 10.1038/nbt.1580.

Chen J., Lu Z., Cheng D., Peng S., Wang H. (2011). Isolation and characterization of porcine amniotic fluid-derived multipotent stem cells. *PLoS One* 6 (5):e19964. doi: 10.1371/journal.pone.0019964.

Chu Z., Niu B., Zhu H., He X., Bai C., Li G., Hua, J. (2015). PRMT5 enhances generation of induced pluripotent stem cells from dairy goat embryonic fibroblasts via down-regulation of p53. *Cell Prolif, 48*(1), 29-38. doi: 10.1111/cpr.12150

Civin C. L., Trischmann T. M., Fackler M. J., et al. (1989). Summary of CD34 cluster workshop section. In W. Knapp et al. (Eds.), Leucocyte Typing IV - white cell differentiation antigens (pp. 818–825). Oxford: Oxford University Press.

Corradetti B., Lange-Consiglio A., Barucca M., Cremonesi F., Bizzaro D. (2011). Size-sieved subpopulations of mesenchymal stem cells from intervascular and perivascular equine umbilical cord matrix. *Cell Prolif* 44 (4): 330-42. doi: 10.1111/j.1365-2184.2011.00759.x.

Corradetti B., Meucci A., Bizzaro D., Cremonesi F., Lange Consiglio A. (2013). Mesenchymal stem cells from amnion and amniotic fluid in the bovine. *Reproduction* 145 (4):391-400. doi: 10.1530/rep-12-0437.

Darabi R., Arpke R. W., Irion S., Dimos J. T., Grskovic M., Kyba M., Perlingeiro R. C.. (2012). Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. *Cell Stem Cell* 10 (5): 610-9. doi: 10.1016/j.stem.2012.02.015.

Darabi R., Pan W., Bosnakovski D., Baik J., Kyba M., Perlingeiro R. C. (2011). "Functional myogenic engraftment from mouse iPS cells." *Stem Cell Rev* 7 (4):948-57. doi: 10.1007/s12015-011-9258-2.

De Schauwer C., Meyer E., Van de Walle G. R., Van Soom A. (2011). Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. *Theriogenology* 75 (8): 1431-43. doi: 10.1016/j.theriogenology.2010.11.008.

Desmarais J. A., Demers S. P., J. Suzuki J. Jr., Laflamme S., Vincent P., Laverty S., Smith L. C. (2011). Trophoblast stem cell marker gene expression in inner cell mass-derived cells from parthenogenetic equine embryos. *Reproduction* 141 (3): 321-32. doi: 10.1530/rep-09-0536.

Dev K., Gautam S. K., Giri S. K., Kumar A., Yadav A., Verma V., Kumar P., Singh B. (2012). Isolation, culturing and characterization of feeder-independent amniotic fluid stem cells in buffalo (Bubalus bubalis). *Res Vet Sci* 93 (2): 743-8. doi: 10.1016/j.rvsc.2011.09.007.

Dominici M., Le Blanc K., Mueller I., Slaper-Cortenbach I., Marini F., Krause D., Deans R., Keating A., Prockop Dj, Horwitz E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8 (4): 315-7. doi: 10.1080/14653240600855905.

Eastham A. M., Spencer H., Soncin F., Ritson S., Merry C. L., Stern P. L., Ward C. M. (2007). Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. *Cancer Res* 67 (23): 11254-62. doi: 10.1158/0008-5472.can-07-2253.

Enwere E. K., Lacasse E. C., Adam N. J., Korneluk R. G. (2014). Role of the TWEAK-Fn14-cIAP1-NF-kappaB Signaling Axis in the Regulation of Myogenesis and Muscle Homeostasis. *Front Immunol* 5:34. doi: 10.3389/fimmu.2014.00034.

Ernst M., Dawud R. A., Kurtz A., Schotta G., Taher L., Fuellen G. (2015). Comparative computational analysis of pluripotency in human and mouse stem cells. *Sci Rep* 5:7927. doi: 10.1038/srep07927.

Esteban M. A., Xu J., Yang J., Peng M., Qin D., Li W., Jiang Z., Chen J., Deng K., Zhong M., Cai J., Lai L., Pei D. (2009). Generation of induced pluripotent stem cell lines from Tibetan miniature pig.. *J Biol Chem* 284 (26): 17634-40. doi: 10.1074/jbc.M109.008938.

Ezashi T., Telugu B. P., Alexenko A. P., Sachdev S., Sinha S., Roberts R. M. (2009). Derivation of induced pluripotent stem cells from pig somatic cells. *Proc Natl Acad Sci U S A* 106 (27): 10993-8. doi: 10.1073/pnas.0905284106.

Filioli Uranio M., Valentini L., Lange-Consiglio A., Caira M., Guaricci A. C., L'Abbate A., Catacchio C. R., Ventura M., Cremonesi F., Dell'Aquila M. E. (2011). Isolation, proliferation, cytogenetic, and molecular characterization and in vitro differentiation potency of canine stem cells from foetal adnexa: a comparative study of amniotic fluid, amnion, and umbilical cord matrix. *Mol Reprod Dev* 78 (5):361-73. doi: 10.1002/mrd.21311.

Fong C. Y., Richards M., Manasi N., Biswas A., Bongso A. (2007). Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. *Reprod Biomed Online, 15*(6), 708-718.

Friedenstein A. J., Chailakhyan R. K., Gerasimov U. V. (1987). Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* 20 (3): 263-72.

Fujishiro S. H., Nakano K., Mizukami Y., Azami T., Arai Y., Matsunari H., Ishino R., Nishimura T., Watanabe M., Abe T., Furukawa Y., Umeyama K., Yamanaka S., Ema M., Nagashima H., Hanazono Y. (2013). Generation of naive-like porcine-induced pluripotent stem cells capable of contributing to embryonic and fetal development. *Stem Cells Dev* 22 (3): 473-82. doi: 10.1089/scd.2012.0173.

Gao Y., Zhu Z., Zhao Y., Hua J., Ma Y., Guan W. (2014). "Multilineage potential research of bovine amniotic fluid mesenchymal stem cells." *Int J Mol Sci* 15 (3):3698-710. doi: 10.3390/ijms15033698.

Gonzalez F., Barragan Monasterio M., Tiscornia G., Montserrat Pulido N., Vassena R., Batlle Morera L., Rodriguez Piza I., Izpisua Belmonte J. C. (2009). Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. *Proc Natl Acad Sci U S A* 106 (22): 8918-22. doi: 10.1073/pnas.0901471106.

Guangjin P, Thomson J. A. (2007). Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Research* 17 (1): 42-49. doi: doi:10.1038/sj.cr.7310125.

Guest D. J., Ousey J. C., Smith M. R.. (2008). Defining the expression of marker genes in equine mesenchymal stromal cells. *Stem Cells Cloning* 1:1-9.

Hall V. J., Kristensen M., Rasmussen M. A., Ujhelly O., Dinnyes A., Hyttel P. (2012). Temporal repression of endogenous pluripotency genes during reprogramming of porcine induced pluripotent stem cells. *Cell Reprogram* 14 (3): 204-16. doi: 10.1089/cell.2011.0089.

Hamilton B., Feng Q., Ye M., Welstead G. G. (2009). Generation of induced pluripotent stem cells by reprogramming mouse embryonic fibroblasts with a four transcription factor, doxycycline inducible lentiviral transduction system. *J Vis Exp* (33). doi: 10.3791/1447.

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

Heffernan C., Sumer H., Verma P. J. (2009). Generation of clinically relevant induced pluripotent stem (iPS) cells. In Pluripotent Stem Cells, Rosales D. W.and Mullen Q. N., Eds. (pp. 81–112). Nova Publishing, New York, NY, USA, 1st edition.

Horwitz E. M., Le Blanc K., Dominici M., Mueller I., Slaper-Cortenbach I., Marini F. C., Deans R. J., Krause D. S., Keating A. (2005). Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 7 (5): 393-5. doi: 10.1080/14653240500319234.

Hou P., Li Y., Zhang X., Liu C., Guan J., Li H., Zhao T., Ye J., Yang W., Liu K., Ge J., Xu J., Zhang Q., Zhao Y., Deng H. (2013). Pluripotent stem cells induced from mouse

somatic cells by small-molecule compounds. *Science* 341 (6146): 651-4. doi: 10.1126/science.1239278.

Hoynowski S. M., Fry M. M., Gardner B. M., Leming M. T., Tucker J. R., Black L., Sand T., Mitchell K. E. (2007). Characterization and differentiation of equine umbilical cord-derived matrix cells. *Biochem Biophys Res Commun* 362 (2): 347-53. doi: 10.1016/j.bbrc.2007.07.182.

Hu J., Zhang X., Zhou L., Zhang Y. (2013). Immunomodulatory properties of colonic mesenchymal stem cells. *Immunol Lett* 156 (1-2): 23-9. doi: 10.1016/j.imlet.2013.09.006.

Huang B., Li T., Alonso-Gonzalez L., Gorre R., Keatley S., Green A., Turner P., Kallingappa P. K., Verma V., Oback B. (2011). A virus-free poly-promoter vector induces pluripotency in quiescent bovine cells under chemically defined conditions of dual kinase inhibition. *PLoS One* 6 (9): e24501. doi: 10.1371/journal.pone.0024501.

Iacono E., Brunori L., Pirrone A., Pagliaro P. P., Ricci F., Tazzari P. L., Merlo B. (2012a). Isolation, characterization and differentiation of mesenchymal stem cells from amniotic fluid, umbilical cord blood and Wharton's jelly in the horse. *Reproduction* 143 (4): 455-68. doi: 10.1530/rep-10-0408.

Iacono E., Cunto M., Zambelli D., Ricci F., Tazzari P. L., Merlo B. (2012b). Could fetal fluid and membranes be an alternative source for mesenchymal stem cells (MSCs) in the feline species? A preliminary study. *Vet Res Commun* 36 (2): 107-18. doi: 10.1007/s11259-012-9520-3.

Iacono E., Rossi B., Merlo B. (2015). Stem Cells from Foetal Adnexa and Fluid in Domestic Animals: An Update on Their Features and Clinical Application. doi: 10.1111/rda.12499. [Epub ahead of print]

Jung D. W., Kim W. H., Williams D. R. (2014). Reprogram or reboot: small molecule approaches for the production of induced pluripotent stem cells and direct cell reprogramming. *ACS Chem Biol* 9 (1):80-95. doi: 10.1021/cb400754f.

Kavanagh D. P., Robinson J., Kalia N. (2014). Mesenchymal stem cell priming: fine-tuning adhesion and function. *Stem Cell Rev* 10 (4): 587-99. doi: 10.1007/s12015-014-9510-7.

Kaviani A., Guleserian K., Perry T. E., Jennings R. W., Ziegler M. M., Fauza D. O. (2003). Fetal tissue engineering from amniotic fluid. *J Am Coll Surg* 196 (4):592-7.

Kaviani A., Perry T. E., Dzakovic A., Jennings R. W., Ziegler M. M., Fauza D. O. (2001). The amniotic fluid as a source of cells for fetal tissue engineering. *J Pediatr Surg* 36 (11): 1662-5.

Kim J., Lee Y., Kim H., Hwang K. J., Kwon H. C., Kim S. K., Cho D. J., Kang S. G., You J. (2007). Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells. *Cell Prolif* 40 (1): 75-90. doi: 10.1111/j.1365-2184.2007.00414.x.

Klemmt P. A., Vafaizadeh V., Groner B. (2011). The potential of amniotic fluid stem cells for cellular therapy and tissue engineering. *Expert Opin Biol Ther* 11 (10): 1297-314. doi: 10.1517/14712598.2011.587800 [doi].

Koh S., Piedrahita J. A. (2014). From "ES-like" cells to induced pluripotent stem cells: A historical perspective in domestic animals. *Theriogenology* 81 (1): 103-111. doi: 10.1016/j.theriogenology.2013.09.009.

Kuang S., Rudnicki M. A. (2008). The emerging biology of satellite cells and their therapeutic potential. *Trends Mol Med* 14 (2): 82-91. doi: 10.1016/j.molmed.2007.12.004.

Lancaster C. (2015). The Acid Test for Biological Science: STAP Cells, Trust, and Replication. *Sci Eng Ethics*. doi: 10.1007/s11948-015-9628-2.

Li Y., Cang, M., Lee, A. S., Zhang, K., Liu, D. (2011). Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors. *PLoS One, 6*(1), e15947. doi: 10.1371/journal.pone.0015947

Liang C. C., Park A. Y., Guan J. L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2 (2): 329-33. doi: 10.1038/nprot.2007.30.

Liao J., Cui C., Chen S., Ren J., Chen J., Gao Y., Li H., Jia N., Cheng L., Xiao H., Xiao L. (2009). Generation of induced pluripotent stem cell lines from adult rat cells. In *Cell Stem Cell*, 11-5. United States.

Liao J., Wu Z., Wang Y., Cheng L., Cui C., Gao Y., Chen T., Rao L., Chen S., Jia N., Dai H., Xin S., Kang J., Pei G., Xiao L. (2008). Enhanced efficiency of generating induced pluripotent stem (iPS) cells from human somatic cells by a combination of six transcription factors. In *Cell Res*, 600-3. China.

Liu H., Zhu F., Yong J., Zhang P., Hou P., Li H., Jiang W., Cai J., Liu M., Cui K., Qu X., Xiang T., Lu D., Chi X., Gao G., Ji W., Ding M., Deng H. (2008). Generation of

induced pluripotent stem cells from adult rhesus monkey fibroblasts. In *Cell Stem Cell*, 587-90. United States.

Lovati A. B., Corradetti B., Lange Consiglio A., Recordati C., Bonacina E., Bizzaro D., Cremonesi F. (2011). Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived progenitor cells. *Vet Res Commun* 35 (2): 103-21. doi: 10.1007/s11259-010-9457-3.

Ma T., Xie, M., Laurent, T., Ding, S. (2013). Progress in the reprogramming of somatic cells. *Circ Res* (Vol. 112, pp. 562-574). United States.

Malaver-Ortega, L. F., Sumer H., Liu J., Verma P. J. (2012). The state of the art for pluripotent stem cells derivation in domestic ungulates. *Theriogenology* 78 (8):1749-62. doi: 10.1016/j.theriogenology.2012.03.031.

Malgieri A., Novelli G., Sangiuolo F. (2011). Potential Clinical Applications of Embryonic Stem Cells, Embryonic Stem Cells - Recent Advances in Pluripotent Stem Cell-Based Regenerative Medicine, Chapter 2. Prof. Craig Atwood (Ed.), ISBN: 978-953-307-198-5, *InTech*, DOI: 10.5772/14886.

Masuda S., Wu J., Hishida T., Pandian G. N., Sugiyama H., Izpisua Belmonte J. C. (2013). Chemically induced pluripotent stem cells (CiPSCs): a transgene-free approach. *J Mol Cell Biol* 5 (5):354-5. doi: 10.1093/jmcb/mjt034.

Miyazaki Y., Imoto H., Chen L. C., Wandless T. J. (2012). Destabilizing domains derived from the human estrogen receptor. *J Am Chem Soc* 134 (9): 3942-5. doi: 10.1021/ja209933r.

Mizuno H., Hyakusoku H. (2003). Mesengenic potential and future clinical perspective of human processed lipoaspirate cells. *J Nippon Med Sch* 70 (4): 300-6.

Montserrat N., Bahima E. G., Batlle L., Hafner S., Rodrigues A. M., Gonzalez F., Izpisua Belmonte J. C. (2011). Generation of pig iPS cells: a model for cell therapy. *J Cardiovasc Transl Res* 4 (2):121-30. doi: 10.1007/s12265-010-9233-3.

Montserrat N., de Onate L., Garreta E., Gonzalez F., Adamo A., Eguizabal C., Hafner S., Vassena R., Izpisua Belmonte J. C. (2012). Generation of feeder-free pig induced pluripotent stem cells without Pou5f1. *Cell Transplant* 21 (5): 815-25. doi: 10.3727/096368911x601019.

Nagy K., Sung H. K., Zhang P., Laflamme S., Vincent P., Agha-Mohammadi S., Woltjen K., Monetti C., Michael I. P., Smith L. C., Nagy A. (2011). Induced pluripotent stem cell lines derived from equine fibroblasts. *Stem Cell Rev* 7 (3): 693-702. doi: 10.1007/s12015-011-9239-5.

Nakagawa M., Koyanagi M., Tanabe K., Takahashi K., Ichisaka T., Aoi T., Okita K., Mochiduki Y., Takizawa N., Yamanaka S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 26 (1): 101-6. doi: 10.1038/nbt1374.

Obokata H., Wakayama T., Sasai Y., Kojima K., Vacanti M. P., Niwa H., Yamato M., C. Vacanti A. (2014a). Stimulus-triggered fate conversion of somatic cells into pluripotency. *Nature* 505 (7485): 641-7. doi: 10.1038/nature12968.

Obokata H., Sasai Y., Niwa H., Kadota M., Andrabi M., Takata N., Tokoro M., Terashita Y., Yonemura S., Vacanti C. A., Wakayama T.. (2014b). Retraction: Bidirectional developmental potential in reprogrammed cells with acquired pluripotency. In *Nature*, 112. England.

Obokata H, Sasai Y, Niwa H. (2014c.). Essential technical tips for STAP cell conversion culture from somatic cells. *Protocol Exchange*.

Okada M., Oka M., Yoneda Y. (2010). Effective culture conditions for the induction of pluripotent stem cells. *Biochim Biophys Acta* 1800 (9): 956-63. doi: 10.1016/j.bbagen.2010.04.004.

Pant D., Keefer C. L. (2009). Expression of pluripotency-related genes during bovine inner cell mass explant culture. *Cloning Stem Cells* 11 (3): 355-65. doi: 10.1089/clo.2008.0078.

Pappa K. I., Anagnou N. P. (2009). Novel sources of fetal stem cells: where do they fit on the developmental continuum? *Regen Med* 4 (3): 423-33. doi: 10.2217/rme.09.12.

Park J. K., Kim H. S., Uh K. J., Choi K. H., Kim H. M., Lee T., Yang B. C., Kim H. J., Ka H. H., Kim H., Lee C. K. (2013). Primed pluripotent cell lines derived from various embryonic origins and somatic cells in pig. *PLoS One* 8 (1): e52481. doi: 10.1371/journal.pone.0052481.

Perin L., Sedrakyan S., Da Sacco S., De Filippo R.. (2008). Characterization of human amniotic fluid stem cells and their pluripotential capability. *Methods Cell Biol* 86: 85-99. doi: 10.1016/s0091-679x(08)00005-8.

Prusa A. R., Marton E., Rosner M., Bernaschek G., Hengstschlager M. (2003). Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? *Hum Reprod* 18 (7):1489-93.

Rainaldi G., Pinto B., Piras A., Vatteroni L., Simi S., Citti L. (1991). Reduction of proliferative heterogeneity of CHEF18 Chinese hamster cell line during the progression toward tumorigenicity. *In Vitro Cell Dev Biol* 27a (12): 949-52.

Reed S. A., Johnson S. E. (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 (2): 329-36. doi: 10.1002/jcp.21312.

Ren J., Pak Y., He L., Qian L., Gu Y., Li H., Rao L., J. Liao, C. Cui, X. Xu, J. Zhou, H. Ri, and L. Xiao. (2011). Generation of hircine-induced pluripotent stem cells by somatic cell reprogramming. In *Cell Res*, 849-53. England.

Rodriguez A., Allegrucci C., Alberio R. (2012). Modulation of pluripotency in the porcine embryo and iPS cells. *PLoS One* 7 (11):e49079. doi: 10.1371/journal.pone.0049079.

Roubelakis M. G., Bitsika V., Zagoura D., Trohatou O., Pappa K. I., Makridakis M., Antsaklis A., Vlahou A., Anagnou N. P. (2011). In vitro and in vivo properties of distinct populations of amniotic fluid mesenchymal progenitor cells. *J Cell Mol Med* 15 (9):1896-913. doi: 10.1111/j.1582-4934.2010.01180.x [doi].

Ruan W., Han, J., Li, P., Cao, S., An, Y., Lim, B., Li, N. (2011). A novel strategy to derive iPS cells from porcine fibroblasts. *Sci China Life Sci, 54*(6), 553-559. doi: 10.1007/s11427-011-4179-5.

Reecy J. M., Miller S. A, Webster M. (2003). Recent Advances That Impact Skeletal Muscle Growth and Development Research. Journal of Animal Science, 81, E1-E8 doi:/2003.8113_suppl_1E1x

Rudnicki M. A., Le Grand F., McKinnell I., Kuang S. (2008). The molecular regulation of muscle stem cell function. *Cold Spring Harb Symp Quant Biol* 73:323-31. doi: 10.1101/sqb.2008.73.064.

Sartore S., Lenzi M., Angelini A., Chiavegato A., Gasparotto L., De Coppi P., Bianco R., Gerosa G. (2005). Amniotic mesenchymal cells autotransplanted in a porcine model

of cardiac ischemia do not differentiate to cardiogenic phenotypes. *Eur J Cardiothorac Surg* 28 (5):677-84. doi: 10.1016/j.ejcts.2005.07.019.

Sartori C., DiDomenico A. I., Thomson A. J., Milne E., Lillico S. G., Burdon T. G., Whitelaw C. B. (2012). Ovine-induced pluripotent stem cells can contribute to chimeric lambs. *Cell Reprogram* 14 (1): 8-19. doi: 10.1089/cell.2011.0050.

Shelton M., Metz J., Liu J., Carpenedo R. L., Demers S. P., Stanford W. L., Skerjanc I. S. (2014). Derivation and expansion of PAX7-positive muscle progenitors from human and mouse embryonic stem cells. *Stem Cell Reports* 3 (3): 516-29. doi: 10.1016/j.stemcr.2014.07.001.

Shi G., Jin. Y. (2010). Role of Oct4 in maintaining and regaining stem cell pluripotency. *Stem Cell Res Ther* 1 (5): 39. doi: 10.1186/scrt39.

Siegel N., Rosner M., Hanneder M., Freilinger A., Hengstschlager M. (2008). Human amniotic fluid stem cells: a new perspective. *Amino Acids* 35 (2): 291-3. doi: 10.1007/s00726-007-0593-1.

Sinowatz F. (2010). Chapter 16: Musculo-skeletal system. In Hyttel P., Sinowatz F., Vejlsted M. Essentials of Domestic Animal Embryology (pp. 286-316). Elsevier, Saunders.

Smith A. (2014). "Cell biology: Potency unchained." *Nature* 505 (7485):622-3. doi: 10.1038/505622a.

Sugawara T., Nishino K., Umezawa A, Akutsu H. (2012). Investigating cellular identity and manipulating cell fate using induced pluripotent stem cells. *Stem Cell Research & Therapy* 3 (2):8. doi: info:pmid/22405125.

Sumer H., Liu J., Malaver-Ortega L. F., Lim M. L., Khodadadi K., Verma P. J. (2011). NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. *J Anim Sci* 89 (9):2708-16. doi: 10.2527/jas.2010-3666.

Sutradhar B. C., Hong G., Ge Z., Kim G. (2012). Enhancement of chondrogenesis via co-culture of bovine chondrocytes with Demineralized bone matrix in chitosan-alginate beads. Journal of Medical and Biological Engineering, 33(5): 518–525.

Takahashi, K., Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126 (4): 663-76. doi: 10.1016/j.cell.2006.07.024.

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

Tanaka A., Woltjen K., Miyake K., Hotta A., Ikeya M., Yamamoto T., Nishino T., Shoji E., Sehara-Fujisawa A., Manabe Y., Fujii N., Hanaoka K., Era T., Yamashita S., Isobe K., Kimura E., Sakurai H. (2013). Efficient and reproducible myogenic differentiation from human iPS cells: prospects for modeling Miyoshi Myopathy in vitro. *PLoS One* 8 (4): e61540. doi: 10.1371/journal.pone.0061540.

Tang M. K., Lo L. M., Shi W. T., Yao Y., Lee H. S., Lee K. K. (2014). Transient acid treatment cannot induce neonatal somatic cells to become pluripotent stem cells. *F1000Res* 3:102. doi: 10.12688/f1000research.4092.1.

Telugu B. P., Ezashi T., Roberts R. M. (2010). The promise of stem cell research in pigs and other ungulate species. *Stem Cell Rev* 6 (1):31-41. doi: 10.1007/s12015-009-9101-1.

Telugu B. P., Ezashi T., Sinha S., Alexenko A. P., Spate L., Prather R. S., Roberts R. M. (2011). Leukemia inhibitory factor (LIF)-dependent, pluripotent stem cells established from inner cell mass of porcine embryos. *J Biol Chem* 286 (33):28948-53. doi: 10.1074/jbc.M111.229468.

Thelie A., Papillier P., Pennetier S., Perreau C., Traverso J. M., Uzbekova S., Mermillod P., Joly C., Humblot P., Dalbies-Tran R. (2007). Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation in vitro and in vivo. *BMC Dev Biol* 7:125. doi: 10.1186/1471-213x-7-125.

Tsai M. S., Lee J. L., Chang Y. J., Hwang S. M. (2004). Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum Reprod* 19 (6): 1450-6. doi: 10.1093/humrep/deh279.

Underwood M. A., Gilbert W. M., Sherman M. P. (2005). Amniotic fluid: not just fetal urine anymore. *J Perinatol* 25 (5): 341-8. doi: 7211290 [pii]10.1038/sj.jp.7211290 [doi].

Wakao S., Kitada M., Kuroda Y., Ogura F., Murakami T., Niwa A., Dezawa M. (2012). Morphologic and gene expression criteria for identifying human induced pluripotent stem cells. *PLoS One* 7 (12): e48677. doi: 10.1371/journal.pone.0048677.

West F. D., Terlouw S. L., Kwon D. J., Mumaw J. L., Dhara S. K., Hasneen K., Dobrinsky J. R., Stice S. L. (2010). Porcine induced pluripotent stem cells produce chimeric offspring. *Stem Cells Dev* 19 (8):1211-20. doi: 10.1089/scd.2009.0458.

Wilschut K. J., Jaksani S., Van Den Dolder J., Haagsman H. P., Roelen B. A. (2008). Isolation and characterization of porcine adult muscle-derived progenitor cells. *J Cell Biochem* 105 (5): 1228-39. doi: 10.1002/jcb.21921.

Wu Z., Chen J., Ren J., Bao L., Liao J., Cui C., Rao L., Li H., Gu Y., Dai H., Zhu H., Teng X., Cheng L., Xiao L. (2009). Generation of pig induced pluripotent stem cells with a drug-inducible system. *J Mol Cell Biol* 1 (1):46-54. doi: 10.1093/jmcb/mjp003.

Yi T. Song S. U. (2012). Immunomodulatory properties of mesenchymal stem cells and their therapeutic applications. *Arch Pharm Res* 35 (2): 213-21. doi: 10.1007/s12272-012-0202-z.

Yin H., Price F., Rudnicki M. A.. (2013). Satellite cells and the muscle stem cell niche. *Physiol Rev* 93 (1):23-67. doi: 10.1152/physrev.00043.2011.

You Q., Cai L., Zheng J., Tong X., Zhang D., Zhang Y. (2008). Isolation of human mesenchymal stem cells from third-trimester amniotic fluid. *Int J Gynaecol Obstet* 103 (2):149-52. doi: 10.1016/j.ijgo.2008.06.012.

You Q., Tong X., Guan Y., Zhang D., Huang M., Zhang Y., Zheng J. (2009). The biological characteristics of human third trimester amniotic fluid stem cells. *J Int Med Res* 37 (1):105-12.

Zagoura D. S., Trohatou O., Bitsika V., Makridakis M., Pappa K. I., Vlahou A., Roubelakis M. G., Anagnou N. P. (2013). AF-MSCs fate can be regulated by culture conditions. *Cell Death Dis* 4: e571. doi: 10.1038/cddis.2013.93.

Zia S., Toelen J., Mori da Cunha M., Dekoninck P., de Coppi P., Deprest J. (2013). Routine clonal expansion of mesenchymal stem cells derived from amniotic fluid for perinatal applications. *Prenat Diagn* 33 (10): 921-928. doi: 10.1002/pd.4162.

Zuk P. A. (2009). The intracellular distribution of the ES cell totipotent markers OCT4 and Sox2 in adult stem cells differs dramatically according to commercial antibody used. *J Cell Biochem* 106 (5): 867-77. doi: 10.1002/jcb.22054.

ACKNOWLEDGEMENTS

Thanks are due to Prof. Cesare Galli, that supervised me during all my PhD, and to Prof. Eleonora Iacono and Prof. Barbara Merlo, with whom I worked day by day and that led me into the *stem cell* world. Special thanks are also due to Dr. Ramiro Alberio, that supervised me during all my work at University of Nottingham.

Research on bovine AF-MSCs was funded by PRIN 2009 from MIUR and by a fellowship from the Department of Veterinary Medical Sciences. Some experiments were carried out at Avantea, Cremona, Italy, so thanks are due to all the lab team, in particular Dr. Giovanna Lazzari and Dr. Silvia Colleoni. I wish to thank Dr. Pier Luigi Tazzari and Dr. Francesca Ricci for cytofluorimetric analysis. I also wish to thanks Professor Giuseppe Sarli (Pathological Anatomy Service, DIMEVET) for Vimentin clone 9 (DAKO M0725) antibody, and Professor Alessandra Scagliarini (Public Health and Animal pathology, DIMEVET) for positive controls for immunostaining analysis on CK protein. Thanks are also due to Dr. Chiara Sartori for the help given with first molecular analyses and to the students Valentina Montanari and Maria Chiara Corlianò for the help given during the culture and the differentiation of the samples.

Research on bovine iPSCs was carried out at Istituto Clinico Humanitas (Rozzano, Milano) in collaboration with Dr. Paolo Vezzoni's group, in particular with Dr. Giovanni Pacchiana. Thanks are also due to Dr. Elisa Di Pasquale (Istituto Clinico Humanitas), for introducing myself into the iPSCs field, and to Professor Maria Laura Bacci (Veterinary Physiology, DIMEVET) who let me attend her lab to train myself working on iPSCs cultures.

Research on STAP, porcine iPSCs and musculo-skeletal differentiation was carried out at University of Nottingham, School of Biosciences, Division of Animal Sciences, under the supervision of Dr. Ramiro Alberio. Thanks are due to Dr. Xinyun Li, who worked at the project along with me.