THE UNIVERSITY *of* EDINBURGH

GENERATION OF OVINE INDUCED PLURIPOTENT STEM CELLS

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Alla mia famiglia

Abstract

Embryonic stem cells (ESCs) are pluripotent cells derived from the early embryo and are able to differentiate into cells belonging to the three germ layers. They are a valuable tool in research and for clinical use, but their applications are limited by ethical and technical issues.

In 2006 a breakthrough report described the generation of induced pluripotent stem cells (iPSCs). IPSCs are ESC-like cells generated from somatic cells by forcing the ectopic expression of specific transcription factors. This circumvents the ethical issues about the use of embryos in research and provides multiple opportunities to understand the mechanisms behind pluripotency.

The aim of this project was to generate sheep iPSCs and characterise them. In order to learn the technique I initially repeated the original iPSC methodology: the putative mouse iPSCs I have generated display a morphology typical of ESCs, characterised by a high nuclear to cytoplasmic ratio, and form colonies with neat edges and smooth domes. These cells are positive to Nanog, a marker of pluripotency, and can give rise to cells belonging to the mesodermal and the ectodermal lineages when differentiated *in vitro*. Since the main aim of the thesis was the derivation of sheep pluripotent cells, once established the protocol in mouse, I then moved to the generation of ovine iPSC colonies. The cells I have generated have a morphology similar to that of mouse ESCs, express markers of pluripotency such as alkaline phosphatase and Nanog and can differentiate *in vitro* and *in vivo* into cells belonging to the three germ layers. Additionally, these ovine iPSCs can contribute to live born chimeric lambs, although at low level.

"I have always believed that in the lives of individuals, just as in society at large, the profoundest changes take place within a very reduced time frame. When we least expect it, life sets us a challenge to test our courage and willingness to change; at such a moment, there is no point in pretending that nothing has happened or in saying that we are not yet ready.

The challenge will not wait. Life does not look back. A week is more than enough time for us to decide whether or not to accept our destiny."

The Devil and Miss Prym
Author's note

-Paulo Coelho-

I declare that the work presented in this thesis is my own, unless otherwise stated.

The work described in this thesis has not been submitted for any other degree or professional qualification.

CHIARA SARTORI

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Abbreviations

-1° ab	Minus primary antibody
2D10 D	2D10 differentiated
AP	Alkaline phosphatase
ASCs	Adult stem cells
bFGF	Basic fibroblast growth factor
bHLH/LZ	Basic helix-loop-helix leucine zipper
BMP4	Bone morphogenetic protein 4
CAG	Cytomegalovirus early enhancer element and chicken βactin
cMyc	Myelocytomatosis cellular oncogene
D1 D	Differentiated D1
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EB	Embryoid body
EMEM	Eagle's minimum essential medium
enJSRV	Endogenous Jaagsiekte sheep retrovirus
EpiSCs	Epiblast stem cells
ESCs	Embryonic stem cells
FCS	Foetal calf serum
FGF4	Fibroblast growth factor 4
G2 D	Differentiated G2
gKlf	Gut-enriched Krüppel-like factor
gp130	Glycoprotein 130
GSK3β	Glycogen synthase kinase 3β
H&E	Hematoxylin and eosin
h	Hour/hours
hCG	Human chorionic gonadotropin
HEK	Human embryo kidney
HMG	High mobility group
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
JAK/STAT	Janus kinase/signal transducer and activator of transcription
Klf4	Krüppel-like factor 4
Klf4-MFFs	Klf4 transduced MFFs
KO-DMEM	Knock Out DMEM
LIF	Leukaemia inhibitory factor
LIFR	LIF receptor
LTR	Long terminal repeat

M	Molar
mESCs D	mESCs differentiated
mESCs	Mouse ESCs
MFFs	Mouse foetal fibroblasts
mg	Milligram/milligrams
min	Minute/minutes
ml	Millilitre/millilitres
mM	Millimolar
MoML retrovirus	Moloney Murine leukaemia retrovirus
MoMLV vector	Moloney Murine leukaemia viral vector
Neo	Neomycin phosphotransferase
NTC	No template control
Oct4	Octamer-binding transcription factor 4
Oct4-MFFs	Oct4 transduced MFFs
OFFs	Ovine foetal fibroblasts
pdt	Population doubling time
PFFs	Porcine foetal fibroblasts
PGK	Phosphoglycerate kinase
PI3K	Phosphoinositide-3 kinase
p-STAT3	Phospho-STAT3
rad	Radiation absorbed dose
Ras/ERK	Extracellular-signal-related kinase
rpm	Rotations per minute
RSV	Rous sarcoma virus
RT-PCR	Reverse transcriptase - polymerase chain reaction
s	Second/seconds
SCID	Severe combined immunodeficient
SCNT	Somatic cell nuclear transfer
Sox2	Sex determining region Y box 2
Sox2-MFFs	Sox2 transduced MFFs
SR	Knock-out serum replacement
TBS	Tris buffered saline,
TBST	Tris buffered saline, tween 20
U	Unit/units
Ubi	Ubiquitin
v/v	volume/volume
w/v	weight/volume
µg	Microgram/micrograms
µl	Microlitre/microlitres
µM	Micromolar

CHAPTER 1

General introduction

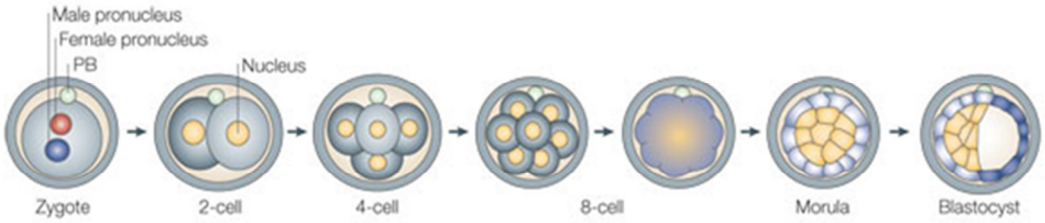
1.1 The embryo

The embryo is generated by the fecundation of the female gamete (oocyte) by the male gamete (sperm). Approximately 24 hours after fertilization the so generated zygote starts dividing exponentially until the morula stage, when compaction happens and cells on the outer part of the morula become bound tightly together with the formation of desmosomes and gap junctions; previously discrete cells become indistinguishable. A central cavity, the blastocoele, begins to form and the embryo becomes a hollow ball of cells, the blastocyst. At the morula-blastocyst stage the first event of differentiation occurs: two distinct cell populations arise; the cells remaining in contact with the outside are destined to form the trophoblast, while the cells inside will form the inner cell mass, ICM (Fig. 1). The trophoblast will give rise to most of the extra embryonic tissues, while the ICM will give rise to the whole animal and some more embryonic tissues. While the embryo develops, the ICM cells continue dividing and are responsible for the second event of differentiation: the cells facing the blastocoele become primitive endoderm, which will form the extraembryonic endoderm, while the inner cells will give rise to the epiblast, which is the precursor of the whole individual (Zernicka-Goetz, 2005).

1.2 Embryonic stem cells

1.2.1 What are stem cells?

Stem cells are defined as undifferentiated cells presenting two main characteristics: self-renewal and the ability to differentiate into somatic cell types (Fig. 2).



Zernicka-Goetz, 2005. Nature Reviews Molecular Cell Biology, 6: 919-928.

Figure 1. A representation of the early stages of embryo development: once fertilised, the zygote starts dividing becoming morula first and then blastocyst. All stages are shown in the same orientation. In the zygote, the female pronucleus is labelled in red, while the male pronucleus is labelled in blue. The second polar body is present at the animal pole. From the 2-cell stage to the beginning of the 8-cell stage all blastomeres are round, but then they flatten giving rise to the process of compaction and undergo apical–basal polarization (shown by blue to yellow gradient). Yellow circles represent the cell nuclei, which are shown only up to the 8-cell-stage. At the morula, the embryo consists of both inside (yellow) and outside (light blue) cells. At the blastocyst, inner cells form the inner cell mass (yellow), which is surrounded by polar trophectoderm (light blue) whereas the mural trophectoderm (darker blue) surrounds the cavity.

Self-renewal is the ability of the cells to go through numerous cycles of division while maintaining the undifferentiated state; differentiation occurs when the undifferentiated cells give rise to one or more specialized cells. According to the types of differentiated cells that can be generated from them, stem cells are classified into different categories:

- totipotent stem cells: cells that can differentiate into all embryonic and extra embryonic cell types (e.g. fertilized egg);
- pluripotent stem cells: they can develop into any cell of the three germ layers (endoderm, mesoderm, ectoderm), but are not able to generate extraembryonic tissues (e.g. cells from the epiblast);
- multipotent stem cells: cells that can give rise to cell types only within one particular lineage (e.g. hematopoietic stem cells);
- unipotent stem cells: they can only differentiate into one cell type, but have the property of self-renewal which distinguishes them from terminally differentiated cells (e.g. keratinocyte stem cells).

Stem cells are also generally divided into two overall classes: embryonic stem cells (ESCs) and adult stem cells (ASCs). As the names suggest, ESCs are stem cells derived from the embryo, while ASCs are present in the postnatal animal, where they are responsible for the replacement of tissues during the normal turnover or as a result of injury (Dor and Melton, 2004). ASCs have a restricted differentiation potential compared with ESCs, since they can often give rise only to cells belonging to one specific tissue. While ASCs are present in an organism for most of its life,

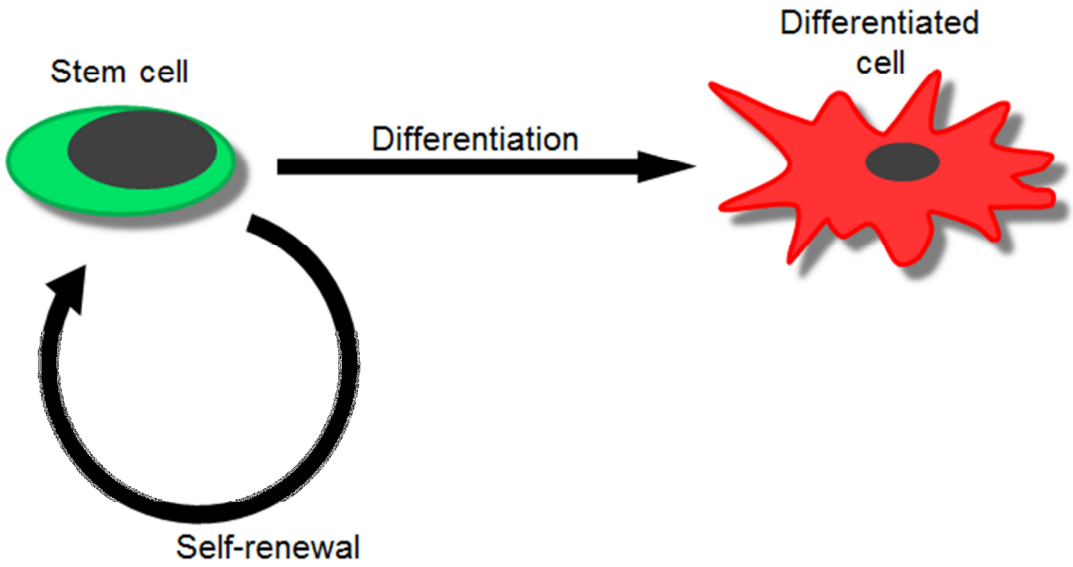


Figure 2. A schematic diagram of the key features of stem cells: stem cells (green in the image, are round and are characterised by a high nuclear-to-cytoplasmic ratio) can proliferate maintaining their undifferentiated status or differentiate towards more specialised cells (red in the figure, usually have a more irregular shape and are characterised by a bigger cytoplasm).

ESCs exist only transiently in the blastocyst, meaning that they do not exist as cells *in vivo*, but are only defined as a cell line *in vitro*.

1.2.2 Embryonic stem cells

ESCs belong to the class of pluripotent stem cells and can give rise to any cell type of an adult animal (Smith, 2006). ESCs are cell lines derived from the ICM of the blastocyst.

ESC isolation was the outcome of earlier observations about the ability of the embryo to give rise to tumours composed of different cell types (teratocarcinomas) after a graft into a mouse (Stevens, 1968; Solter, 1970; Stevens, 1970) and was supported by the evidence that embryonal carcinoma cell lines cultured in the presence of fibroblasts were characterised by a broad differentiation potential.

Although ESCs derive from the ICM, they are not identical to those cells: *in vivo* pluripotent cells exist only transiently, while *in vitro* ESCs represent cell lines which can be kept indefinitely. The maintenance in culture changes ESC properties; ESCs are therefore a useful model for the understanding of the processes unfolding in the early embryo, but are not entirely equivalent to the cells in the ICM.

After isolation, ESCs are normally characterised following a precise protocol (Robertson, 1987):

- morphological criteria: colonies must be round and domed, with defined edges; cells must have a high nuclear to cytoplasmic ratio (Robertson, 1987);

- immunocytochemistry and/or RT-PCR to evaluate the expression of markers of the pluripotency (e.g. Nanog, Oct4, Sox2) and the absence of markers typical of more differentiated cells (Robertson, 1987);
- *in vitro* differentiation followed by immunocytochemistry and/or RT-PCR to confirm that the cells can give rise to differentiated cells belonging to all three germ layers (Doetschman, 1985);
- *in vivo* differentiation to verify whether the cells can differentiated into the three lineages even in a more complex biological system;
- injection into blastocysts to show that ESCs can contribute to different tissues of an adult animal, forming chimeras (Nagy, 1990);
- germ line transmission: it means that, after generating a chimera, ESCs are found in the germ cell population of the adult animal and can generate offspring;
- tetraploid complementation: ESCs are inoculated in tetraploid embryos; the foetus will be exclusively derived from the ESCs, while the extraembryonic tissues are exclusively derived from the tetraploid cells (Nagy, 1993).

The last two evidences are considered definitive proof of pluripotency.

Authentic ESCs have been derived only from a few species; mouse ESCs were generated in 1981 and were the first pluripotent stem cells to be maintained in culture (Evans and Kaufman, 1981; Martin, 1981); rat ESCs were obtained much later, in 2008 (Buehr, 2008; Li, 2008). ESCs in both species have demonstrated their ability

to grow for virtually unlimited time and have passed the key test for pluripotency: the germline transmission (Bradley, 1984; Bradley, 1987; Li, 2008).

Human ESCs have been derived too; yet their characteristics and culture conditions significantly differ from those employed for mouse and rat. This has raised doubts about the true nature of their pluripotency, but, for obvious reasons, chimera formation, germline transmission or tetraploid complementation have never been assessed.

Many putative ESCs from other animals (e.g. pig, sheep, cow, horse, rabbit, dog, cat) have been described (Chen, 1999; Wang, 2005; Wobus and Boheler, 2005; Dattena, 2006; Li, 2006; Vackova, 2007; Wang, 2007; Hayes, 2008; Gómez, 2010) but they lack of the final evidence of their pluripotency. Many ESC-like cells have been isolated either, but those cells, although pluripotent in the first few passages, soon differentiated (Saito, 1992; Dattena, 2006; Gómez, 2010).

Because of their unique features, ESCs have drawn a lot of attention and have become a valuable tool in research and for clinical application. They represent a robust model to study the mechanism underlying pluripotency and can be used to better understand biochemical pathways that drive differentiation and senescence. The knowledge of how the cell decide their fate represents the first step for the comprehension of developmental biology; this will eventually lead to gaining the ability to manipulate stem cells to achieve our goals.

ESCs can provide an *in vitro* model of mammalian development and can represent a valuable system for drug discovery and for toxicology tests and in the future may reduce animal testing. Because of their limitless proliferation ability, ESCs

theoretically provide a limitless source of cell types which could be used to treat degenerative diseases such as type I diabetes or Parkinson's disease (Gardner, 2007). These cells have revolutionized the field of transgenesis allowing the selection of mutant cells before senescence sets in, thus making possible the creation of cell lines carrying specific mutation typical of different disease (Gossler, 1986; Liew, 2005; Alenzi, 2010). Due to their ability to contribute to adult animals and go germline, ESCs have made also possible the generation of knockout or knock-in animals as models of human disease (Ben-Nun and Benvenisty, 2006) or to produce humanized tissues for xenotransplantation (Yamada, 2005; Oropeza, 2009).

ESCs, however, undergo several limitations due to the difficulty of their derivation. True ESCs have been derived from mouse (Evans and Kaufman, 1981; Martin, 1981), rat (Buehr, 2008; Li, 2008) and human (Thomson, 1998), even though controversies regard the exact definition of the latter (Brons, 2007; Tesar, 2007). The derivation of ESCs from other species have been hampered mainly by technical problems: the conditions for the isolation of ESCs vary among species; in order to find the correct cocktail of cytokines and growth factors able to sustain pluripotency many attempts must be carried out. So a large number of embryos must be employed in order to pursue and identify the suitable conditions; this can be easy in case of the small laboratory animals, such as mouse and rat, that produce a large amount of embryos with little cost and little time limitations. However, the generation of embryos from large animals is time consuming and very expensive and often the blastocysts recovered are very few; furthermore many large animals such as sheep or

horse, are seasonal, so the access to their embryos is limited only to a part of the year.

In parallel the use of human ESCs has given rise to an important debate about moral and religious issues: since these cells are created from an embryo, for those who believe that the embryo has the moral status of a person from the moment of conception, research or any other activity on the blastocyst means the destruction of a human life and is therefore ethically wrong.

The use of ESCs as a source of tissues for the use in therapy is hampered by the difficulty to differentiate the cells towards a specific fate, by issues such as immune rejection and by the risks associated with the difficult selection of the differentiated cells to be injected for the therapy: even a little carry-over of pluripotent cells in the organism might induce cancer.

1.3 Mouse embryonic stem cells

The first mouse ESCs were derived in 1981 by two independent groups, which reported the direct isolation of pluripotent stem cell lines from the mouse embryo (Evans and Kaufman, 1981; Martin, 1981). Mouse ESCs were first derived by plating ICMs isolated from expanded blastocyst on a feeder layer composed of mitotically inactivated fibroblasts in medium conditioned by teratocarcinoma stem cells.

1.3.1 LIF/BMP4 medium

While conditioned medium was first used to derive and grow ESCs, with time the requirements for the maintenance of pluripotency were studied and better

understood: the feeder layer was found to be dispensable when the cells were cultured in medium containing leukaemia inhibitory factor, LIF (Smith, 1988; Williams, 1988) and, in 2003, the first defined conditions for mouse ESC culture were established. A serum-free medium containing the supplements N2 and B27 (Ying, 2003) and supplemented with LIF and BMP4 was sufficient for the derivation and propagation of pluripotent ESCs.

Leukaemia inhibitory factor. LIF, a member of the IL6 cytokine family, fulfils its role by binding to the glycoprotein 130 (gp130) which then heterodimerizes with LIF receptor (LIFR); both gp130 and LIFR are members of the cytokine receptor family. This interaction leads to the recruitment and subsequent activation of at least three different pathways: JAK/STAT, Ras/ERK and PI3K (Fig. 3).

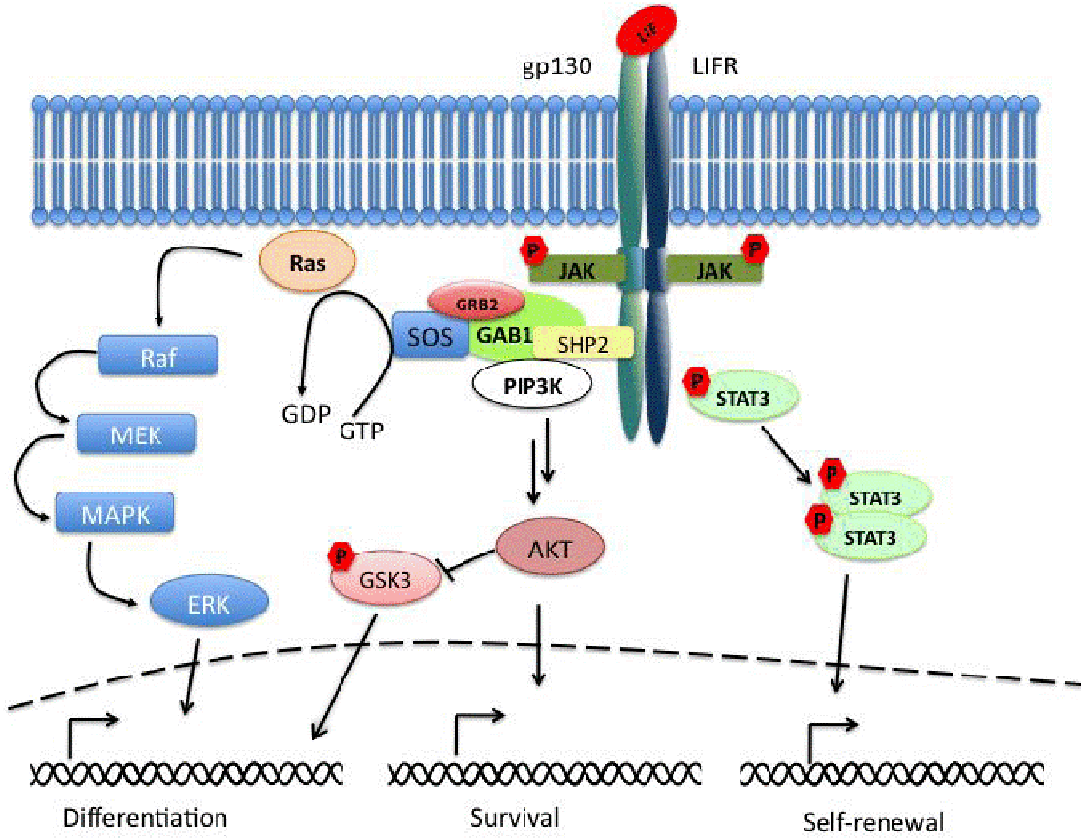
JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway: the activation and autophosphorylation of the tyrosine kinase JAK promotes STAT3 phosphorylation and dimerization. STAT3 homodimers translocate to the nucleus where they bind to a consensus DNA sequence in the promoter of its target genes to regulate transcription and promote self-renewal.

Although many STAT3 target genes still remain elusive, some of them have been identified; cMyc (Kiuchi, 1999) for example has a significant role in self-renewal: its constitutive expression causes ESC self-renewal to be independent from LIF (Cartwright, 2005). Myc also confers increased proliferative capacity to the cells, acting through the induction of the regulatory subunit of the telomerase and being responsible for changes in chromatin organization (Eilers, 1991; Adhikary and

Eilers, 2005). Another identified target of STAT3 is the transcription factor Klf4 (Li, 2005), which is a zinc finger transcription factor that contributes to self-renewal through a dual mechanism: Klf4 increases expression of Oct4 (Jiang, 2008), which is a master regulator of pluripotency, and downregulates p53 expression (Rowland, 2005), which, in turns, blocks the expression of Nanog (Lin, 2005), another key regulator of pluripotency (Zindy, 1998).

Ras/ERK (extracellular-signal-related kinase) pathway: in addition to STAT3, LIF can also activate the Ras/ERK pathway, which starts from the activation of the GTPase Ras, with subsequent activation of Raf, MEK and finally ERK, whose role is the stimulation of the transcription of target genes. In contrast to STAT3, the Ras/ERK pathway is responsible for cellular differentiation through inhibition of Nanog expression (Burdon, 1999; Hamazaki, 2006). Despite this, ESCs can maintain their pluripotency because the Ras/ERK pathway is suppressed by BMP4 (Qi, 2004).

PI3K (phosphoinositide-3 kinase) pathway: when activated by the dimerization of gp130 and LIFR, PI3K maintains self-renewal by activating Akt (Watanabe, 2006), a protein kinase B (PKB) that inhibits glycogen synthase kinase 3β (GSK3β). GSK3β is a major inhibitor of the Wnt/β-catenin pathway, which is involved in the maintenance of pluripotency (Sato, 2004; Hao, 2006; Ogawa, 2006) furthermore, GSK3β activates p53 which, as already mentioned, promotes differentiation of mouse ESCs by suppressing Nanog expression (Lin, 2005; Liu, 2007b). AKT is also involved in survival and proliferation because it promotes a quick G1 to S phase transition by facilitating the formation of cyclin/CDK complexes (Brazil, 2004).



Modified from Graf et al., 2011. Genes, 2: 280-297.

Figure 3. The figure summarises the main pathways that are stimulated by the interaction between LIF with its receptor in mouse ESCs. The binding of LIF to the LIFR induces its heterodimerization with gp130. The formation of this complex results in the activation of the JAKs and finally in the recruitment of Src homology-2 (SH2) domain containing proteins such as STAT3. When bound to the receptor, STAT3 directly targets gene promoters by homodimerizing and translocating to the nucleus of the cell, without the need for second messengers.
In parallel to the activation of the STAT3-pathway, the binding of LIF to the its receptor leads to the activation of the mitogen-activated protein kinase (MAPK) and the PI3K pathways. Active gp130 receptor associates with the protein tyrosine phosphatase SHP-2 and leads to the recruitment of GRB2-associated-binding protein 1 (Gab1). The so formed complex starts a phosphorylation cascade that ends in the activation of the ERK kinases.
LIF binding to the LIFRβ/gp130 receptor also induces the activation of PI3Ks, which mediate signal transduction through downstream effector molecules including the serine/threonine protein kinase B AKT. The PI3K/AKT-pathway control the GSK3β phosphorylation thereby regulating its inactivation.

Bone morphogenetic protein 4. Mouse ESCs grown in the presence of LIF, but in the absence of serum differentiate towards the neuronal lineage. This means that LIF does not inhibit neural differentiation, but an additional signal is required to suppress the differentiation and sustain self-renewal. This signal is provided by BMP4, which acts inhibiting the Ras/ERK pathway and activating the Smad transcription factors. This leads to the expression of the inhibitor of differentiation protein, which blocks the neural differentiation (Ying, 2003; Gerrard, 2005).

1.3.2 3i medium

For almost thirty years the medium supplemented with LIF and serum or BMP4 had been the only culture condition known to maintain pluripotency in mouse ESCs. In 2008, however, Ying et al (Ying, 2008) reported that ESCs do not require any external signalling, but have an intrinsic plan for the maintenance of pluripotency. This was evidenced with the introduction of the 3i medium, which is based on N2B27 and is supplemented with three inhibitors (PD184352, SU5402 and CHIR99021) is able to maintain ESC self-renewal without the addition of LIF or BMP4. The medium bases its effect solely upon inhibition of the differentiation pathways and the stimulation of the proliferation. PD184352 and SU5402 both act on the ERK pathway: the former is a MEK inhibitor, while the latter operates upstream, blocking the activation of the FGF receptor in response to the binding with its ligand. This prevents the cells to respond to FGF signalling cascade, which consists in the activation of the ERK and PI3K pathways. While PD184352 and SU5402 maintain pluripotency, CHIR99021 improves the proliferation ability of the cells, inhibiting GSK3.

Later an optimised medium named 2i medium, based on only two inhibitors (PD0325901 and CHIRON99021) was developed: in the new medium PD184352 and SU5402 were replaced by a more potent MEK inhibitor, which alone proved to be sufficient to switch off the ERK pathway.

Surprisingly the STAT3 signalling, which was considered the key pathway involved in the maintenance of pluripotency, is dispensable when ESCs are grown in 3i or 2i media, as demonstrated by the derivation of mouse ESCs from STAT3-null homozygous mutant embryos (Ying, 2008).

1.3.3 Transcriptional network controlling pluripotency

Pluripotency is sustained through the tight regulation of gene expression which is coordinated by a small group of key transcription factors: Oct4, Sox2 and Nanog (Fig. 4).

Oct4 and Sox2 form a complex which can bind enhancer elements close to each other in the promoter of target genes. One of the most important targets of Oct4-Sox2 complexes is Nanog (Rodda, 2005). When Oct4 is expressed at physiological levels Nanog transcription is activated, whereas when Oct4 is overexpressed Nanog is not transcribed (Rodda, 2005; Chambers and Tomlinson, 2009). Additionally both Sox2 and Oct4 promoters contain the Oct4-Sox2 complex recognition elements; this means that they can reciprocally regulate their own expression (Tomioka, 2002; Chew, 2005). Sox2 also sustains Oct4 transcription by indirectly regulating the expression of other transcription factors that affect Oct4. Oct4 regulates itself too: when its expression levels rise above the steady state level, Oct4 represses its own promoter in a negative feedback regulation loop (Pan, 2006).

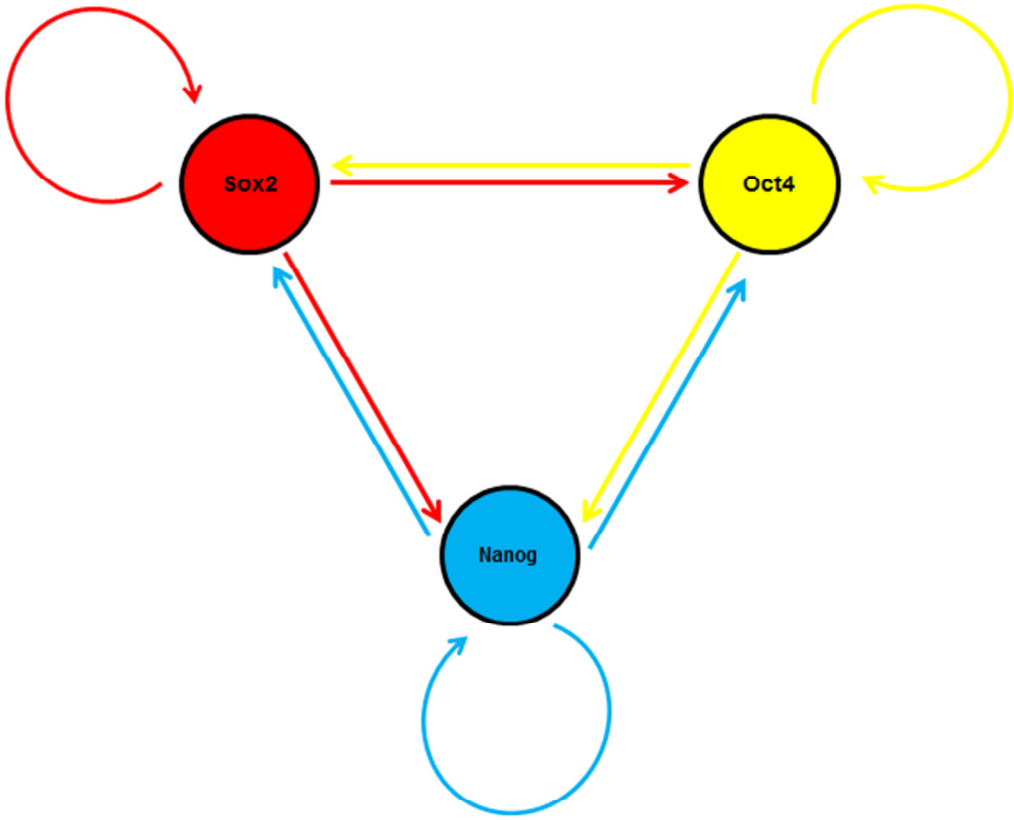


Figure 4. Oct4 (in the yellow circle), Nanog (in the blue circle) and Sox2 (in the red circle) are the master regulators of pluripotency and act in a complex network regulating their own and each other expression (auto-regulatory and cross-regulatory transcriptional loops are shown with arrows; the interactions carried out by Oct4 are represented by the yellow arrows, the interactions carried out by Nanog are represented by the blue arrows and the interactions carried out by Sox2 are represented by the red arrows). The balance of their activation and inhibition maintains ESCs in the pluripotent state.

This inhibits Oct4 expression, which in turn represses Nanog transcription. Conversely, Nanog and FoxD3 function as activators for Oct4 expression.

Oct4 (octamer-binding transcription factor 4) belongs to the family of POU (Pit, Oct, Unc) transcription factor, which is characterized by a bipartite DNA binding domain called POU domain (Scholer, 1991a; Herr and Cleary, 1995). The POU domain consists of two structurally independent subdomains: an N-terminal POU specific region (POUs), which is unique to each POU factor, and a C-terminal homeodomain (POUh) (Scholer, 1991a). These two domains are connected by a variable linker, both can make specific contacts with DNA through a helix-turn-helix structure and have a role in transactivation (Vigano and Staudt, 1996; Brehm, 1997), but while the activity of the C-domain is cell type specific and is regulated through phosphorylation, the activity of the N-domain is not (Brehm, 1997; Brehm, 1998; Brehm, 1999).

In mouse Oct4 is expressed specifically in pluripotent and totipotent cells: *in vivo* it is expressed in the embryo from the embryonic transcriptional activation till the ICM and then the epiblast of the blastocyst stage; Oct4 expression is later restricted to the primordial germ cells and undergoes downregulation during oogenesis and spermatogenesis (Pesce, 1998b; Pesce and Scholer, 2001). *In vitro* Oct4 is upregulated in ESC lines and carcinoma cell lines (Pan, 2002).

Studies suggest that the expression pattern and function of Oct4 are very similar between mouse and human cells (Hansis, 2000; Hansis, 2001; Hay, 2004).

Oct4 expression is continuously required for the maintenance of pluripotency: Oct4 downregulation is associated with ESC differentiation and Oct4 reactivation occurs

in somatic cells after cell fusion with ESCs (Tada, 2001; Kimura, 2004) or after nuclear transfer into oocytes (Boiani, 2002; Bortvin, 2003). Oct4 controls the pluripotency of stem cells in a quantitative fashion and very small changes in its expression level can have significant impact on cell fate and growth properties (Niwa, 2000; Niwa, 2001; Hay, 2004): Oct4 maintains pluripotency in ESCs when expressed at steady level, but promotes cellular differentiation into endoderm and mesoderm when expressed 1.5 fold higher than normal; a greater than 0.5 fold reduction of Oct4 level causes cells to differentiate into trophoblast instead (Niwa, 2000). This is consistent with the observation that embryos lacking Oct4 form outgrowths consisting entirely of trophoblast-like cells when plated on gelatin (Nichols, 1998). Although Oct4 is a master regulator of pluripotency, its expression alone is not sufficient: removal of LIF results in differentiation even if Oct4 is maintained at a proper level by an inducible transgene (Niwa, 2001).

Oct4 is a transcription factor that can both activate and repress target genes (Pan, 2002). Oct4 promotes transcription through two main mechanisms: by directly binding to POU sites immediately upstream to the promoter, where it can synergize with other transcription factors, or by binding sites located at a considerable distance from the target gene; in this case adaptor proteins must be involved to bridge Oct4 to the basic transcription machinery near the target gene. Oct4 represses gene expression either directly, by binding to promoters or indirectly, by neutralizing activators such as FOXD3.

Oct4 target genes are classified into three groups based on their response to Oct4: activated, repressed or activated and repressed. The last group includes those genes

that are activated at physiological levels, but are repressed at high levels of Oct4 (Scholer, 1991b).

Many Oct4 target genes have been identified: fibroblast growth factor 4 (FGF4), osteopontin, Sox2, Utf1, Rex1, Fbx15, Nanog, Cdx2, INFτ and both α and ß forms of human chorionic gonadotropin (hCG) (Pesce, 1998a; Okumura-Nakanishi, 2005; Liu, 2007a).

FGF4 is expressed by the ICM and is necessary for development shortly after implantation (Feldman, 1995); Oct4 activates this element synergistically with Sox2 (Yuan, 1995).

Oct4 together with Sox2 also regulates Nanog expression: analyses of Nanog 5' promoter region have revealed a composite Oct4/Sox2 site upstream of the transcription start which is important for Nanog regulation (Kuroda, 2005; Rodda, 2005): when Oct4 is at a physiological level, Nanog transcription is activated, whereas when Oct4 is above the normal level Nanog expression is repressed.

Oct4 acts as a repressor of the genes coding for α and ß hCG (Liu, 1997) and for Cdx2 (Niwa, 2005), a marker of trophoblast; indeed when Oct4 is inhibited in ESCs, the cells differentiate towards the trophoblast lineage (Niwa, 2005; Pan, 2006). When Cdx2 is overexpressed, however, differentiation in trophoblast happens even though the levels of Oct4 are physiological; this also suggests that that loss of Oct4 expression is not required for differentiation.

Finally, Oct4 regulates itself: when its expression levels rise above steady state levels, Oct4 represses its own promoter in a negative feedback regulation loop (Pan, 2006). This prevents the increase of Oct4 expression, thus sustaining pluripotency.

Sox2 (Sex determining region Y box 2) is a member of the SRY-related high mobility group (HGM) box family of transcription factors (Gubbay, 1990; Sinclair, 1990; Wegner, 1999).

Sox2 expression pattern is similar to that of Oct4; with Sox2 detectable from the oocyte to the blastocyst stage, and later becomes restricted to the ICM and the epiblast and finally to the germ cells and the neural stem cells (Li, 1998; Zappone, 2000; Avilion, 2003). Sox2 downregulation correlates with a commitment to differentiate, since this protein is no longer expressed in cell types with restricted developmental potential (Avilion, 2003).

Sox2 protein interacts with DNA through the highly conserved HMG domain. Whereas the overall conformation of the HMG domain remains unaltered upon DNA binding, a large conformational change is induced into target DNA, such that its minor groove follows the concave binding surface of the HMG domain perfectly. As a consequence, DNA bound by Sox proteins is helically unwound relative to classical B-DNA (Ferrari, 1992; Weiss, 2001).

Sox2 protein regulates different sets of target genes depending on the cell type in which the protein is expressed: it acts as a transcriptional activator after forming a protein complex with other proteins such as Oct4, Nanog and Rex1; such partner proteins are specifically expressed in different tissues, therefore, depending on the tissue, different sets of genes will be activated (Kamachi, 2000; Smith, 2001b; Shi, 2006).

Nanog was identified independently by two groups; through a functional cDNA screening for genes conferring LIF-independent self-renewal (Chambers, 2003) or

through differential expression analysis in the other (Mitsui, 2003). Nanog is expressed first in the morula, then in the ICM of the blastocyst. Nanog levels decrease in the late blastocyst where it become restricted to the epiblast. Nanog is also detected in primordial germ cells during the migration and the colonisation of the genital ridges, but is later downregulated.

Nanog is a homeobox domain-containing protein expressed specifically in pluripotent cells. It acts as both transcription repressor of genes important in differentiation such as Gata4 and Gata6 (Chambers, 2003; Mitsui, 2003) and transcription activator of genes essential for self-renewal like Rex1(Shi, 2006) and Oct4 (Pan, 2006).

Before the development of the 3i medium, Nanog was thought to hold a key role in maintaining the pluripotency of mouse ESCs. Indeed when Nanog is downregulated, ESCs acquire a morphology and express markers typical of endoderm, while its overexpression alone is able to sustain the undifferentiated state of the cells without the supplementation of the culture medium with LIF. This ability, however, requires a continued physiological expression of Oct4, as the downregulation of Oct4 leads to differentiation of the cells even when Nanog is upregulated (Cavaleri and Schöler, 2003; Chambers, 2003). Although LIF is dispensable for pluripotency when Nanog is overexpressed, self-renewal occurs with higher efficiency when the cells are also stimulated with LIF; this indicates that Nanog and LIF function in parallel (Chambers, 2003).

Although Nanog null cells retain the ability to contribute to chimeras, they cannot contribute to the germline, suggesting that Nanog may play a role in establishing the pluripotent state during normal development. In ESCs Nanog function might involve

the support of self-renewal by protecting the pluripotent state of the cells (Chambers, 2007). This is consistent with the reduction in spontaneous differentiation observed upon forced expression of Nanog (Chambers, 2003).

1.4 Epiblast stem cells

In 2007 a novel type of stem cells, named epiblast stem cells (EpiSCs), was derived from the mouse embryo. While mouse ESCs are derived from the ICM of the pre-implantation blastocyst, EpiSCs were isolated from the post-implantation epiblast (Brons, 2007; Tesar, 2007). The EpiSCs are able to self-renew, express numerous markers of pluripotency such as Oct4, Nanog and Sox2 at the same level of ESCs and can differentiate *in vitro* when grown in suspension as aggregates (Brons, 2007; Tesar, 2007); moreover EpiSCs can form, after injection into SCID mice, teratomas composed of many differentiated cell types, (Brons, 2007; Tesar, 2007). These results indicate that EpiSCs have a wide developmental ability.

Many features of EpiSCs, however, differ considerably from those of ESCs: EpiSC colonies are large and grow flat, while mouse ESCs typically grow in small, compact, domed colonies; unlike ESCs, EpiSCs are Rex1 negative and express FGF5 and Nodal; while both X chromosomes are active in ESCs (Brons, 2007), EpiSCs correspond to a stage when X inactivation has already been initiated (Wutz, 2011). Most importantly, chimeric mice can be efficiently generated after injection of mouse ESCs into the pre-implantation embryo, while EpiSCs can generate chimeras at very low efficiency and germline transmission has not been observed yet (Brons, 2007).

EpiSCs rely on the supplementation of basic fibroblast growth factor (bFGF) and activin to proliferate and maintain their undifferentiated marks (Fig. 5). Removal of one of these molecules, in fact, leads to differentiation promoting rapid neural induction and subsequent neurogenesis (Greber, 2010; Sterneckert, 2010). Activin pathway promotes self-renewal of EpiSCs via activation of Nanog through activation of the SMAD2/3 transcription factors; on the other hand bFGF acts by activating the ERK pathway, which, instead of promoting differentiation as in mouse ESCs, inhibits lineage commitment (Greber, 2010).

Initially it was believed that the differences observed between EpiSCs and ESCs were due to the developmental stage from which they were derived, but recently Najm et al (Najm, 2011) reported the isolation of EpiSCs from a pre-implantation embryo when culturing the ICM in bFGF and activin instead of LIF and BMP4 (Najm, 2011). Additionally post-implantation-derived EpiSCs are able to revert into ESCs in response to LIF signalling (Bao, 2009). This process is characterised by the erasure of key properties of epiblast cells, resulting in DNA demethylation, X reactivation and expression of E-cadherin; furthermore the reverted cells are able to give chimera contribution in both somatic tissues and germ cells (Bao, 2009).

1.5 Human embryonic stem cells

Mouse ESCs had been the only pluripotent cell line available for many years, until 1998, when Thomson et al (Thomson, 1998) developed a technique to isolate human ESCs. Initially human ESCs were grown in conditions very similar to those employed in the first mouse ESC derivation, however it was soon clear that human

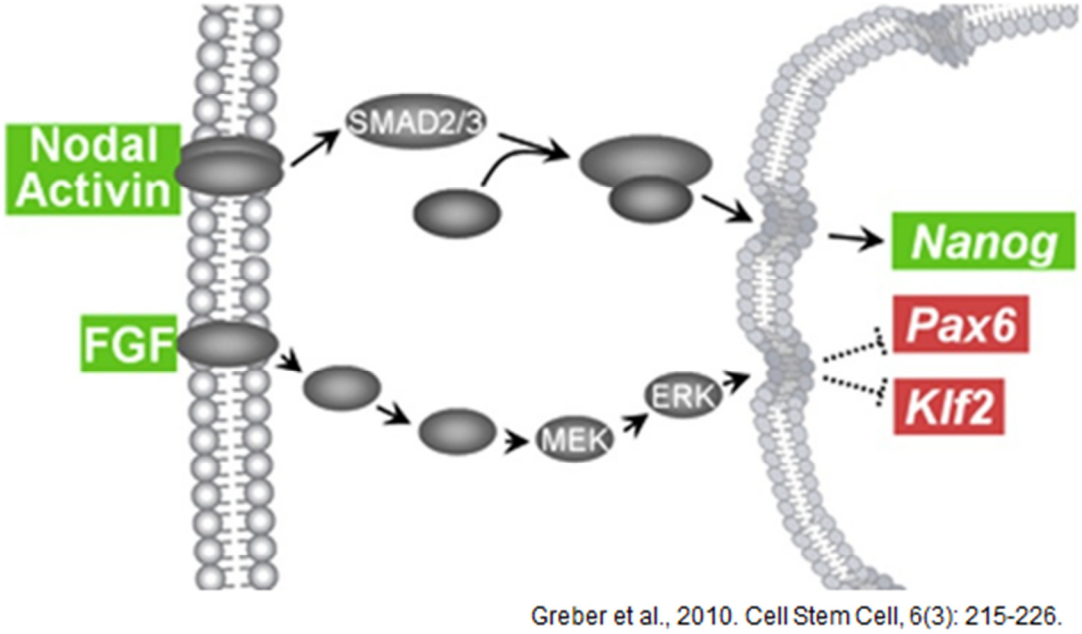


Figure 5. The main pathways activated in mouse EpiSCs: activin promotes Nanog expression, while FGF stimulates proliferation and inhibits cellular differentiation: the Activin pathway promotes self-renewal of EpiSCs via direct activation of Nanog, whereas inhibition of this pathway induces neuroectodermal differentiation, like in hESCs. FGF signalling appears to stabilize the epiblast state by inhibiting the differentiation towards neuroectoderm the reversion to a mouse embryonic stem cell-like state.

ESCs could not be maintained in feeder-free culture utilising the same mouse conditions: indeed the supplementation of medium with LIF was not able to inhibit the differentiation (Thomson, 1998; Reubinoff, 2000; Daheron, 2004; Humphrey, 2004). Currently, when human ESCs are grown in absence of feeder layer the medium employed consist in a serum-free culture medium supplemented with bFGF and activin (Beattie, 2005).

Like mouse ESCs, human ESCs have the ability to self-renew for an undefined time and to differentiate towards somatic cells belonging to different lineages. They are both characterised by a high nuclear to cytoplasmic ratio (Wobus, 2001). They also have a similar expression pattern: they both show high AP levels, high telomerase activity and Nanog, Oct4 and Sox2 expression.

Although there are many properties in common between mouse and human ESCs, many other features are different. Human ESCs, unlike mouse ESCs, form large, flattened colonies and although many cellular markers overlap with those of mouse ESCs, others are different. Murine ESCs express Rex1 and SSEA1, while human ESCs express these markers upon differentiation (Draper and Fox, 2003). On the other hand human ESCs express SSEA3 and SSEA4, while their mouse counterparts do not. In addition human ESCs survive poorly when dissociated into single cells and exhibit X chromosome inactivation, while in mouse ESCs tolerate clonal densities and both X chromosomes are active.

Those differences between human and mouse ESCs have raised many questions regarding the equivalence of the cells coming from the two species and in particular about the true stemness of human ESCs. The gold standard for pluripotency is

contribution of the ESCs to chimeras and germ cells, but for human this cannot be tested.

The derivation of EpiSCs has underlined how human ESCs more closely resemble to these new cells than to mouse ESCs: human ESCs proliferate under the same culture conditions employed for EpiSCs and have an expression profile, a morphology and epigenetic marks more similar to that of those cells.

These observations have led to the belief that human ESCs and mouse ESCs represent two distinct pluripotency states: whereas mouse ESCs are the early, naïve, state, the human counterparts, as much as mouse EpiSCs, correspond to a later developmental stage.

1.6 Induced pluripotent stem cells

1.6.1 Reprogramming differentiated cells

Although ESCs are very useful tools in research and for clinical applications, the technical and ethical limitations related to their derivation have hampered their routine use.

In order to avoid these concerns, attempts to create pluripotent cells without the use of any embryo have been pursued. For a long time it had been uncertain whether it were possible to completely reverse the changes that occur during differentiation.

Somatic cell nuclear transfer (SCNT) before (Campbell, 1996) and cell fusion later (Cowan, 2005) have clearly demonstrated that the cytoplasm of totipotent and pluripotent cells contain sufficient information to modify the nucleus of somatic cells to convert them back to a pluripotent state. Reprogramming requires that epigenetic marks of the differentiated cells are modified, allowing the expression of genes that have been silenced during development and, vice versa, impeding the translation of genes switched on when the differentiation process had started.

1.6.2 Induced pluripotent stem cells

SCNT and fusion results have revealed that ESCs and oocyte cytoplasms contain proteins able to reprogram a somatic nucleus. Based on this assumption, Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) hypothesized that overexpression of specific pluripotent genes in differentiated cells could induce them to acquire embryonic properties. Twenty four factors, which were chosen among transcription factors, genes frequently upregulated in tumours as well as genes specifically expressed in ESCs, were selected as candidates for the direct reprogramming strategy. These genes were introduced in different combinations into mouse foetal fibroblasts (MFFs) and mouse tail tip fibroblasts by retroviral transduction with a Moloney murine leukaemia viral (MoMLV) vector. In order to assess whether the cells were able to acquire a more pluripotent state, fibroblasts were isolated from mice expressing a drug selection cassette (ßgeo) under the control of an ESC specific promoter (Fbx15), so that, if reprogramming had taken place, the fibroblasts would have shown β-galactosidase activity and become resistant to high concentrations of neomycin.

Among the 24 genes tested, 4 were found to be essential for the induction of pluripotency in somatic cells: cMyc, Klf4, Oct4 and Sox2. Colonies with morphology similar to that of ESCs and resistant to neomycin were generated. These cells, referred to as induced pluripotent stem cells (iPSCs), were tested for the salient features of authentic ESCs (Takahashi and Yamanaka, 2006). As ESCs, iPSCs exhibited immortal growth characteristics and were able to differentiate into the three germ layers *in vitro* and *in vivo*.

1.6.3 The four genes

Oct4 and Sox2 features and functions within the pluripotency network have been widely described above. During direct reprogramming the role of the exogenous expression of Oct4 and Sox2 might be to stimulate the basic transcriptional backbone necessary for the acquisition of the ESC-like properties. Klf4 is proposed to act in reprogramming as an upstream regulator of a larger loops addressing Oct4 and Sox2 (Li, 2005); furthermore Klf4 is thought to be a regulator of Nanog and a potential regulator of Myc, as it binds the cMyc promoter (Kim, 2008a). Klf4 also inhibits p53, regulaing cMyc induced apoptosis (Rowland, 2005). Additionally Klf4 maintains Nanog levels high, since p53 is a repressor of Nanog expression (Zindy, 1998). The last of the four reprogramming factors, cMyc, is not greatly involved in the activation of pluripotency regulators, but it plays an important role in the initial phases of reprogramming, in particular it is required to improve the efficiency of derivation of iPSCs, probably acting through epigenetic mechanisms (Fig.6).

cMyc (myelocytomatosis cellular oncogene) belongs to the Myc family of transcription factors, which also includes L-myc, N-myc, S-Myc and B-Myc.

Myc is characterized by an N-terminal transcription regulatory domain and by a C-terminal basic Helix-Loop-Helix Leucine Zipper (bHLH/LZ) domain, where the bHLH domain interacts with the DNA, while the LZ domain allows the dimerization of cMyc with other proteins (Steiger, 2008).

Myc transcription is activated upon various mitogenic signals via the ERK pathway, the Wnt pathway and the STAT pathway and works as a transcription regulator both activating and repressing transcription of its target genes (Henriksson and Luscher, 1996; Amati, 2001).

In vivo, cMyc needs to be associated with Max (Kretzner, 1992) in order to carry out its role: the heterodimerization is required for both activation and inhibition of transcription. Activation is achieved through the binding to enhancer box consensus sequences, while inhibition is realised through protein-protein interactions with other DNA-binding proteins (Roy, 1993; Shrivastava, 1993; Peukert, 1997; Gartel, 2001; Izumi, 2001; Staller, 2001; Herold, 2002; Mao, 2003).

Among its key target genes are p21, p15, p18, p27, p19, Bcl-x and Bcl2 (Zindy, 1998; Eischen, 2001; Adhikary and Eilers, 2005; Gartel and Radhakrishnan, 2005).

The protein p21, also known as CDKN1A, is inhibited by cMyc; p21 is upregulated by p53 after exposure to DNA damage and inhibits the activity of cyclin-CDK2 or cyclin-CDK4 complexes, preventing the cells to progress to S phase of the cell cycle (Gartel and Radhakrishnan, 2005). In addition, cMyc also represses the genes encoding for INK4 inhibitors (p15 and p18) and the gene encoding for p27; this leads to a strong enhancement of cyclin D-CDK4 followed by progression of cells to phase

G1 (Adhikary and Eilers, 2005). The suppression of the proteins involved in the control of the cell cycle is responsible for the proliferation of cells with pre-tumorigenic mutations as a result of the failure of the cells to go towards apoptosis after DNA damage (Herold, 2002; Seoane, 2002). Myc also blocks the anti-apoptotic Bcl-x and Bcl2 proteins, contributing to the release of cytochrome c (Eischen, 2001).

Myc activates several genes that encode for ribosomal proteins (Boon, 2001), translation factors (Rosenwald, 1993), proteins involved in the biogenesis and processing of ribosomal RNA (Greasley, 2000; Menssen and Hermeking, 2002), ribosomal RNA (Poortinga, 2004; Arabi, 2005; Grandori, 2005; Grewal, 2005), RNA-pol-III-dependent genes (Gomez-Roman, 2003) and non-coding RNA molecules (Cawley, 2004). Myc also probably acts through the induction of the regulatory subunit of the telomerase (Wang, 1998).

Beside its direct role in activation and repression of transcription, cMyc can also modulate the epigenetic state: it recruits histone acetyltransferases such as TIP60, GCN5 (McMahon, 1998), CREB-binding protein and p300 (Vervoorts, 2003) to its target genes (Bouchard, 2001; Frank, 2001). This enrolment is probably required for the transcriptional activation of these genes. Myc also recruits a DNA methyltransferase (DNMT-3a) to the Myc-Miz1 complex, indicating that Myc-dependent gene repression could at least partly be mediated by methylation of its target promoters (Brenner, 2005).

Klf4 (Krüppel-like factor 4), also known as gut-enriched Krüppel-like factor (gKLF), belongs to the Kruppel-like factor family, which is an evolutionarily conserved family of zinc finger transcription factors.

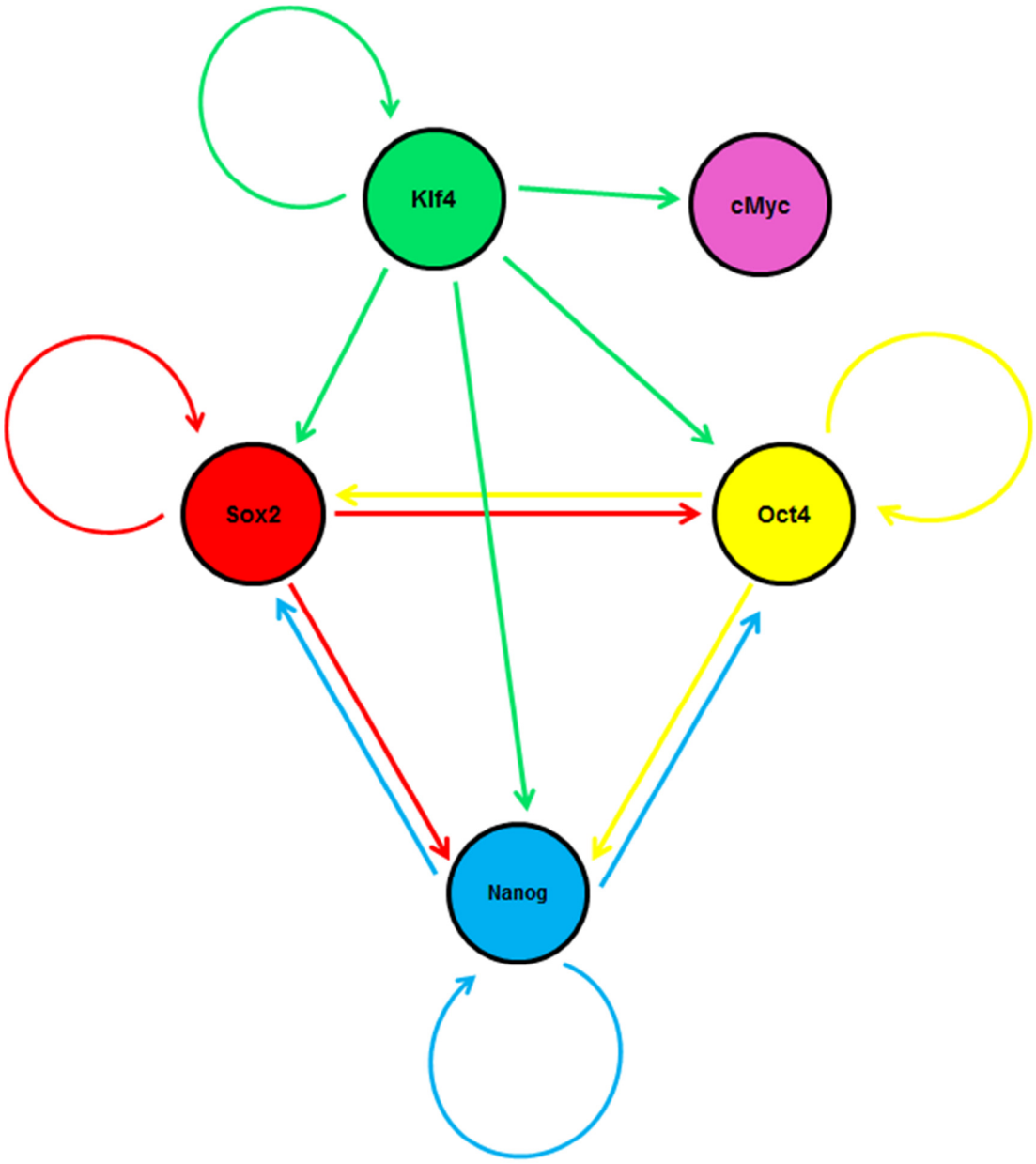


Figure 6. The figure shows how the reprogramming factors interact with each other to induce pluripotency in somatic cells. Oct4 (in the yellow circle) and Sox2 (in the red circle) act in a complex network regulating their own and each other expression; Klf4 (in the green circle) stimulates the key pluripotency genes such as Oct4, Sox2 and Nanog (in the blue circle); cMyc (in the purple circle) is responsible of the augmented proliferation and loss of senescence of the cells (auto-regulatory and cross-regulatory transcriptional loops are shown with arrows; the interactions carried out by Oct4 are represented by the yellow arrows, the interactions carried out by Nanog are represented by the blue arrows, the interactions carried out by Sox2 are represented by the red arrows and the interactions carried out by Klf4 are represented by the green arrows). The induced expression of cMyc, Klf4, Oct4 and Sox2 allows somatic cells to be reprogrammed to a pluripotent state.

Like cMyc, Klf4 is a downstream target of STAT3 in ESCs (Lewitzky and Yamanaka, 2007). Previous reports have shown that Klf4 is expressed in many tissues e.g. gut (Shields, 1996; Zhang, 2006), skin (Segre, 1999), thymus (Panigada, 1999), adipocytes (Birsoy, 2008), fibroblasts (Zhang, 2000). Furthermore, it is detectable in the mouse embryo, with the highest expression occurring in the later stages (Garrett-Sinha, 1996; Ton-That, 1997). Klf4 is responsible for cell differentiation in some tissues and cell growth in other tissues (Matsumoto, 1998; Yet, 1998; Adam, 2000; Swamynathan, 2007). The mechanisms of this bi-functional action in differentiation and proliferation are not fully known, however it is known that Ras has a determinant function (Rowland, 2005).

When it is overexpressed, Klf4 acts by activating p21 and repressing p53 (Rowland, 2005); these proteins are both inhibitors of proliferation, consequently it is the background environment that decides the fate of the signalling: when p53 prevails the cells are driven toward proliferation; vice versa, when p21 activation overcomes p53 inhibition differentiation prevails and the consequence is cell cycle arrest. The addition of Ras, which inhibits p21, changes the equilibrium allowing Klf4 to repress p53 preventing apoptosis and eventually leading to enhancement of proliferation.

1.6.4 Advances in the technology

Although the iPSCs generated by Takahashi and Yamanaka shared many crucial pluripotency features with ESCs, they were not able to contribute to an adult organism after injection in a blastocyst: they were shown to be part of embryos only up to 13.5dpc. The absence of any contribution to postnatal animals following blastocyst injection, the distinct transcriptional signature and an epigenetic state that

was intermediate between somatic and ESCs indicated that Takahashi and Yamanaka's iPSCs were not identical to ESCs: even though the nuclear reprogramming observed was substantial, it was not complete.

In this first study, the choice of the Fbx15 promoter was revealed to be the main problem in the failure of a complete reprogramming: although specifically expressed in mouse ESCs and early embryos, Fbx15 is dispensable for the maintenance of pluripotency, so better markers should have been used to replace of the nonessential Fbx15. This is the strategy employed in three following reports (Maherali, 2007; Okita, 2007; Wernig, 2007), in which the key improvement was the selection of iPSCs using Oct4 and/or Nanog promoters as indicators of reprogramming. The iPSCs derived with this approach are functionally equivalent to ESCs, can give rise to chimeras (Maherali, 2007; Okita, 2007; Wernig, 2007), are able to contribute to the germline (Okita, 2007; Wernig, 2007) and are capable of generating viable, fertile offspring by tetraploid complementation (Zhao, 2009).

Further improvements to the iPSC isolation method have later been documented. For example, in murine cells it was demonstrated that reprogrammed pluripotent cells can be isolated from genetically unmodified somatic donor cells solely based upon morphological criteria (Meissner, 2007) and without the requirement of cMyc (Nakagawa, 2008; Park, 2008). Induced pluripotent stem cell isolation was reported by several independent groups employing different combination of factors including Oct4, Sox2, cMyc, Klf4, Nanog, Lin28, SV40L-T and H-TERT (Takahashi, 2007b; Yu, 2007; Park, 2008). Additionally, it was found that Klf4 and cMyc are dispensable for reprogramming, increasing only the efficiency of the process (Kim,

2008b; Nakagawa, 2008; Lyssiotis, 2009). In neural stem cells, where Sox2 is expressed at high levels, Oct4 alone is able to induce pluripotency (Duinsbergen, 2008; Eminli, 2008; Kim, 2009c).

In the initial study, the reprogramming genes were delivered to differentiated cells using retroviral transduction. New strategies for delivering the exogenous factors have been developed with time (Table 1). Lentiviruses were employed, initially carrying the transgenes under the control of a constitutive promoter; then, since differentiation of these cells was severely impaired by the continuative expression levels of the reprogramming factors (Brambrink, 2008; Sommer, 2010), the lentiviral vectors were modified using inducible promoters, so that the expression of the reprogramming factors could be switched off when reprogramming had occurred.

Despite the efficiency of retroviral and lentiviral vectors as reprogramming tools, the resulting iPS clones contain proviral integrations, the significance of this being the risk of insertional mutagenesis and/or spontaneous transgene reactivation, either of which could result in tumour formation (Okita, 2007). Several methods have been investigated to mitigate these risks, including the removal of the integrated transgenes by the Cre/LoxP technology (Chang, 2009; Soldner, 2009; Sommer and Mostoslavsky, 2010) or the use of the piggyBac transposon/transposase system (Woltjen, 2009), which is inserted in the genome of the host cells, but is characterized by a precise self-excision mechanism.

Reprogramming without genomic integration has been reported: a number of studies have described the successful generation of mouse iPSCs by using transient

REPROGRAMMING TYPE	DELIVERY METHODS	ADVANTAGES	DISADVANTAGES	REFERENCES
Integrative	Retrovirus	-Good efficiency -Silenced transgenes in iPSCs	-Multiple genomic integrations	(Takahashi and Yamanaka, 2006; Okita, 2007; Wernig, 2007)
	Constitutive lentivirus	-Generally good efficiency	-Multiple genomic integrations -Differentiation potential may be affected by incomplete transgene silencing	(Brambrink, 2008)
	Inducible lentivirus	-Good efficiency -Controlled expression	-Multiple genomic integrations	(Brambrink, 2008; Stadtfeld, 2008a; Sommer, 2009)
	piggyBac transposon	-Good efficiency -No vector sequence left behind	-Analysis of transposase-mediated excision can be demanding	(Kaji, 2009; Woltjen, 2009; Yusa, 2009)
Non-integrative	Plasmid transfection	-No genome modification	-Low efficiency -Possible integration -Screening for possible integration can be demanding	(Okita, 2008)
	Minicircle			(Jia, 2010)
	Episomal vector			(Yu, 2009)
	Adenovirus			(Stadtfeld, 2008c; Zhou and Freed, 2009)
DNA-free	Sendai vector	-No genome modification	-Requires continuous passaging to eliminate virally encoded transgenes from iPSCs	(Fusaki, 2009)
	ESC protein extract		-Low efficiency -Bacterial protein scheme requires VPA	(Cho, 2010)
	HEK293 expressed transducible protein Bacterially expressed transducible protein			(Kim, 2009a; Zhou, 2009)

Table 1. A summary of the different methods employed to reprogram somatic cells: the most efficient protocols for reprogramming are carried out employing retroviruses and lentiviruses. This however integrate in the iPSCs, making them unsuitable *in vivo* applications. On the other side, non-integrative methods are safer, but their efficiency is very low.

expression of plasmids (Okita, 2008; Gonzalez, 2009) or by using adenoviral vectors (Stadtfeld, 2008c) providing proof of principle that insertions are not necessary for iPSC generation. Most recently, expression of reprogramming factors from a non-viral minicircle vector proved capable of converting human adipose stem cells to pluripotency (Jia, 2010). Likewise, Sendai virus RNA based vectors achieved reprogramming of human somatic cells and were able to produce transgene-free iPSCs by antibody-mediated selection (Fusaki, 2009). Alternatively an oriP/EBNA1 episomal vector has been utilized to reprogram human fibroblasts (Yu, 2009).

Two groups achieved the generation of iPSCs through the delivery of reprogramming proteins. In order to enable cellular uptake, the reprogramming factors were tagged with a poly-arginine protein transduction domain and produced either in *E. coli* and purified (Zhou, 2009) or in HEK293 cells, where the whole-protein extract was then employed for reprogramming (Kim, 2009a). Finally, a recent report demonstrated that a single transfer of ESC-derived proteins is able to induce pluripotency in adult mouse fibroblasts (Cho, 2010). The main methods employed for reprogramming are summarised in Table 1.

While improving different ways of delivering the reprogramming genes, efforts have also been made to locate molecules capable of substituting the reprogramming factors or enhancing the reprogramming efficiency. In agreement with the knowledge that nuclear reprogramming to pluripotency is a gradual process that involves the conversion of the epigenetic state of a differentiated cell into that of an ESC-like cell (Jaenisch and Young, 2008), many of the compounds which proved to be useful for direct reprogramming inhibit the activity of chromatin remodelling factors. For

example in the presence of the DNA methyltransferase inhibitor 5'-azacytidine or the histone deacetylase inhibitor valproic acid, the efficiency of reprogramming can be enhanced by approximately 10-fold and 100-fold, respectively (Huangfu, 2008a). Other molecules found to increase the efficiency of reprogramming are the ROCK inhibitor Y27632 (Park, 2008), BIX-01294, a G9a histone methyltransferase inhibitor (Shi, 2008a; Shi, 2008b), BayK8644, a L-type calcium channel agonist (Shi, 2008a), ALK5 (Ichida, 2009; Maherali and Hochedlinger, 2009) and RepSox, both inhibitors of TGFβ signalling (Ichida, 2009), kenpaullone, a kinase inhibitor (Lyssiotis, 2009), vitamin C (Esteban, 2010), butyrate, a natural small fatty acid and histone deacetylase inhibitor (Liang, 2010), apigenin and luteolin (Chen, 2010).

Induced pluripotent stem cells were initially derived from embryonic and adult fibroblast, but many other cell types have been reprogrammed in the following years (summarised in Table 2). In particular, both ASCs and terminally differentiated cells have been turned into pluripotent cells; also, the reprogramming process has been successful on cells deriving from each of the three germ layers (Table 2). The direct reprogramming process proved to be also effective not only in mouse, but in many different species, going from human, to pig, to rat (Table 2). This indicates that the key mechanisms of regaining pluripotency are not specific to a particular cell type or species, but are shared among a number of tissues from many different species.

1.6.5 Importance of induced pluripotent stem cells

As discussed above, ESCs are viewed as a promising resource for medical applications and a platform for basic research, but their use is hampered by the

CELL TYPE	SPECIES	REFERENCES
Adipose stem cells	Mouse, human	(Sun, 2009; Tat, 2010)
Amnion-derived cells	Human	(Zhao, 2010)
B lymphocytes	mouse	(Hanna, 2008)
Bone marrow cells	Human, rat, pig	(Liao, 2009; Wu, 2009; Kunisato, 2011)
Cord blood derived cells	Human	(Giorgetti, 2009; Haase, 2009)
Dental pulp stem cells	Human	(Yan, 2010)
Fibroblasts	Mouse, human, pig, rat, dog, primates, sheep, bovine and horse	(Takahashi and Yamanaka, 2006; Takahashi, 2007b; Liu, 2008; Esteban, 2009; Liao, 2009; Shimada, 2010; Han, 2011; Li, 2011; Nagy, 2011)
Hematopoietic stem cells	Human	(Loh, 2009)
Hepatocytes	Human	(Liu, 2010)
Keratinocytes	Human	(Aasen, 2008)
Liver cells	Mouse, rat	(Aoi, 2008; Stadtfeld, 2008c; Li, 2009)
Malignant cells	Mouse, human	(Utikal, 2009; Carette, 2010)
Melanocytes	Mouse and human	(Utikal, 2009)
Meningiocytes	Mouse	(Qin, 2008)
Neural progenitor cells	Mouse, human	(Eminli, 2008; Kim, 2008b; Kim, 2009a)
Pancreatic B-cells	Mouse	(Stadtfeld, 2008a)
Smooth muscle cells	Human	(Lee, 2010)
Stomach cells	Mouse	(Aoi, 2008)

Table 2. Somatic cell types and species from which iPSCs have been derived.

technical difficulties encountered in their derivation in large animals; besides, although the derivation of human ESCs has been achieved, major ethical concerns about the destruction of human embryos have limited their derivation and their use.

Induced pluripotent stem cells share the key feature of ESCs, but are derived from differentiated cells. This circumvents the moral and technical problems linked to the use of embryos. Being ESC-like, iPSCs maintain the same applications of ESCs: they can be a tool for the screening of new drugs or toxics, represent a model for the study of pluripotency and differentiation, are a source of tissues for regenerative medicine and can be exploited for the creation of animals carrying specific disease. Additionally, iPSCs will open opportunities beyond those provided by ESCs: iPSCs will help to better understand the mechanisms of regaining pluripotency and will be a further tool in medicine: patient-specific iPSCs might be created, *in vitro* modified and differentiated toward a specific cell type and reinserted into the patient, with little risk of immune rejection.

Induced pluripotent stem cells have great promise, but their generation by employing integrating viral vectors to drive the expression of the reprogramming factors does not allow full exploitation of the potential of iPSCs in research and in particular in the clinical field: the resulting iPS clones contain proviral integrations, the significance of this being the risk of insertional mutagenesis and/or spontaneous transgene reactivation, either of which could result in tumour formation (Okita, 2007). Although several methods have been developed without the use of integrating

systems in order to mitigate the risks, their efficiency is still much lower than that of viral transduction.

1.7 A step forward: transdifferentiation

The idea that the nucleus of somatic cells could be reprogrammed to pluripotency through overexpression of specific transcription factors typical of ESCs has raised the question whether reprogramming could be achieved by directly converting one differentiated cell type into another differentiated cell type. This has opened the door to studies where factors were identified that can drive the transdifferentiation of readily available cells, such as fibroblasts, to therapeutically desirable cells, such as neurons (Vierbuchen, 2010; Caiazzo, 2011), cardiomyocytes (Ieda, 2010), progenitors of blood cells (Szabo, 2010) or hepatocytes (Huang, 2011; Sekiya and Suzuki, 2011).

One of the major issues in the employment of ESCs or iPSCs in therapy regards the concern about the potential tumorigenicity of these cells: although the cells employed in clinics are differentiated cells derived from ESCs, the selection methods to avoid a carry-over of pluripotent cells are not completely efficient. Induced transdifferentiated cells do not go through the pluripotent step; therefore they are seen as a safer way for the production of patient specific cells for medical purposes; even though there may be issues of scale up.

Despite their relevance, these cells, likewise ESCs, are produced by viral transduction. Safer methods such as transient expression of plasmids or the use of

cocktails of proteins should be used for generating induced transdifferentiated cells for clinical applications. Furthermore it must be shown that induced transdifferentiated cells have undergone a stable conversion of cell fate. Likewise induced transdifferentiated cells should be assayed to determine their suitability for transplantation and applicability in regenerative medicine (Nicholas and Kriegstein, 2010).

1.8 Aim of the project

I had two main goals: 1) establishing the protocol to induce pluripotency in our institute and 2) applying it to the ovine species.

Initially MFFs were reprogrammed through retroviral transduction employing the four original factors: cMyc, Klf4, Oct4 and Sox2. Once the protocol was optimised in mouse, it was translated to ovine somatic cells. This species was chosen because it is a recognised model for human disease and it takes further relevance for those syndromes where the mouse model have failed, such as cystic fibrosis (Harris, 1997; Abraham, 2008). When putative ovine iPSCs were derived, they were assessed for the main features of pluripotency, such as pluripotency marker expression, transgene silencing and ability to differentiate *in vitro* and *in vivo*. Once the cells demonstrated an iPSC phenotype they were inoculated into ovine blastocysts to test their ability to contribute to offspring.

CHAPTER 2

Materials and methods

2.1 DNA and RNA manipulation and analysis

2.1.1 Transformation of bacteria

The Subcloning Efficiency DH5α Competent Cell kit (Invitrogen, 18265-017) was employed for the routine propagation of all plasmids. The amount of 50µl of DH5α cells was thawed on ice and incubated on ice with 1µg of DNA for 30min. Cells were then heat-shocked for 20s at 42°C and placed on ice again for 2min; 950µl of pre-warmed LB medium were added and tubes were incubated at 37°C for 1h in a shaker at 225rpm. From 20µl to 200µl of each transformation were spread on 90mm LB-agar plates containing 100µg/ml ampicillin and incubated overnight at 37°C. The following day colonies of bacteria containing the plasmid would appear.

2.1.2 Extraction of plasmid DNA from bacteria

Large-scale extraction of plasmid DNA from bacteria was performed using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, K2100-07): one individual bacterial colony was picked from a selective plate and inoculated into a 15ml Falcon tube containing 5ml of LB medium supplemented with 100µg/ml ampicillin. Bacteria were then incubated for 4-5h at 37°C at 225rpm in a shaker. Starter culture was diluted (1:250) into 250ml of LB complemented with 100µg/ml ampicillin in a 1l conical flask and grown overnight at 37ºC in a shaker at 225rpm. The following day the bacterial culture was transferred into a 250ml dry-spin bottle (Sorvall) and harvested by at 4000g for 30min at 4ºC. After removing the supernatant, the bacterial pellet was resuspended in 10ml of resuspension buffer R3 (50mM Tris-HCl pH8.0, 10mM EDTA, 20mg/ml RNase A) and transferred to a 50ml Falcon tube. In order to

lyse the bacteria, 10ml of lysis buffer (0.2M NaOH, 1% SDS) were added and the tube was mixed gently by inverting the tube.

The lysis reaction was carried out at RT for 5min and then 10ml of precipitation buffer (3.1M potassium acetate pH5.5) were added. The sample was mixed immediately by inverting the tube until the mixture was homogeneous and was centrifuged at 13400g for 10min at RT.

The supernatant was loaded onto a column previously equilibrated with 30ml of equilibration buffer EQ1 (0.1M Sodium acetate pH5.0, 0.6M NaCl, 0.15% (v/v) TritonR X-100) and allowed to drain by gravity flow; the column was then washed with 60ml of wash buffer (0.1M Sodium acetate pH5.0, 825mM NaCl) and 15ml of elution buffer (100mM Tris-HCl pH8.5, 1.25M NaCl) were added to the column to elute the DNA. The resulting DNA was precipitated with 10.5ml of isopropanol and centrifuged at 15,000g for 30min at 4°C.

The supernatant was discarded, the DNA pellet resuspended in 5ml of 70% ethanol and centrifuged again at 15000g for 5min at 4°C. The supernatant was carefully removed and the DNA pellet was air-dried, resuspended in 500µl of ddH2O, quantified by NanoDrop (ND1000 Thermo Scientific) and stored at -20°C. Plasmid DNA was usually tested by restriction enzyme digestion.

2.1.3 Extraction of genomic DNA from cells

Genomic DNA (gDNA) from cells and embryos was extracted using the DNeasy Blood and Tissue Kit (Qiagen, 69504).

While passaging cells, aliquots were pelleted at 300g for 5min, the medium was removed and the cells were stored at -20°C. Before starting the extraction, frozen cell

pellets were allowed to thaw, dislodged by gently flicking the tube and were resuspended in 200µl of PBS. In order to lyse the cells and digest the proteins, 200µl of buffer AL and 20µl of proteinase K were added. Samples were mixed thoroughly by vortexing and incubated at 56°C for 10min. After adding 200µl of 96% ethanol, the samples were then mixed thoroughly by vortexing to yield a homogeneous solution and transferred into a DNeasy Mini spin column. The mixture was centrifuged at more than 6000g for 1min, washed with 500µl of buffer AW1 and centrifuged again for 1min at more than 6000g. The column was washed a second time with 500µl of buffer AW2 and centrifuged for 3min at 20000g; the flow-through was discarded and the column was centrifuged again for 1min at full speed to allow the membrane to dry. The DNeasy Mini spin column was placed in a clean 1.5ml microcentrifuge tube and 200µl of buffer AE were added directly onto the DNeasy membrane; the column was incubated at RT for 1min and then centrifuged for 1min at 6000g to elute. The elution step was repeated a second time using a clean 1.5ml microcentrifuge tube. Genomic DNA was quantified on the NanoDrop (ND1000 Thermo Scientific) and stored at 4°C.

2.1.4 Extraction of genomic DNA from tissues

Extraction of gDNA from tissues was performed using the DNeasy Blood and Tissue Kit (Qiagen, 69504): samples were cut into small pieces and placed in a 1.5ml microcentrifuge tube with 180µl of Buffer ATL and 20µl of proteinase K, mixing thoroughly by vortexing and incubating at 56°C until the completely lysed. During the incubation the samples were occasionally vortexed to disperse them. After complete digestion, the tubes were vortexed for 15s and 200µl of buffer AL were

added; the samples were then mixed thoroughly by vortexing and, after adding 200µl of 96% ethanol, were mixed again by vortexing to yield a homogeneous solution and were transferred into a DNeasy Mini spin column. The mixture was then centrifuged at more than 6000g for 1min, washed with 500µl of buffer AW1 and centrifuged again for 1min at 6000g. The column was washed a second time with 500µl of buffer AW2 and centrifuged for 3min at 20000g. The flow-through was discarded and the column was centrifuged again for 1min at full speed to allow the membrane to dry. The DNeasy Mini spin column was placed in a clean 1.5ml microcentrifuge tube, 200µl of buffer AE were added directly onto the DNeasy membrane and the column was incubated at RT for 1min and then centrifuged for 1min at 6000g to elute the DNA. The elution step was repeated a second time using a clean 1.5ml microcentrifuge tube. Genomic DNA was quantified on the NanoDrop (ND1000 Thermo Scientific) and stored at 4°C.

2.1.5 Extraction of RNA from cells

RNA was extracted using RNeasy Mini kit (Qiagen, 74104): while passaging the cells, aliquots were pelleted at 300g for 5min. The medium was removed and the cells were stored at -80°C. On the day of the extraction, frozen cell pellets were loosened by flicking the tube and 350 or 600µl of buffer RLT were added. The solution was mixed by pipetting, loaded into a QIAshredder spin column (Qiagen, 79654) and centrifuged for 2min at full speed to homogenize the lysate. One volume of 70% ethanol was added to the homogenized lysate, mixed well by pipetting, transferred to an RNeasy spin column and centrifuged for 15s at more than 8000g.

In order to wash the column membrane 350µl of buffer RW1 were added and the RNeasy spin column was centrifuged for 15s at 8000g. At this point on-column DNA digestion was performed using RNase-Free DNase Set (Qiagen, 79254): 10µl of DNase I stock solution were added to 70µl of buffer RDD, mixed by gently inverting the tube, added directly to the RNeasy spin column membrane and placed on the bench top (20–30°C) for 15min; 350µl of buffer RW1 were added and the column was centrifuged for 15s at more than 8000g. The membrane was washed with 500µl of buffer RPE, centrifuged for 15s at more than 8000g, washed again with 500µl of buffer RPE, centrifuged for 2min at more than 8000g and centrifuged again at full speed for 1min. The RNeasy spin column was placed in a new 1.5ml collection tube and 30 to 50µl of RNase-free water were added directly to the membrane. The column was centrifuged for 1min at more than 8000g to elute the RNA. The elution step was repeated a second time using a clean 1.5ml microcentrifuge tube.

RNA was quantified by NanoDrop (ND1000 Thermo Scientific) and immediately used for a second DNase digestion or stored at -80°C.

2.1.6 DNase digestion

Before reverse transcription, the RNA was treated a second time with DNase in order to degrade completely the gDNA. The kit used was RQ1 RNase-Free DNase (Promega, M6101). The reaction was set up as follows:

8µl	RNA
1µl	10x Reaction Buffer 10x
1µl	RNase-free DNase (1U/µg RNA)
10µl	

The digestion was incubated at 37°C for 30min, then 1µl of RQ1 DNase Stop Solution was added and the sample was incubated at 65°C for 10min to inactivate the DNase and terminate the reaction.

The DNase digested RNA was immediately used for reverse transcription or stored at -80°C.

2.1.7 Reverse transcription

The reverse transcription reaction was performed employing SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen, 18080-051). The reaction was set up as follows:

8µl	DNase digested RNA
1µl	Oligo$(dT)_{20}$ 50µM
1µl	dNTP mix 10mM
10µl	

The mixture was incubated at 65°C for 5min, placed on ice for at least 1min and the following reagents were added:

2µl	RT buffer 10x
2µl	DTT 0.1M
4µl	$MgCl_2$ 25mM
1µl	RNase OUT (40U/µl)
2µl	SuperScript III RT (200U/µl) or H_2O
21µl	

Alongside the reverse transcription, in order to ensure the absence of contaminant gDNA, a control reaction was always performed in which the reverse transcriptase was replaced by water. The reaction tubes were gently mixed, solutions collected by

brief centrifugation and incubated for 50min at 50°C. The reaction was terminated at 85°C for 5min and chilled on ice. The cDNA was used for PCR reaction or stored at -20°C.

2.1.8 Polymerase chain reaction

Polymerase chain reaction was performed using the Taq DNA Polymerase kit (Roche, 11146173001). A typical setting for DNA amplification can be summarized as follows:

1-16µl	DNA
3µl	buffer 10x
3µl	dNTPs 2mM
6µl	primers enJSRV F + enJSRV R) 2.5µM
1µl	Taq
1-16µl	Water
30µl	

The reaction mix and the DNA were mixed together in 0.2ml thermo-strip tubes (Abgene) and the samples were placed in gradient thermal cycler (Biometra TProfessional gradient). Primers and cycling parameters were adjusted for every individual experiment (see table 3). The PCR results were analysed by agarose gel electrophoresis.

2.1.9 Restriction enzyme analysis

Restriction enzyme digestions of plasmid were performed according to the manufacturer's instructions. DNA was usually digested with a 10-fold excess of enzyme with a final volume of glycerol not exceeding 5% (v/v).

GENE	APPLICATION	PRIMERS	CYCLING CONDITIONS
cMyc	gDNA and cDNA from cells	Forward 5'-GGA TCC CAG TGT GGT GGT ACG-3' Reverse 5'-TTA TGC ACC AGA GTT TCG AAG CTG TTC G-3'	95°C 5min, 35x 94°C 1min, 65°C 1min, 72°C 1min
cMyc genotyping	gDNA from lambs	Forward 5'-TAG AAC CTC GCT GGA AAG GA-3' Reverse 5'-CAG CTC GAA TTT CTT CCA-3'	95°C 5min, 45x 94°C 1min, 58°C 1min, 72°C 1min
enJSRVs	gDNA from cells	Forward 5'-ATA AAG AGA GGG GAG CTG CG-3' Reverse 5'-GGA AGG ATC TGA AAC GTG GA-3'	95°C 5min, 35x 94°C 1min, 59°C 1min, 72°C 1min
Klf4	gDNA and cDNA from cells	Forward 5'-GGA TCC CAG TGT GGT GGT ACG-3' Reverse 5'-TTA GGC TGT TCT TTT CCG GGG CCA CGA-3'	95°C 5min, 35x 94°C 1min, 65°C 1min, 72°C 1min
Klf4 genotyping	gDNA from lambs	Forward 5'-TAG AAC CTC GCT GGA AAG GA-3' Reverse 5'-GTG GGT TAG CGA GTT GGA AA-3'	95°C 5min, 45x 94°C 1min,58°C 1min, 72°C 1min
LIF	cDNA from cells	Forward 5'- CCC CTG TAA ATG CCA CCT GT -3' Reverse 5'- CTT CTC TGT CCC GTT GCC AT -3'	95°C 5min, 35x 94°C 1min,57°C 1min, 72°C 30s
Oct4	gDNA and cDNA from cells	Forward 5'-GGA TCC CAG TGT GGT GGT ACG-3' Reverse 5'-CTG TAG GGA GGG CTT CGG GCA CTT-3'	95°C 5min, 35x 94°C 1min, 65°C 1min, 72°C 1min
Oct4 genotyping	gDNA from lambs	Forward 5'-GGA TCC CAG TGT GGT GGT ACG-3' Reverse 5'-TGC GGG CGG ACA TGG GGA GAT CC-3'	95°C 5min, 45x 94°C 1min, 65°C 1min, 72°C 1min
OvHPRT	cDNA from cells and gDNA from lambs	Forward 5'-TGA GGA TTT GGA GAA GGT GTT C-3' Reverse 5'-CCA TCT TTG GTT TAT GCT TCT TG-3'	95°C 5min, 30x 94°C 1min, 55°C 1min, 72°C 1min
Sox2	gDNA and cDNA from cells	Forward 5'-GGA TCC CAG TGT GGT GGT ACG-3' Reverse 5'-TCA CAT GTG CGA CAG GGG CAG-3'	95°C 5min, 35x 94°C 1min, 65°C 1min, 72°C 1min
βactin	cDNA form cells	Forward 5'-TGA CAG GAT GCA GAA GGA GA -3' Reverse 5'-GTA CTT GCG CTC AGG AGG AG -3'	95°C 5min, 25x 94°C 1min, 60°C 1min, 72°C 1min

Table 3. List of the primers employed, their sequence and cycling conditions.

2.1.10 Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose at 0.5-2% (w/v) in TAE buffer by heating the suspension in a microwave oven. Ethidium bromide at 0.5µg/ml (Sigma) or 1x Sybr Safe (Invitrogen, S33102) were added to the solution and poured into a gel tray. Once set, the gel was submerged in TAE buffer in a gel tank; a DNA loading buffer (6x solution composed with 30% glycerol, ddH^2O and bromophenol blue or xylene cyanol) were was added to the sample and the mix was loaded into the wells. DNA was visualized by illumination on a UV light box and photographed. The size of the DNA fragment was examined by comparison of their mobility to that of restriction fragments of known size, typically 1kb DNA ladder (Fermentas).

2.2 Cell cultures

2.2.1 Cells

The cells employed in this project were human embryo kidney HEK 293T/17 cell line (ATCC, CRL-11268), mouse foetal fibroblasts, ovine foetal fibroblasts (OFFs, prepared by Dr Alison Thomson), immortalised MFFs (10T1/2 cell line, ECACC, 99072801), SNL 76/7 cell line (ECACC, 07032801), H9 cell lines, provided by Dr Alex DiDomenico (Thomson, 1998), HM1 mouse ESC line (provided by Dr Alex DiDomenico (Magin, 1992), Bruce4 mouse ESC line (Kontgen, 1993), human Caucasian colon adenocarcinoma LS174T cells (ECACC, 87060401).

2.2.2 Coating of flasks and plates for cell culture

Gelatin-coated flasks and plates were prepared dissolving gelatin from porcine skin (Sigma G2500) in water to obtain a 0.1% solution which was autoclaved twice. Prior to seeding the cells, the 0.1% gelatin solution was poured in the flasks or plates and left at RT; after 1h the gelatin was removed and the flasks or plates were washed once in PBS in order to remove the excess of gelatin.

Matrigel (Becton Dickinson) stored at -20°C was thawed at 4ºC for at least 2h to avoid the formation of a gel and was then diluted 1:100 in cold Knock Out Dulbecco's modified Eagle medium (KO-DMEM, Invitrogen) using a chilled pipette. T25cm^2 flasks were normally coated with 3ml of the Matrigel solution. Flasks were either incubated at RT for 1h-2h before use or stored at 4ºC for no longer than 2 weeks. Before use, the Matrigel solution was removed from the flasks and washed with KO-DMEM.

Poly-L-lysine (Sigma, P4832) was poured in flasks or plates and left for 1h at 37°C. It was then removed and flasks or plates were rinsed once with PBS.

2.2.3 General maintenance of cells (thawing, passaging and freezing)

Vials containing frozen cells were placed into a 37ºC water bath. When the cells were thawed, in order to remove the freezing mix 5ml of appropriate medium were added to the content of a vial in a 15ml falcon tube and centrifuged at 200g for 5min. Supernatant was discarded and pellet was resuspended in an appropriate volume of medium for plating out.

Cells were grown in the appropriate culture medium at 5%CO_2, 37°C and, when confluent, were passaged by trypsinization: the medium was removed and cells were

washed in warm PBS; trypsin-EDTA solution (Sigma, T3924) was added and cells were placed at 37°C until a single cell suspension was obtained. The appropriate culture medium was added to stop the enzymatic activity and the remaining clumps of cells were disaggregated by pipetting. Cells were collected by centrifugation for 5min at 200g, the supernatant was discarded and pellets were resuspended either in the appropriate culture medium and seeded again or prepared to be frozen.

For freezing cells were resuspended in an appropriate volume of freezing mix composed of foetal calf serum (FCS, PAA), 10% dimethyl sulfoxide (DMSO, Sigma) and the suspension was gently mixed and aliquoted into pre-chilled cryovial tubes. Vials were stored at -80ºC for 24h and then transferred to -150ºC.

2.2.4 Foetal fibroblasts

For the preparation of MFFs, pregnant mice were sacrificed on day 13 or 14 post coitum. The abdomens were swabbed with 70% ethanol and the abdominal cavities were dissected to expose the uterine horns. The foetuses were removed and placed into a 10cm Petri dish containing PBS supplemented with 5000µg/ml penicillin and 5000µg/ml streptomycin (Penicillin-Streptomycin Solution, Invitrogen, 15140-130). Placentas and membranes were removed and heads and soft tissues were separated from individual embryos. The foetuses were then washed twice in PBS supplemented with Penicillin-Streptomycin Solution and placed individually into tubes containing 2ml of Trypsin-EDTA. Tubes were finally incubated for at least 20min at 37ºC and vortexed every 5min. When the embryos were well disaggregated, 3ml of MFF medium were added, tubes were vortexed again and allowed to stand for 5min. When large masses settled, the top 4ml of liquid from each tube were removed and

transferred to a gelatin-coated 75cm^2 flask with 10ml of (DMEM, Sigma), 10% FCS (fibroblast medium). MFFs were then incubated at 37ºC, 5%CO_2 and the medium was changed the following day to remove cellular debris.

Sub-confluent 75 cm^2 flasks were washed once with PBS and incubated with 2ml of Trypsin-EDTA solution at 37ºC for 2-3min. When cells were in single cell suspension, 8ml of MFF medium were added, cells were centrifuged and supernatant removed. Cells were then frozen in FCS, 10% DMSO and stored at -80ºC or plated for immediate use.

MFFs could be expanded for up to 6 passages before senescence set in.

For the preparation of OFFs pregnant ewes were sacrificed on day 35 post coitum. The preparation of the ovine cells was performed employing the same protocol employed for MFFs.

2.2.5 SNL 76/7 cell line and feeder layer preparation

The SNL 76/7 cell line was clonally-derived from a STO cell line and express LIF and neomycin phosphotransferase (Neo). This cell line can be used as a feeder layer to support the growth of mouse ESCs and iPSCs (McMahon and Bradley, 1990).

Cells were purchased frozen from ECACC and were thawed in a 37°C water bath. The content of the vial was transferred to a 15ml centrifuge tube containing 5ml of DMEM, 10% FCS, 2mM L-Glutamine (Invitrogen). Cells were pelleted by centrifugation at 300g for 5min. The supernatant was discarded and cells were resuspended in 10ml of warm medium, plated in a gelatin-coated 25cm^2 flasks and incubated at 37°C, 5% CO_2.

Medium was changed every 2-3 days and when subconfluent, the cells were washed once with PBS and incubated with 1ml of Trypsin-EDTA solution at 37ºC for 2-3min. When cells were in single cell suspension, 4ml of medium were added, cells were centrifuged and supernatant removed. Cells were then stored in FCS, 10% DMSO at -160ºC or plated for expansion.

In order to prepare the feeder layer, SNL cells were expanded typically in nine gelatin-coated 150cm^2 flasks. When subconfluent, cells were collected by trypsinization in 50ml Falcon tubes and mitotically inactivated by irradiation at 10000rad (Yi, 1999). Feeder cells were then frozen in working aliquots and stored at -80ºC. Plating density of the SNL feeders is summarised in table 4.

2.2.6 Human embryonic stem cell line H9

Human ESCs H9 were grown on Matrigel-coated flasks in filtered conditioned medium supplemented with 4ng/ml human basic fibroblast growth factor (bFGF, Peprotech, 100-18B) at 37°C, 5% CO_2. In order to prepare conditioned medium, MFFs were grown in 150 cm^2 flask and fibroblast medium was replaced with 50ml of serum-free human ESC medium: 80% knock-out DMEM (KO-DMEM, Invitrogen), 20% knock-out serum replacement (SR, Invitrogen), 0.1mM non-essential amino acids (Invitrogen), 2mM L-glutamine, 0.1mM 2-mercaptoethanol (Invitrogen) and 4ng/ml bFGF; conditioned medium was collected from the flask the following day and stored at –20º C.

The medium was changed every day. When cells reached 80-90% confluence, the conditioned medium was removed and the cells were washed once with KO-DMEM. Typically, for a 25cm2 flask, cultures were rinsed with PBS incubated

Plate/Dish/Flask	SNL plating density
96 well plate	$2x10^4$
48 well plate	$5x10^4$
24 well plate	$1.7x10^5$
12 well plate	$3x10^5$
6 well plate	$7x10^5$
10cm dish	$5x10^6$
T25cm2 flask	$2x10^6$
T75cm2 flask	$6x10^6$

Table 4. Plating density of the SNL feeder layer.

with 1ml of Trypsin-EDTA at 37ºC until cells were detached, then 5ml of KO-DMEM were added to the cells and a single-cell suspension was obtained by pipetting vigorously. Cells were then transferred to a Falcon tube and centrifuged at 200g for 5min. The supernatant was discarded, the pellet was resuspended in fresh filtered conditioned medium supplemented with 4ng/ml bFGF and seeded in a Matrigel-coated flask.

2.2.7 Mouse pluripotent cells

Mouse ESCs (Bruce4 and HM1) and putative mouse iPSCs were maintained in mouse ESC medium: DMEM, 15% FCS, 2mM L-glutamine, 0.1mM non-essential amino acids, 0.1mM 2-mercaptoethanol, 1000 U/ml mouse LIF (Millipore, ESG1107). Medium was renewed daily. When at 70-80% confluent the cells were passaged at 1:3 to 1:5 ratio; the medium was renewed and around 2-3h later cells were rinsed with PBS, trypsinized for 2 to 5min at 37°C and collected in a Falcon tube after adding some culture medium in order to inactivate the trypsin-EDTA. The cells were then centrifuged at 200g for 5min, the supernatant was removed and the cell pellet was resuspended in culture medium and plated. Bruce4 and putative mouse iPSCs were typically grown on a feeder layer of irradiated SNL cells, while HM1 cells were normally grown in gelatin-coated plates or flasks.

2.2.8 Ovine induced pluripotent stem cells

Ovine iPSCs were derived on γ-irradiated SNL feeder layer employing two different media: mouse ESC medium or human ESC medium (KO-DMEM, 20% SR, 2mM L-glutamine, 0.1mM non-essential amino acids, 0.1mM 2-mercaptoethanol, 8ng/ml

human bFGF). Medium was renewed daily. When the cells were around 70-80% of confluence, medium was renewed and after 2 to 3h the cells were rinsed with PBS, trypsinized for 2 to 5min at 37°C, resuspended in culture medium and plated for 15-20 min in gelatin-coated plates in order to separate them from the feeder layer: feeder cells would adhere tightly, while the iPSCs would adhere weakly and differentiated cells and dead feeders would remain afloat. The loosely adhering ESCs were detached from the dish by vigorously pipetting with a 5ml pipette (protocol modified from (Tompers and Labosky, 2004) and plated on feeder layer. Typical passaged ratio was between 1:3 to 1:5.

2.2.9 Maintenance of other cell lines

Immortalised MFFs were grown in DMEM, 10% FCS. OFFs were cultured in 0.1% gelatin-coated plates in medium composed of DMEM, 10% FCS. HEK 293T/17 were grown in DMEM, 10% FCS, 2mM L-glutamine, 0.1mM non-essential amino acids. LS174T were maintained in Eagle's minimum essential medium (EMEM, EBSS), 10% FCS, 2mM L-Glutamine, 0.1mM non-essential amino acids.

2.2.10 *In vitro* differentiation of pluripotent cells

For the differentiation of mouse ESCs and putative mouse iPSCs, clones were grown in one well of six well plate in mouse ESC medium on SNL feeder layer until subconfluent (50-70% confluence). On day 0 cells from a T25cm^2 flask were fed and, after 2-3h, washed with warm PBS and trypsinized for around 2min at 37°C in order to get small clumps of cells instead of a single cell suspension. Cells were then plated in a gelatin-coated T25cm^2 flask for 15-20min to separate them from the

feeders. The loosely adhering cells were detached by vigorously pipetting with a 5ml pipette and the clumps of cells were collected and grown in suspension in a 9cm bacterial Petri dish in mouse EB medium (mouse ESC medium without LIF).

On day 2 floating EBs were washed: the medium containing the cells was collected in a tube and the EBs were let to sink at the bottom of the tube. The supernatant was removed and the cells were resuspended in fresh EB medium and plated again in a new Petri dish. On day 5 EBs were washed again and on day 7 they were plated in gelatin-coated wells of 24 well plate with 1 to 10 EBs per well. Differentiating cells were fed every 3-4 days and, when confluent, were rinsed twice in PBS, fixed in 4% PFA for 20min, rinsed again twice in PBS and stored at 4°C in PBS.

The protocol performed for the differentiation of the ovine iPSCs was similar, with only few modifications: before differentiation putative ovine iPSCs were grown in human ESC medium; on day 0 cells were seeded again in human ESC conditions, while on day 1 they were resuspended in human EB medium (KO-DMEM, 10% FCS, 2mM L-glutamine, 0.1mM non-essential amino acids and 100μM β-mercaptoethanol). EBs were then plated in gelatin-coated wells of a 24 well plate or in poly-L-lysine-coated plates or glass chambers.

2.3 Viral transduction and reprogramming

2.3.1 Moloney Murine Leukaemia viral and lentiviral vector packaging

A total of $3x10^6$ cells HEK 293T/17 cells were plated in a gelatin-coated 10cm dish in HEK medium. When at 70% of confluence, they were transfected using FuGENE HD Transfection Reagent (Roche, 04709691001) according to the manufacturer's recommendation. The transfection was carried out as follows:

MoMLV:

- Expression vector	8 µg
- Packaging vector	8 µg
- OPTI-MEM I Reduced Serum Medium (Invitrogen 31985) to a final volume of	600 µl

Oct4-EGFP and CMV-EGFP:

- Expression vector	6.5µg
- pLP1	5 µg
- pLP2	2.5µg
- pVSV-G	2 µg
- OPTI-MEM I Reduced Serum Medium to a final volume of	600µl

The solution was mixed and 68µl of Fugene HD were added. The mix was left for 20min at RT and added dropwise to the cells. The day after the medium was removed and replaced with 6ml of fresh HEK medium. After 24h the supernatant was taken, filtered with a 0.45µm filter and either immediately used for transduction or frozen -20°C in 1ml aliquots.

The packaging vectors employed for MoMLV packaging were pCL-Eco Retrovirus Packaging Vector (Imgenex, 10045P) or pCL-10A1 Packaging Vector (Imgenex, 10047P). The expression vectors utilized for MoMLV packaging were pMXs-cMyc, pMXs-Klf4, pMXs-Oct4, pMXs-Sox2 (Addgene) and pCLXSN-GFP (provided by Gillian Parham). The packaging vectors employed for lentiviral packaging were pLP1, pLP2 and pVSV-G (Invitrogen).The expression vectors utilized for lentiviral packaging were Oct4-EGFP and CMV-EGFP (provided by Dr Debiao Zhao).

2.3.2 Transduction of ovine induced pluripotent stem cells

PGK-GFP (pRRL-PGK-eGFP-WPRE, Genethon), CAG-GFP (provided by Dr Simon Lillico) and Ubi-EGFP (provided by Dr Feifei Song) viruses were transduced

in ovine iPSCs in two different ways in order to assess which protocol resulted in a higher number of green cells. Cells were either transduced when subconfluent or transduced just after passage.

For the first protocol cells were plated in one well of 12 well plate on feeders and, when subconfluent, the medium was renewed. After 3h medium was removed and 500µl of medium containing 25µ of virus were added to the cells. After other 3h 2.5ml of medium were included and the day after medium was renewed. Cells were looked at under the fluorescence microscope in order to estimate the number of green cells.

According to the second protocol, when cells were subconfluent, medium was changed and, after 3h, cells were trypsinized, feeders were removed and cells were seeded again in a new well of 12 well plate on feeders with 200µl of medium supplemented with 25µl of virus. After other 3h 2.5ml of medium were included and the day after medium was renewed. Cells were looked at under the fluorescence microscope in order to estimate the number of green cells.

2.3.3 Reprogramming transduction

For the reprogramming of mouse cells, a total of $1.3x10^5$ passage 2 MFFs were seeded in each well of a 6 well plate on $2x10^5$ SNL feeder layer in 2ml fibroblast medium. The day after (day 0), the medium was removed and 1ml of each fresh viral vector carrying a different reprogramming gene (cMyc, Klf4, Oct4 or Sox2) was added to the MFFs, after being supplemented with 4µg/ml of polybrene. One well of MFFs was not transduced to be used as negative control. After 5h, 2ml of fresh HEK medium was added. The same viral transduction was repeated the day after. At day 2

the HEK medium containing the viral vectors was replaced with MFF medium. At day 3 fibroblast medium was replaced with mouse ESC medium.

For the reprogramming of ovine cells the protocol was slightly modified: $1.3x10^5$ OFFs at passage 6 were plated in each of 5 gelatin-coated wells of a 6 well plate in 2ml of fibroblast medium. At day 0 and day 1 the cells were transduced with the four viral vectors (1ml of each) supplemented with 4µg/ml of polybrene. One well of MFFs was transduced with pCLXSN-GFP for the double aim of being used as negative control and of verifying the ability of the amphotropic viral vector to transduce OFFs. On day 2 the ovine fibroblasts were passaged to a new 6 well plate ($4x10^4$ cells/well) on SNL feeder cells. On day 4 the fibroblast medium was replaced with mouse ESC medium in 2 wells and human ESC medium in the other 2 wells.

2.3.4 Picking colonies

In order to pick colonies ESC medium was removed from the plates and cells were washed twice with warm PBS. A thin layer of PBS was left in the plated in order to avoid the cells to dry. The colonies were dislodged from the feeders by pipetting up and down with a Gilson pipette and each colony was transferred in a round bottom well of a 96 well plate. To disaggregate the cells 30µl of trypsin were added and left for 10min. Afterwards, 70µl ESC medium were added and colonies were transferred to gelatin-coated wells of 96 well plate on SNL feeder layer ($5x10^3$ cells/well) in ESC medium (150µl/well).

2.4 Analyses

2.4.1 Giemsa staining

Medium was removed and cells were washed twice in PBS. A 3% Giemsa's stain improved R66 solution Gurr (VWR) was added to the cells and left for 20min at RT. Wells were then rinsed with water and left to dry.

2.4.2 Alkaline Phosphatase staining

Alkaline Phosphatase (AP) staining of pluripotent colonies was performed with Leukocyte Alkaline Phosphatase Kit (Sigma, 86R-1KT).

Before the staining cells were washed twice with PBS, fixed with 4%PFA for 20min at RT, washed again twice with PBS. For a 24 well plate 0.2ml of Sodium Nitrite Solution were added to 0.2ml of FRV-Alkaline Solution, then mixed by gentle inversion and allowed to stand for 2min. Nine millilitres of deionized water were added, the solution was mixed and 0.2ml Naphthol AS-BI Alkaline Solution was included. At this point the tube was wrapped in foil because the resulting solution is light-sensitive. After mixing thoroughly, 0.35ml were added into each well. Plates were wrapped in foil and left overnight. The following day the stain was removed and cells were rinsed with PBS. Positive cells were pink.

2.4.3 Immunofluorescence

For immunocytochemistry against intracellular markers cells were permeabilized for 20min at RT. For both intracellular and surface markers, cells were blocked from 1h to 2h at RT. Primary antibodies were diluted in the blocking solution and applied to the cells overnight at 4˚C. Plates were then washed four times for 5min in washing

buffer and blocked again for 15min at RT. The appropriate FITC conjugated secondary antibody was diluted in washing solution (in blocking buffer for Nanog) and added to the cells for 1h to 2h at RT in the dark. Cells were later washed four times for 5min in PBST and the nuclei were stained with 1µg/ml of DAPI (Sigma). The markers, their corresponding antibodies and the condition employed for each antibody are listed in table 5.

2.4.4 Metaphase spreads

Subconfluent cells growing in a T25cm^2 flask were passaged at 1:3 ratio in a gelatin-coated well of 6wp, without separating cells from the feeders. The day after the medium was renewed and 5ml of fresh medium supplemented with 0.1µg/ml of KaryoMAX Colcemid Solution (Invitrogen, 15210-040) for 1.5-2h at 37°C. Medium was then removed, cells were washed once with PBS and dissociated with trypsin. Trypsin was not quenched with serum, but diluted in 5ml of PBS. The suspension was pipetted to disaggregate the cells, transferred into a Falcon tube and centrifuged at 250g for 8min. The supernatant was discarded and the tube flicked to release the pellet into the remaining supernatant. The amount of 10ml of 75mM KCl were added, and the suspension was mixed and incubated for 8-15min at 37°C. One77millilitre of ice-cold 3:1 methanol/acetic acid was added at RT, the cells were centrifuge at 200g for 5min and the supernatant was removed. The pellet was resuspended in 5ml of ice-cold 3:1 methanol/acetic acid, the cells were then centrifuged at 200g for 5min and the supernatant was discarded. This last step was repeated two more times. The cells were then suspended in 1ml of ice-cold 3:1

MARKER	PERMEAB. SOLUTION	BLOCKING SOLUTION	WASHING SOLUTION	PRIMARY ANTIBODY (DILUTION)	SECONDARY ANTIBODY (DILUTION)
Nanog	PBST	PBST, 10% goat serum, 1% BSA	PBST	Abcam, ab21603 (1:200)	Sigma, F0382 (1:200)
SSEA1	\	PBS, 2% BSA	PBS, 0.05% Tween	DSHB, MC480 (1:100)	Invitrogen, A11017 (1:500)
SSEA4	\	PBS, 2% BSA	PBS, 0.05% Tween	DSHB, MC-813-70 (1:100)	Invitrogen, A11017 (1:500)
αFP	PBST	PBS, 2% BSA, 0.05% Tween, 0.05% Triton	PBS, 0.05% Tween	R&D Systems, MAB1368 (1:500)	Invitrogen, A11017 (1:500)
CK18	PBST	PBS, 2% BSA	PBS, 0.05% Tween	Sigma C8541, clone CY 90 (1;500)	Invitrogen, A11017 (1:1000)
βIII tub	PBST	PBST, 2% goat serum, 1% BSA	PBS, 0.05% Tween	Sigma, T 8660 (1:400)	Invitrogen, A11017 (1:1000)
Vim	PBST	PBS, 2% BSA, 0.05% Tween, 0.05% Triton	PBS, 0.05% Tween	Abcam, ab8978 (1:300)	Invitrogen, A11017 (1:500)
Trop T	PBST	PBST, 2% goat serum, 1% BSA	PBST	NeoMarkers, MS 295 PO (1:50)	Sigma, F 5262 (1:500)

Table 5. Antibodies and solutions employed in immunocytochemistry (PBST: PBS, 0.3% Triton).

methanol/acetic acid and spread on a glass microscope slide refrigerated at 4°C in 70% Ethanol: 10-20µl were dropped onto the slide and blown briskly. Slides were kept at 70-80°C overnight and stained with 10% Giemsa.

2.4.5 Population doubling time

Ovine iPSCs were seeded at low density ($3.55x10^4$ cells per well of a 48 well plate) and counted in triplicates every 12h for 96h. Using MiniTab, a linear regression was performed on the exponential phase of the growth curve to establish population doubling time. After evaluation of the data with MiniTab, two outliers (mouse ESCs at 48h and G2 cells at 72h) were excluded from the analysis.

2.4.6 STAT3 induction assay

OFFs, MFFs, ovine iPSCs and mouse ESCs were seeded each on gelatin-coated wells of a 6 well plate, in the appropriate culture medium. When subconfluent, the medium was removed and fresh medium without LIF, bFGF, FCS or SR (starvation media) was added to starve the cells. After 4h one well for each cell line was trypsinized and cells were counted; starving media supplemented with mouse LIF (1000U/ml) but without SR or FCS were added to the remaining wells (200µl/well). Cells were incubated at 37°C for 10min, then medium was removed, cells washed with PBS and 125µ of SDS lysis buffer (10% Glycerol, 3% SDS, 62.5mM TrisHCl pH 6.8, 0.005% Bromophenol Blue, 3% β mercaptoethanol) were added directly into the wells. The plates were incubated on a rocking table for 20min at 4°C on ice. Remaining on ice, the wells were scraped and the lysates collected

with a p1000 and transferred in 1.5ml tubes. Samples were frozen at -20°C and later used for western blot.

2.4.7 Western immunoblotting

Reagents employed for Western Blot are listed in table 6. Samples were thawed in a 37°C heating block, placed on ice and sonicated for 8min. Tubes were flicked and if the samples were still viscous they were sonicated again. Just before loading, the samples were heated to 98°C for 5min, vortexed and centrifuged for 30s. NuPAGE Novex 7% Tris-Acetate Gel 1.0 mm, 10 well (Invitrogen, EA0355) was inserted into the running chamber; 10µl of Precision Plus Protein All Blue Standards (BioRad, 161-0373) were loaded into the gel. The samples were loaded at different volumes, which were calculated in order to load the amount of protein extracted from the same amount of cells ($26.56x10^4$ cells) for all cell lines.

A total of 500µl of antioxidant was added in cold 200ml running buffer (NuPage Tris-Acetate SDS Running Buffer, Invitrogen, LA0041), the mix was poured into the running chamber and the gel was allowed to run for 1h at 200V.

The gel was then blotted onto a nitrocellulose membrane: materials previously immersed in transfer buffer were assembled with the gel in the following order into a cassette: sponge, paper, membrane, gel, paper and sponge. The cassette was assembled into the transfer tank with the protein side of the gel facing the positive pole of the tank. The transfer was carried out at 28V for 2h 30min.

The sandwich was then dismantled and the membrane, with the protein side up, was washed in TBST 4 times for 15min. Twenty millilitres of blocking solution were

Transfer buffer stock solution (10x)
29.3g Glycine 58g Tris 18.8ml SDS 20% Bring to 1l with dH_2O
Transfer buffer
100ml transfer buffer stock solution 10x 200ml EtOH 100% 700ml dH_2O
TBS 10x
24.2g Tris 80g NaCl Bring to 1l with dH_2O pH 7.6
TBST
1x TBS Tween 0.1% dH_2O
Blocking solution
5% dried skimmed milk (Marvel) 1x TBST
Transfer buffer stock solution (10x)
29.3g Glycine 58g Tris 18.8ml SDS 20% Bring to 1l with dH_2O
Transfer buffer
100ml transfer buffer stock solution 10x 200ml EtOH 100% 700ml dH_2O

Table 6. Solutions employed in the Western immunoblotting assay.

then added and the membrane was incubated over night at 4°C on a rocking plate. The day after the blocking solution was removed and an anti-phoshpoSTAT3 primary antibody (Cell signalling, 9131), diluted 1:1000 in TBST, 5% BSA was added onto the membrane and incubated over night at 4°C on a rocking plate. The following day the membrane was rinsed 4 times for 15min in 20ml TBST on a rocking plate. An anti-rabbit secondary antibody (Cell signalling, 7074) diluted 1:5000 in TBST, 10% blocking solution was added on the membrane and incubated 1h at RT on a rocking plate. The membrane was then rinsed 4x15 min in 20ml TBST on a rocking plate. The Amersham ECL detection systems (GE Healthcare, RPN2109) was employed for detection: the ECL Detection reagents A + B were mixed at 1:1 ratio, added on the membrane and incubated for 5min.

The excess of reagents was drained and the blot was placed between two transparency sheets side up in an X-ray film cassette and the film was exposed for 3min. The blot was then rinsed in TBST for few minutes and 10ml of stripping buffer (Thermo Scientific, 21059) were added. The membrane was placed at 37°C for 20min on a rocking plate. The blot was then washed 4 times for 15min in TBST. An anti-β actin antibody (Sigma A3854) diluted 1:50000 in 5% dried skimmed milk (Marvel), TBST was added to the membrane and incubated for 1h at RT on a rocking plate. The detection step was performed again and the blot was exposed to the film for 30s. The membrane was stripped again and incubated with an anti-STAT3 primary antibody (DB Transduction Laboratories, 610190) diluted 1:1000 in TBST, 5% BSA over night at 4°C on a rocking plate. The blot was rinsed 4 times for 15min in 20ml TBST on a rocking plate and an anti-mouse secondary antibody (Cell signalling, 7076) diluted 1:5000 in TBST, 10% blocking solution was added. The

secondary antibody was incubated 1h at RT on a rocking plate. The detection step was performed and the blot was exposed to the film for 3min for STAT3.

2.4.8 LIF withdrawal assay

Ovine iPSCs and mouse ESCs were seeded at low density ($1.3x10^5$ cells/well in a 12 well plate) on SNL feeder layer or gelatin in the appropriate media (mouse ESC medium or human ESC medium) in the presence or absence of mouse LIF. The medium was renewed daily and after 10 days the cells were fixed with 4% PFA and assessed for AP activity.

2.4.9 *In vivo* differentiation of mouse and putative ovine pluripotent cells

Ovine iPSCs resuspended in 100µl PBS were intramuscularly injected in the right leg of SCID mice (10^6 cells/mouse). After 3 to 5 weeks, mice were sacrificed. Tumours were collected and either enclosed in OCT embedding medium (Tissue-Tek) and snap frozen in liquid nitrogen for 2h or fixed in a formal saline solution (ddH2O, 4% w/v formaldehyde, 0.9% w/v NaCl) and paraffin embedded. Sometimes bones were included in the tumours, therefore, before paraffin embedding, the lumps were placed in at least 15 volumes of neutral EDTA solution (10% w/v EDTA, ddH_2O, pH 7.4) which was changed daily in order to decalcify the tissues.

Sections ranging from 3µm to 7µm were cut with the microtome or cryostat for paraffin embedded or frozen tissues respectively. The slides were stained with H&E or against markers of differentiation and histologically analysed.

2.4.10 Hematoxylin and eosin staining

Slides were deparaffinised with 2 changes of xylene for 10min each, then rehydrated in 2 changes of absolute ethanol for 5min each. Sections were then left twice in 90% ethanol for 5min and twice in 70% ethanol for 5min. The slides were washed twice in distilled water for 5min and stained in hematoxylin (Sigma, MHS-16) solution for 5min. They were then washed in running tap water for 5min and stained with 0.5% eosin for 2-3min. Slides were washed again in running tap water for 5min and dehydrated quickly (6 times for 2-3s) in 70% ethanol and 90% ethanol. The samples were then placed twice in 100% ethanol for 5min and once in xylene for 10min. In order to mount the slides, 2-3 drops of DPX mounting (Raymond A. Lamb) were added to the cover slip and the slide was placed on top of cover slip with section side down; the air bubbles where expelled by gently tapping on the cover slip. Slides were placed on a flat surface for at least 24h to allow the mounting solution to set. When looking at the microscope nuclei should be blue and cytoplasms pink to red.

2.4.11 Immunohistochemistry

Sections were cut, fixed in acetone for 2min at RT and then rinsed in 0.1M PBS for 2min. They were post-fixed in fresh Periodate Lysine Paraformaldehyde solution for 8min at room temperature, rinsed in PBS for 2min, rinsed in distilled water for 2min and transferred to Sequenza coverplates using filtered TBST (Tris buffered saline pH 7.5, tween 20). The sections were later washed twice in TBST, incubated with diluted primary antibody for 1h at 25°C, rinsed three times in TBST and blocked with 100µl Dako Real blocker for 20min at room temperature. The slides were washed again three times in TBST and 100µl of Secondary link polymer complex

(Dako Envision kit) were added for 30min at 25°C. The sections were washed five times in TBST and allowed to drain completely. They were then rinsed in distilled water and allowed to drain completely. One hundred microlitres of DAB chromogen were added twice for 5mins at 25°C and the sections were washed once in TBST. Counterstaining was carried out with haematoxylin using the Gemini autostainer).

The primary antibodies utilised were those already employed for immunocytochemistry, with the exception of the anti-cardiac troponin T antibody (Abcam, ab19615) and the anti-neurofilament antibody (Dako, M0762).

2.5 Chimera production

2.5.1 Ovary collection, *in vitro* maturation and *in vitro* fertilization

Ovine ovaries were collected at the abattoir, maintained at 30°C during transport to the laboratory and washed in warm PBS. Follicular fluid was collected using an 18-gauge needle and a 10ml syringe and placed into 15ml centrifuge tubes containing fresh warm wash medium (1x Medium 199, 4.76mM $NaHCO_3$, 12.5mM HEPES, 112.5U/ml Heparin, 10.875µl/ml oestrus sheep serum, H_2O, pH7.4). Good quality oocytes surrounded by a compact cumulus mass were selected and washed again three times in the wash medium and once in fresh maturation medium (1x Medium 199, 6.25mM $NaHCO_3$, 2mM L-glutamine, 5ng/ml ovine FSH, 5ng/ml ovine LH, 0.5ng/ml estradiol, 250µl/ml oestrus sheep serum, H_2O, pH7.6). The oocytes were eventually placed in 800µl of maturation medium in a 4-well Nunc tissue culture plate (40-50 oocytes/well) and incubated for approximately 22h at 5% CO_2, 38.5°C. After this time, oocytes which have been matured to MII stage of development are prepared for fertilisation removing cumulus cells by pipetting in

wash medium. Oocytes surrounded by coronal cells were washed twice in fertilisation medium (SOF medium, 2% oestrus sheep serum, pH7.8) and fertilised with the upper fraction of ram semen pellets stored in liquid nitrogen and previously thawed and activated using a swim up technique (Parrish, 1986). Cells were then incubated at 5% CO_2, 38.5°C and after 22-24h were washed twice in warm SOFaaBSA medium (SOF medium, 1x BME amino acids solution, 1x MEM non-essential amino acids solution, 2mM L-glutamine, 4mg/ml BSA, pH7.4), removing the sperm and any remaining cumulus cells by gently pipetting. Fertilised oocytes were then placed into pre-equilibrated 4-well plates in SOFaaBSA medium and overlaid with mineral oil and culture in a 5% CO_2, 5% O_2 incubator at 38.5°C. After 24h oocytes were checked to assess development and remove any that have not cleaved.

2.5.2 Injection of ovine induced pluripotent stem cells in ovine embryos

Ovine iPSCs were microinjected into day 6 or day 7 *in vitro* produced blastocysts as follows: starting from one subconfluent well of 6 well plate (50-70% confluent), 2-3h before harvesting the medium was changed, ovine iPSCs were trypsinized to obtain a single cell suspension and plated in a gelatin-coated T25cm^2 flask. In order to separate the cells from the feeder layer, the flask was placed for 20min in the incubator at 37°C, 5% CO_2. Immediately prior to microinjection, cells were collected, centrifuged, resuspended in 500µl of medium and stored at room temperature until microinjection was complete.

2.5.3 Synchronization of recipients

Sponges impregnated with 30mg cronolone (flugestone acetate) were inserted into the vagina of recipient ewes. The sponges remained in the vagina for 11 to 16 days. When removed the animals showed oestrus within 36-48h later. The insertion of the sponge was calculated so that the recipient ewes were at 6 days post oestrus when the blastocysts were at day 6.

2.5.4 Embryo transfer

Following microinjection, embryos were cultured until blastocyst stage in 850µl drops of SOFaaBSA under mineral oil at 38.5°C in an atmosphere of 5% CO_2, 5% O_2; SOFaaBSA, Synthetic Ovidictal Fluid (Walker, 1996) plus 1x essential and non-essential amino acids (Sigma, Poole, UK) and 4 mg/ml of fatty acid-free BSA (Sigma, Poole, UK). If these embryos developed into blastocysts they were then transferred in recipient ewes. Blastocysts that were micro-injected were transferred into recipient ewes on the same day as the micro-injection. Recipient ewes were selected from groups of cycling adults in which oestrus was synchronised using intravaginal progestagen sponges (Veramix, Upjohn). Oestrus was detected by introduction of vasectomised males. Animals that had exhibited oestrus 6 or 7 days previous to transfer were selected as recipients for blastocysts. Following a general anaesthetic a midline laparotomy was performed to expose the uterine tract. A small puncture was made in the uterine wall using a blunt 16-gauge needle and a 20ml Drummond positive pipette fitted with sterile ‘fire polished’ capillaries were used for

the transfer. The animals were monitored throughout pregnancy by trans-cutaneous ultrasound examination.

CHAPTER 3

Viral vector strategy for induced pluripotent stem cell induction

3.1 Introduction

Originally, iPSCs were derived by transduction of four reprogramming genes (cMyc, Klf4, Oct4 and Sox2) by employing a MoML retroviral vector (Takahashi and Yamanaka, 2006).

Later, more efficient or safer ways of delivering the reprogramming factors have been achieved: lentiviral vectors (Yu, 2007), piggyBac transposon/transposase systems (Woltjen, 2009), adenoviral vectors (Stadtfeld, 2008c; Zhou and Freed, 2009) or transient expression of plasmids (Okita, 2008; Gonzalez, 2009) have been successfully exploited; additionally three independent groups achieved to generate iPSCs simply delivering the reprogramming proteins instead of employing genetic material (Kim, 2009a; Zhou, 2009; Cho, 2010). Other changes to the method regarded the choice of the genes to be used (Kim, 2009b; Wu, 2009; Bao, 2011) and the supplementation of small compounds able to change the epigenetic state of the DNA (Feng, 2009).

The work described in this thesis had two main goals: repeating the published experiment to establish the protocol in our institute, then applying it to the ovine species. We started one year after the first report about iPSCs (Takahashi and Yamanaka, 2006) and the only published method about the isolation of iPSCs was based on retroviral transduction of the four pluripotency genes cMyc, Klf4, Oct4 and Sox2 (Takahashi and Yamanaka, 2006). We therefore carried out the mouse reprogramming according to this protocol. By the time the ovine experiment started, many new strategies were available, but, since the reprogramming in mouse had been

successful, we decided to replicate the viral vector-based protocol. While the overall method was maintained, we faced two main issues. The first regarded the tropism: in the mouse experiment the packaging vector employed (pCL-Eco) was able to transduce only murine cells, thus in the sheep protocol pCL-Eco was replaced by a similar vector (pCL-10A1) expressing a different envelop protein, known to target most mammalian cells. The second matter referred to the choice of the species of the reprogramming genes.

In this chapter I will describe the rationale behind the reprogramming strategy we utilised for the generation of mouse iPSCs and ovine iPSCs. In addition, I will illustrate in details the expression and the packaging vectors. Their viability and ability to express the proteins of interest will be analysed through immunocytochemical analyses.

3.2 Results

3.2.1 Expression and packaging plasmids

The expression plasmids employed in the isolation of iPSCs were generated by Takahashi et al (Takahashi and Yamanaka, 2006) starting from the MoMLV-based retroviral plasmid pMXs (Kitamura, 2003) by employing the Gateway Technology. Briefly a Gateway cassette rfA was introduced into the retroviral plasmid pMXs and the four reprogramming genes were amplified by RT-PCR, cloned into appropriate plasmids and recombined in the pMXs backbone.

The resulting pMXs reprogramming plasmids (Fig. 7a) harbour 5' and 3' long

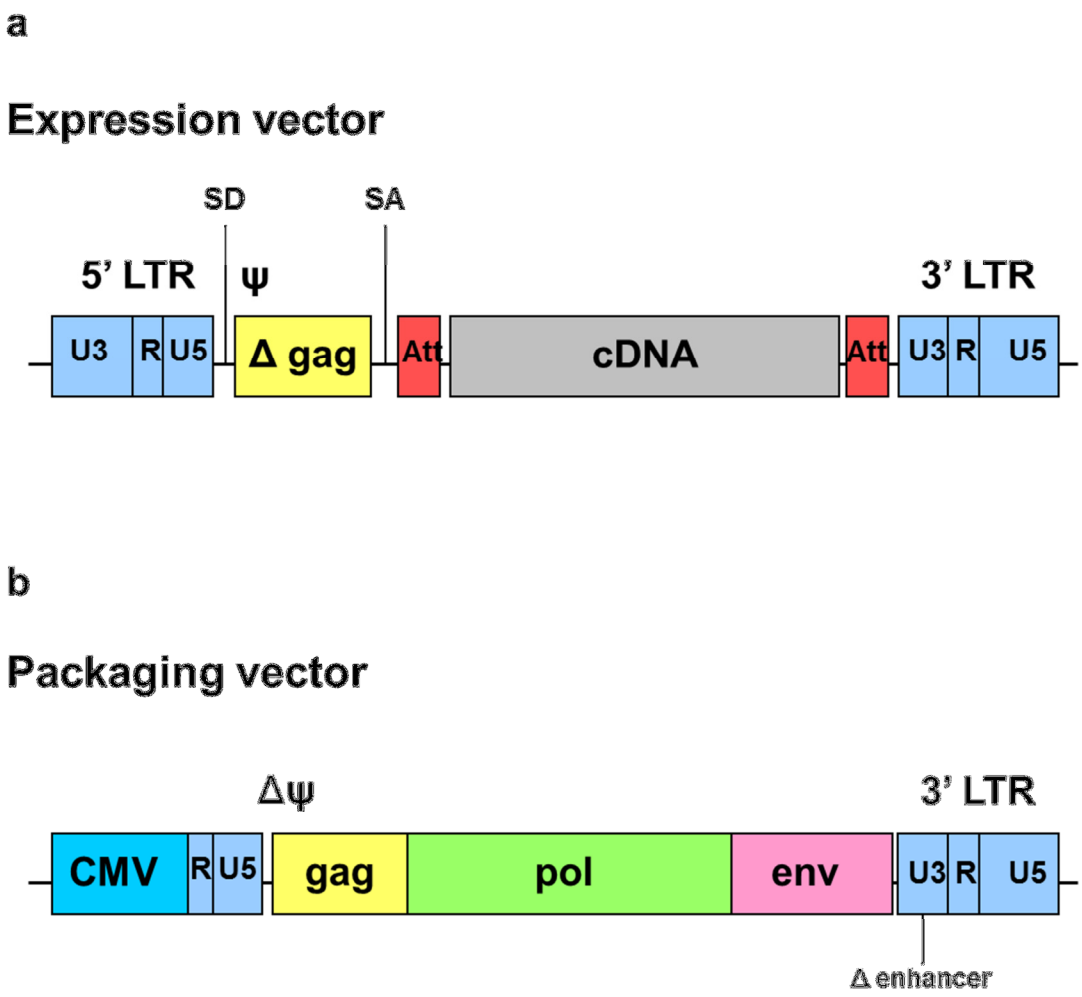


Figure 7. Schematic map of the plasmids employed for the murine and ovine reprogramming. a) The expression vector is composed of the cDNA of one of the reprogramming genes (represented by the grey box) which is under the control of the MoMLV promoter. In order to accommodate the exogenous cDNA and to make the vectors replication-defective pol, env and part of gag were removed. b) The packaging plasmid harbours the gag (yellow box), pol (green box) and env (pink box) genes required for the packaging under the control of a constitutive promoter (aquamarine box), but does not contain the packaging signal, so that the viral transcript will not be able to form a proper virus and does not carry the enhancer in U3 either, so that, once integrated, the retroviral sequence will lack a functional promoter in the 5'LTR. LTR (blue box): long terminal repeats; SD: splice donor site; SA: splice acceptor sit; Ψ: packaging signal; Δgag: truncated gag sequence; att (red box): sites generated by the recombination reaction; CMV (aquamarine box): human cytomegalovirus immediate early enhancer-promoter.

terminal repeats (LTRs) which not only are essential for the integration in the host genome, but also drive the transcription of the viral genome. The expression plasmids also carry an extended packaging signal necessary for the efficient encapsidation of viral genomes and the cDNA of the reprogramming genes; furthermore they have the viral splice donor and acceptor sequences that are required for the generation of the sub-genomic viral mRNA carrying the reprogramming sequences. In order to avoid the production of the viral full-length genome RNA, the ATG start codon for *gag* was disrupted and four stop codons were inserted in different positions through the gag ORF: one downstream of the CTG start codon and the others in the three different reading frames, just before the Gateway site (Kitamura, 2003).

Due to the lack of functional gag, pol and env genes in the expression plasmids, these factors need to be supplied in trans for the correct encapsidation of the viral genome into viral particles. This was obtained through the co-transfection of the pMXs reprogramming vectors and a packaging plasmid in 293T cells. The packaging plasmids employed in the mouse and ovine experiments are pCL-Eco Retrovirus Packaging Vector and pCL-10A1 Packaging Vector (Fig. 7b) respectively. Both of them express the MoMLV gag, pol and env proteins at high levels and with a balanced stoichiometry. The enhancer in U3 and the packaging signal are deleted to make the helper genome transcriptionally inactive and unable to be packaged (Naviaux, 1996). The pCL-Eco and pCL-10A1 vectors differ only for their tropism, which is given by the envelop glycoprotein gp70: in pCL-Eco gp70 is ecotropic, while in pCL-10A1 gp70 is amphotropic.

3.2.2 Viability of Moloney murine leukaemia vectors and expression of the reprogramming proteins

Before the reprogramming experiment, the ability of the viral vectors to express the reprogramming proteins was tested. Due to the lack of any selectable markers or reporter genes in the reprogramming plasmids, the validation of the MoML viral vectors was performed through immunocytochemistry which was carried out on immortalized MFFs transduced with serial dilutions (ranging from 10^{-1} to 10^{-6}) of the reprogramming vectors. The MoML viral vectors were separately packaged (Nakagawa, 2008) in 293T cells and stored at -80°C until use. For the transduction, cells were plated at low density ($3x10^4$ cells/well in a 24 well plate) so that they would not reach confluence in the following 4-5 days. The day after, the virus diluted in MFF medium and supplemented with 4µg/ml polybrene was added to the wells. After 24h medium was replaced and at day 4 or 5 cells were fixed with 4% PFA for immunostaining. Each well was transduced with only one type of virus. In each staining, non-transduced immortalized MFFs were employed as negative control, whereas mouse ESCs were employed as positive control.

As shown in figure 8, Oct4-transduced MFFs were Oct4-positive with the staining restricted to the nucleus which is the typical localization of this protein. The negative control and the sample displayed some background, with a signal localized in the cytoplasm and much fainter than the one observed in the mouse ESCs; nonetheless, in the Oct4-transduced MFFs it was clearly possible to recognize, above the background, few fluorescent cells which are not visible in the non-transduced MFFs. The efficiency of the MoML viral vector expressing Oct4 was calculated as a ratio

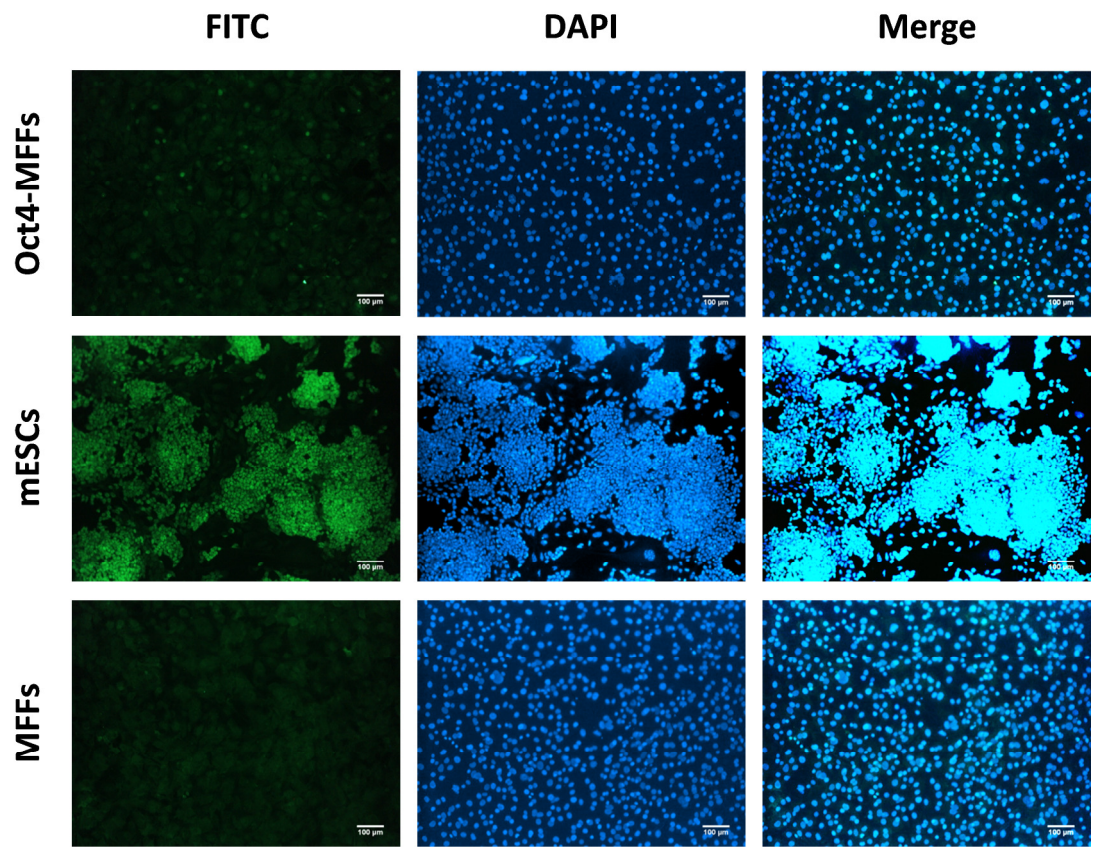


Figure 8. Immunocytochemistry of Oct4 transduced MFFs (Oct4-MFFs). Mouse ESCs (mESCs) represent the positive control, MFFs represent the negative control. Only the 10 time virus dilution is shown. Images were taken at 10x magnification. Scale bars: 100µm.

between the Oct4-positive cells and the total number of cells and was estimated to be around 34%.

Despite some background in Sox2 immunocytochemistry, the signal was brighter and the proportion of green cells much higher (the efficiency was around 73%) than for Oct4 (Fig. 9). Again, while the background gave some fluorescence in the cytoplasm, Sox2 staining was clearly localized in the nucleus.

In contrast to Oct4 and Sox2, Klf4 is expressed also in MFFs, so these cells cannot be a real negative control. However, we assumed that Klf4 expression in transduced MFFs would be significantly increased compared to that in non-transduced MFFs. Indeed, both transduced and non-transduced MFFs were Klf4-positive, but the number of stained cells was higher in transduced MFFs (Fig. 10), suggesting that Klf4 MoMLV was able to produce the Klf4 protein. Since both transduced and non-transduced cells were fluorescent, it was not possible to discriminate between endogenous and exogenous Klf4, therefore the efficiency was not calculated.

Although the transduction to validate the reprogramming plasmids was performed on all the four genes, we were unable to obtain any results about the MoML vector carrying cMyc due to problems with the anti-cMyc antibody. An optimisation for the staining was performed in the laboratory using different dilutions of the primary and secondary antibodies with no success: the negative control was always positive, suggesting a poor specificity of the cMyc antibody.

MoML viral vectors are known to be extremely sensitive to freezing/thawing cycles (Takahashi, 2007a); additionally their viability is substantially reduced when they are filtered after thawing. Both situations happened when the MFFs were transduced for

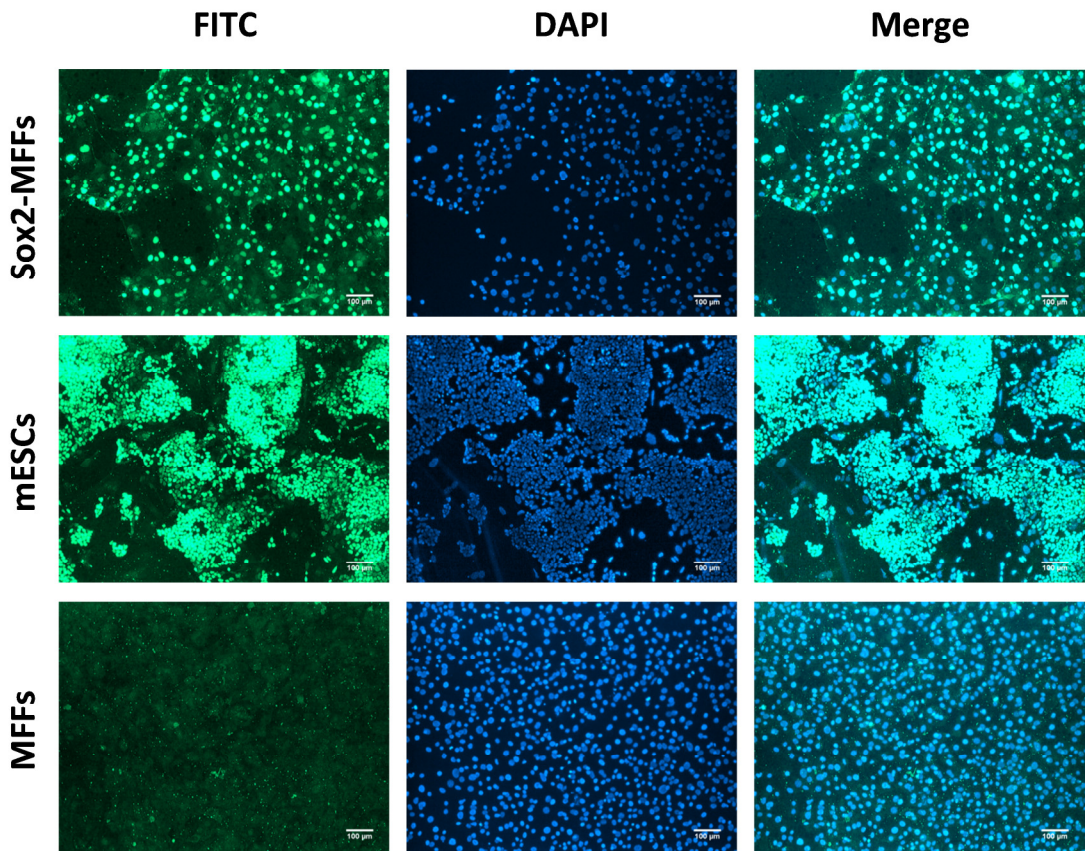


Figure 9. Immunocytochemistry of Sox2 transduced MFFs (Sox2-MFFs). Mouse ES cells (mESCs) represent the positive control, MFFs represent the negative control. Only the 10 time virus dilution is shown. Images were taken at 10x magnification. Scale bars: 100µm.

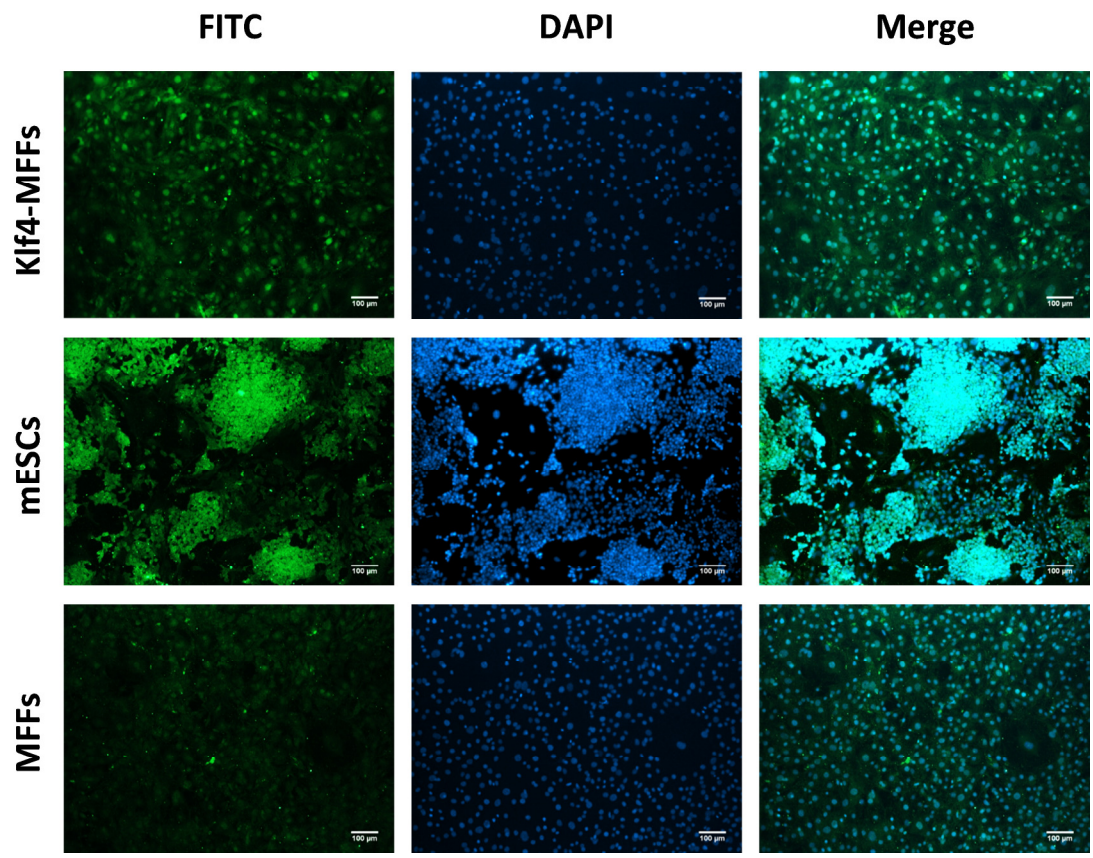


Figure 10. Immunocytochemistry of Klf4 transduced MFFs (Klf4-MFFs). Mouse ESCs (mESCs) represent the positive control. Although positive for Klf4, MFFs represent the negative control because their Klf4 expression is expected to be lower than that in Klf4-transduced MFFs. Only the 10 time virus dilution is shown. Images were taken at 10x magnification. Scale bars: 100µm.

the validation protocol. Therefore Sox2 was chosen among the four genes to be packaged again and transduced fresh into MFFs to assess whether the expression of the exogenous genes could be increased following the new protocol (Fig. 11). This was achieved: indeed the proportion of green cells raised from 73% obtained with the frozen virus to 99.7% with the fresh virus.

3.2.3 Sequence alignment of murine and ovine reprogramming genes

As already mentioned in the introduction to this chapter, for the ovine experiment we had to decide whether to employ the murine reprogramming genes already tested or to clone and use the ovine genes. The use of ovine orthologous genes is intrinsically more appealing but for a number of practical reasons we elected to use the mouse cDNAs. First, the mouse vectors were readily available and available in appropriate expression vectors. Second, the ovine sequences required to be identified, amplified and cloned. Third, the then status of knowledge of the ovine genome sequences was rudimentary; specifically the ovine sequences for Klf4 and Oct4 were not available.

In order to evaluate whether the murine genes could be employed in place of the ovine equivalent genes, the amino acid homology between the proteins of the two different species was investigated (Fig. 12): sheep and mouse cMyc and Sox2 were compared and found 91.8% and 98.1% homologous respectively. Due to the lack of ovine Klf4 and Oct4 sequences and since bovine and ovine genomes are very closely related (Echard, 1994), *B. taurus* orthologs were chosen for the comparison with the mouse proteins as a replacement for the unavailable sheep sequences. We aligned the bovine and murine proteins and found again a high sequence homology (92.7% for Klf4, 81.9% for Oct4).

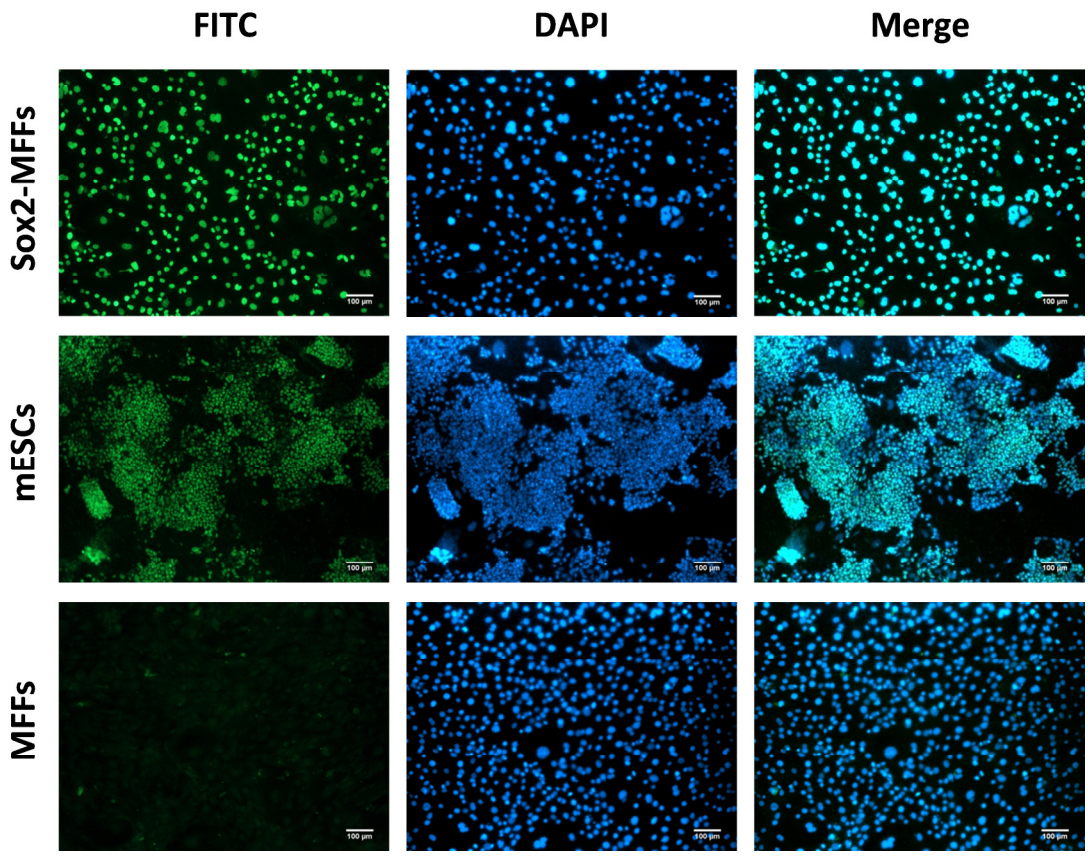


Figure 11. Additional immunocytochemistry of Sox2 transduced MFFs (Sox2-MFFs). Mouse ESCs (mESCs) represent the positive control, MFFs represent the negative control. The Sox2 staining was performed on MFFs transduced with fresh virus. It is evident that the efficiency of MoML viral vectors is strongly increased when the viruses are not stored frozen. Images were taken at 10x magnification. Scale bars: 100µm.

Although electing to work with the mouse genes we initially started to isolate the ovine orthologs. As soon as ESC-like colonies started to appear we decided to focus on these cells rather than continue with isolation of the sheep genes.

3.3 Discussion

Several experimental strategies have been developed for the derivation of iPSCs. While non-integrating methods eliminate the risks associated with genome modifications, thus having broader applications in human regenerative medicine and in those fields where the generation of chimeric animals is required, retroviruses are still one of the most efficient ways for the production of iPSCs (Sommer and Mostoslavsky, 2010).

MoMLV is one of the first viruses employed for the delivery of genetic material into host cells and it is still widely used (Barquinero, 2004) despite some drawbacks: its infectivity is limited to dividing cells (Miller, 1990), thus restricting the range of cell types that can be transduced; additionally MoMLV is known to undergo silencing in ESCs (Jahner, 1982; Wolf and Goff, 2007). The latter characteristics which is considered as a limitation for the application of the MoML viral vector in ESCs, provides a useful tool for direct reprogramming since only clones whose transgene expression has been silenced are considered authentic iPSCs (Maherali and Hochedlinger, 2008). This important feature of MoMLV-based vectors gives them an advantage above lentiviral vectors, a subclass of retroviruses which MoMLV does not belong to: although having a slightly higher efficiency of reprogramming (Stadtfeld, 2008b) and although able to transduce also non-dividing cells (Naldini, 1996), lentiviruses do not undergo silencing.

cMyc

```
91.8% identity in 440 residues overlap; Score: 2062.0; Gap frequency: 0.5%

M.musculus     1 MPLNVNFTNRNYDLDYDSVQPYFICDEEENFYHQQQQSELQPPAPSEDIWKKFELLPTPP
O.aries        1 MPLNVSFANRNYDLDYDSVQPYFYCDEEENFYHQQQQSELQPPAPSEDIWKKFELLPTPP
                 ***** * *************** ************************************

M.musculus    61 LSPSRRSGLCSPSYVAVATSFSPREDDDGGGGNFSTADQLEMMTELLGGDMVNQSFICDP
O.aries       61 LSPSRRSGLCSPSYVAVA-SFSPRGDDDGGGGSFSSADRLEMVTELLGGDMVNQSFICDP
                 ****************** ***** ******* ** ** *** *****************

M.musculus   121 DDETFIKNIIIQDCMWSGFSAAAKLVSEKLASYQAARKDSTSLSPARGHSVCSTSSLYLQ
O.aries      120 DDETLIKNIIIQDCMWSGFSAAAKLVSEKLASYQAARKDGGSPSPARGHGGCSTSSLYLQ
                 **** ************************************  * ******  *********

M.musculus   181 DLTAAASECIDPSVVFPYPLNDSSSPKSCTSSDSTAFSPSSDSLLSS-ESSPRASPEPLV
O.aries      180 DLSAAASECIDPSVVFPYPLNDSSSPKPCASPDSTAFSPSSDSLLSSAESSPRASPEPLA
                 ** ************************ * * *************** ***********

M.musculus   240 LHEETPPTTSSDSEEEQEDEEEIDVVSVEKRQTPAKRSESGSSPSRGHSKPPHSPLVLKR
O.aries      240 LHEETPPTTSSDSEEEQEDEEEIDVVSVEKRQPPAKRSESGSPSAGSHSKPPHSPLVLKR
                 ********************************* *********     *************

M.musculus   300 CHVSTHQHNYAAPPSTRKDYPAAKRAKLDSGRVLKQISNNRKCSSPRSSDTEENDKRRTH
O.aries      300 CHVSTHQHNYAAPPSTRKDYPAAKRAKLDSGRVLKQISNNRKCASPRSSDTEENDKRRTH
                 ******************************************* *****************

M.musculus   360 NVLERQRRNELKRSFFALRDQIPELENNEKAPKVVILKKATAYILSIQADEHKLTSEKDL
O.aries      360 NVLERQRRNELKRSFFALRDQIPELENNEKAPKVVILKKATAYILSVQAEEQKLISEKDV
                 ********************************************** ** * ** ****

M.musculus   420 LRKRREQLKHKLEQLRNSGA
O.aries      420 LRKRREQLKLKLEQIRNSCA
                 ********* **** *** *
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Sox2

```
98.1% identity in 320 residues overlap; Score: 1660.0; Gap frequency: 0.3%

M.musculus     1 MYNMMETELKPPGPQQASGGGGGGG-NATAAATGGNQKNSPDRVKRPMNAFMVWSRGQRR
O.aries        1 MYNMMETELKPPGPQQTSGGGGGGGGNSTAAAAGGNQKNSPDRVKRPMNAFMVWSRGQRR
                 *************** ******** * **** ****************************

M.musculus    60 KMAQENPKMHNSEISKRLGAEWKLLSETEKRPFIDEAKRLRALHMKEHPDYKYRPRRKTK
O.aries       61 KMAQENPKMHNSEISKRLGAEWKLLSETEKRPFIDEAKRLRALHMKEHPDYKYRPRRKTK
                 ************************************************************

M.musculus   120 TLMKKDKYTLPGGLLAPGGNSMASGVGVGAGLGAGVNQRMDSYAHMNGWSNGSYSMMQEQ
O.aries      121 TLMKKDKYTLPGGLLAPGGNSMASGVGVGAGLGAGVNQRMDSYAHMNGWSNGSYSMMQDQ
                 ************************************************************ *

M.musculus   180 LGYPQHPGLNAHGAAQMQPMHRYDVSALQYNSMTSSQTYMNGSPTYSMSYSQQGTPGMAL
O.aries      181 LGYPQHPGLNAHGAAQMQPMHRYDVSALQYNSMTSSQTYMNGSPTYSMSYSQQGTPGMAL
                 ************************************************************

M.musculus   240 GSMGSVVKSEASSSPPVVTSSSHSRAPCQAGDLRDMISMYLPGAEVPEPAAPSRLHMAQH
O.aries      241 GSMGSVVKSEASSSPPVVTSSSHSRAPCQAGDLRDMISMYLPGAEVPEPAAPSRLHMSQH
                 ************************************************************ **

M.musculus   300 YQSGPVPGTAINGTLPLSHM
O.aries      301 YQSGPVPGTAINGTLPLSHM
                 ********************
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Figure 12 (legend in the next page).

Klf4

```
92.7% identity in 477 residues overlap; Score: 2374.0; Gap frequency: 0.6%

M.musculus    10 MAVSDALLPSFSTFASGPAGREKTLRPAGAPTNRWREELSHMKRLPP-LPGRPYDLAA-T
B.taurus       1 MAVSDALLPSFSTFASGPAGREKTLRPAGAPNNRWREELSHMKRLPPVLPGRPYDLAAAT
                 ********************************* *************** ********** *

M.musculus    68 VATDLESGGAGAACSSNNPALLARRETEEFNDLLDLDFILSNSLTHQESVAATVTTSASA
B.taurus      61 VATDLESGGVGAACGSSNPALLPRRETEEFNDLLDLDFILSNSLSHQESVAATVSSSASA
                 ********* **** * ***** ******************** *********  ****

M.musculus   128 SSSSSPASSGPASAPSTCSFSYPIRAGGDPGVAA-SNTGGGLLYSRESAPPPTAPFNLAD
B.taurus     121 SSSSSPSSSGPASAPSTCSFSYPIRAGGDPGVAAPGGAGGGLLYGRESAPPPTAPFNLAD
                 ****** ***************************    ****** ***************

M.musculus   187 INDVSPSGGFVAELLRPELDPVYIPPQQPQPPGGGLMGKFVLKASLTTPGSEYSSPSVIS
B.taurus     181 INDVSPSGGFVAELLRPELDPVYIPPQQPQPPGGGLMGKFVLKASLSAPGSEYGSPSVIS
                 ********************************************  ***** ******

M.musculus   247 VSKGSPDGSHPVVVAPYSGGPPRMCPKIKQEAVPSCTVSRSLEAHLSAGPQLSNGHRPNT
B.taurus     241 VSKGSPDGSHPVVVAPYSGGPPRMCPKIKQEAVSSCTVGRPLEAHLGTGPPLSNGHRPPA
                 ********************************** **** * *****  ** *******

M.musculus   307 HDFPLGRQLPTRTTPTLSPEELLNSRDCHPGLPLPPGFHPHPGPNYPPFLPDQMQSQVPS
B.taurus     301 HDFPLGRQLPSRTTPTLGAEELLSSRDCHPALPLPPGFHPHPGPNYPPFLPDQMQPQVPP
                 ********** ******  **** ****** ************************ ***

M.musculus   367 LHYQELMPPGSCLPEEPKPKRGRRSWPRKRTATHTCDYAGCGKTYTKSSHLKAHLRTHTG
B.taurus     361 LHYQELMPPGSCMPEEPKPKRGRRSWPRKRTATHTCDYAGCGKTYTKSSHLKAHLRTHTG
                 ************ ************************************************

M.musculus   427 EKPYHCDWDGCGWKFARSDELTRHYRKHTGHRPFQCQKCDRAFSRSDHLALHMKRHF
B.taurus     421 EKPYHCDWDGCGWKFARSDELTRHYRKHTGHRPFQCQKCDRAFSRSDHLALHMKRHF
                 ************************************************************
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Oct4

```
81.9% identity in 360 residues overlap; Score: 1519.0; Gap frequency: 2.2%

M.musculus     1 MAGHLASDFAFSPPPGGG-DGSAGLEPGWVDPRTWLSFQGPPGGPGIGPG----SEVLGI
B.taurus       1 MAGHLASDFAFSPPPGGGGDGPGGPEPGWVDPRTWMSFQGPPGGSGIGPGVVPGAEVWGL
                 ****************** **  * ********** ******** *****     ** *

M.musculus    56 SPCPPAYEFCGGMAYCGPQVGLGLVPQVGVETLQPEGQAGARVESNSEGTSSEPCADRPN
B.taurus      61 PPCPPPYDLCGGMAYCAPQVGVGPVPPGGLETPQPEGEAGAGVESNSEGASPDPCAAPAG
                  **** *  ******* **** * **  * ** **** *** ******* *  ***

M.musculus   116 AVKL--EKVEPTPEESQDMKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFGKVFS
B.taurus     121 APKLDKEKLEPNPEESQDIKALQKDLEQFAKLLKQKRITLGYTQADVGLTLGVLFGKVFS
                 * **  ** ** ****** ***** ************************************

M.musculus   174 QTTICRFEALQLSLKNMCKLRPLLEKWVEEADNNENLQEICKSETLVQARKRKRTSIENR
B.taurus     181 QTTICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENR
                 ************* ********** ***************** *****************

M.musculus   234 VRWSLETMFLKCPKPSLQQITHIANQLGLEKDVVRVWFCNRRQKGKRSSIEYSQREEYEA
B.taurus     241 VRGNLESMFLQCPKPTLQQISHIAQQLGLEKDVVRVWFCNRRQKGKRSSSDYSQREDFEA
                 **  ** *** **** **** *** ************************  *****  **

M.musculus   294 TGTPFPGGAVSFPLPPGPHFGTPGYGSPHFTTLYS-VPFPEGEAFPSVPVTALGSPMHSN
B.taurus     301 AGSPFTGGPVSSPLAPGPHFGTPGYGGPHFTTLYSSVPFPEGEVFPSVSVTALGSPMHAN
                  * ** ** ** ** *********** ******** ******* **** ********* *
```

Figure 12. Amino acid alignment of mouse and ovine cMyc and Sox2 and of mouse and bovine Klf4 and Oct4, calculated utilising the SIM alignment tool for protein sequences. The sequence similarity of the orthologs is very high: above 90% for cMyc, Klf4 and Sox2 orthologous, while above 80% for Oct4 orthologous.

At the time of our mouse experiment the MoML retroviral strategy was the only published method. When starting the ovine reprogramming we utilised again the MoML viral vectors for two main reasons: we had already employed these vectors successfully in mouse reprogramming and they carry a major advantage, which is the ability to be silenced in pluripotent cells.

Once selected the vectors, viruses carrying the four reprogramming factors were packaged and their efficiency was analysed. The titre of retrovirus is extremely important for iPSCs generation (Takahashi, 2007a). Often viral vectors carry either a reporter gene, which allows the quantification of the transduced colonies (i.e. with a GFP reporter gene the transduced cells will be green) or an antibiotic resistance gene that permits the selection of the transduced cells. Since the pMXs plasmids did not possess either of those strategies, we decided to evaluate the viability of the vectors through immunocytochemistry, transducing 293T cells with serial dilution of the viruses and staining them against the reprogramming factors. The efficiency of the transduction was calculated as the ratio between positive and total cells in a representative field of the microscope. This calculation does not provide an accurate titre as the result depends upon different factors (e.g. the expression levels driven from the plasmids, the protein stability, the immunocytochemistry protocol); however positive staining gives a clear evidence of the viability of the viruses and their ability to express the proteins of interest in MFFs.

During Takahashi and Yamanaka's reprogramming experiment the integration efficiency of their retroviral vectors was measured with a pMXs-based virus

expressing GFP and was found higher than 70% (Takahashi, 2007a). Initially, when validating my vectors, the efficiency was lower, reflecting the use of vectors from frozen virus stock. After repeating the protocol with fresh virus we notice that the viability of the MoML viral vector carrying Sox2 was greatly increased. Additionally during the validation protocol the viruses were diluted at least 10 times, while in the iPSC experiment they were to be used as a concentrated stock.

Unfortunately, due to problems with the antibody, it was not possible to show the presence of cMyc through immunocytochemistry. Despite at different levels, the other three viral vectors proved to be viable, able to infect MFFs and capable of expressing the reprogramming factors. Since the vector carrying cMyc was the same as the vectors carrying Klf4, Oct4 and Sox2 and since the cMyc virus was packaged according to the same protocol employed for the other retroviral vectors, I assumed that the cMyc MoMLV production was successful. Note also that it was demonstrated that cMyc, although increasing the iPSCs isolation rate, is dispensable for the reprogramming process (Wernig, 2008b).

Combining the above data and conclusions, I resolved that the yield of MoML viral vectors was acceptable to perform the transduction for iPSCs isolation as described in the next chapter.

CHAPTER 4

Derivation and characterization of putative mouse induced pluripotent stem cells

4.1 Introduction

The aims of the current work were twofold: to repeat the work published by Takahashi and Yamanaka regarding the isolation of iPSCs in mouse (Takahashi and Yamanaka, 2006) and to then extend this to the derivation of iPSCs in sheep.

In the first iPSC report, mouse foetal or adult fibroblasts were transduced with retroviruses carrying cMyc, Klf4, Oct4 and Sox2 in order to drive the cells toward a pluripotent state. A selectable marker driven by the promoter of Fbx15, a gene expressed in mouse ESCs, was employed to recognise the reprogrammed cells (Takahashi and Yamanaka, 2006): when the dedifferention had occurred, Fbx15 promoter would have been switched on and the cells would have become resistant to the antibiotic. The expression cassette carrying the selectable marker was integrated in the genome of the experimental fibroblasts. Many ESC-like colonies were generated with this strategy, but none were able to give rise to live chimeras.

In other studies the iPSC generation technique was optimized by placing the selectable marker under the control of Oct4 or Nanog promoters: both genes are present in ESCs, but in contrast to Fbx15 their expression is required for the maintenance of pluripotency (Maherali, 2007; Okita, 2007; Wernig, 2007). In these studies the iPSCs proved their ability to contribute to adult animals after injection in blastocysts (Maherali, 2007; Okita, 2007; Wernig, 2007) and to go germline (Okita, 2007; Wernig, 2007).

During the numerous attempts to derive iPSCs using antibiotic resistance genes, the evidence arose that the later the selection was applied, the more ESC-like colonies would appear (Maherali, 2007; Okita, 2007). This led to the successful attempt to

isolate iPSCs without any antibiotic resistance or reporter genes (Maherali, 2007; Meissner, 2007).

In this chapter I will describe the experimental steps taken towards the derivation of mouse iPSCs. I will additionally illustrate the assays performed in order to characterise the newly generated cells, specifically immunocytochemistry and *in vitro* differentiation.

4.2 Results

4.2.1 Selection strategy for the induced pluripotent stem cell isolation

My initial strategy for the derivation of iPSCs included the use of a marker: a selectable gene or a reporter gene placed under the control of the promoter of a pluripotency-specific gene and integrated into the genomic DNA of the cells that were going to be reprogrammed would have allowed to discern between reprogrammed and non-reprogrammed cells. Yet this required the creation of new chimeric mice carrying an appropriate expression cassette, which is a long process. Mice expressing the neomycin resistance gene under the control of the mouse Oct4 promoter were already available at the Roslin Institute (McWhir, 1996); however these mice were old and not able to give offspring anymore. Consequently, in parallel to the Oct-Neo mouse strategy, I decided to employ a lentiviral vector expressing GFP under the control of the human Oct4 promoter (Fig. 13). This vector would be transduced into MFFs together with the four factors, so that the reprogrammed cells would fluoresce green.

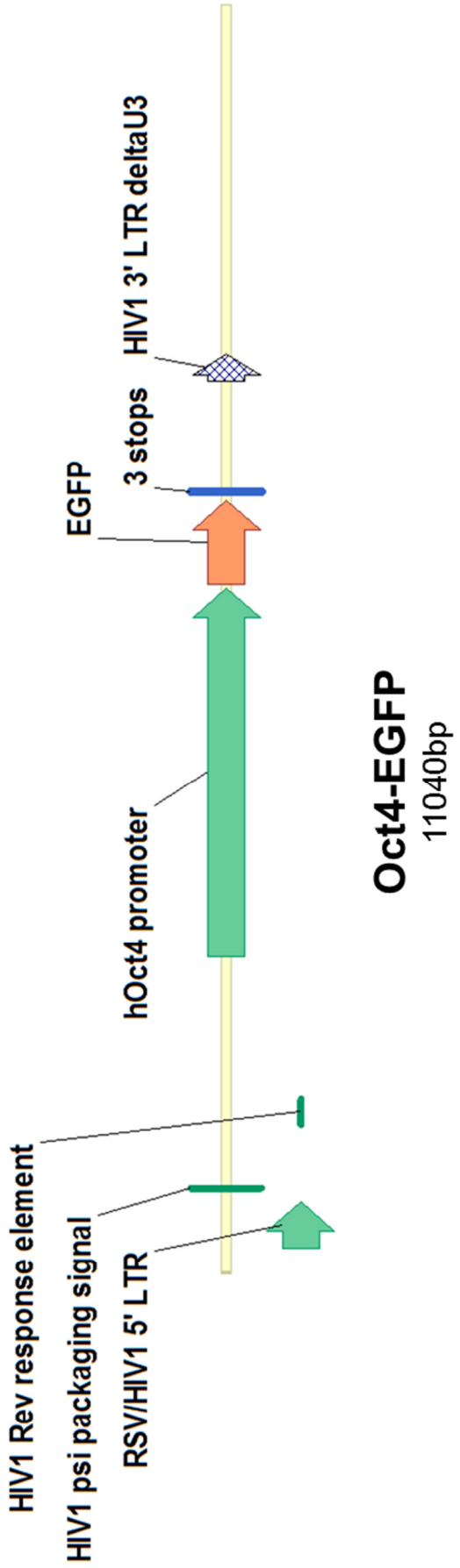


Figure 13. Map of Oct4-EGFP expression plasmid: the EGFP gene is under the control of the human Oct4 promoter. RSV/HIV1 5'LTR is constituted by the Rous Sarcoma Virus (RSV) promoter and the HIV1 R/U5 long terminal repeat. This element provides a high level of expression of the full-length viral transcript in 293T cells, allowing, at the same time, efficient tat-independent production of viral RNA; in fact tat is required for transcriptional activation of the HIV1 promoter, but becomes dispensable in the presence of a constitutively active promoter such as that of the RSV. The rev response element is bound by the rev protein; this bond allows the mRNA to be exported from the nucleus to the cytoplasm. Self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA.

___Derivation and characterization of putative mouse induced pluripotent stem cells

First I verified whether the human Oct4 promoter was active in mouse: mouse ESCs were seeded into a gelatin-coated 6 well plate (10^6 cells/well) and the following day were transfected with 4µg of Oct4-EGFP plasmid; pEGFP-C1, a plasmid expressing GFP under the control of a constitutive promoter, was transfected at the same time as positive control. Even though the transfection of the Oct4-EGFP plasmid was not very efficient, one day after transfection the cells clearly displayed bright green spots (Fig. 14).

After assessing that the species difference did not affect the activation of the promoter, the virus was packaged. In order to monitor the lentiviral production protocol, since Oct4-EGFP is only active in ESCs, we packaged in parallel a lentiviral vector expressing EGFP under the control of the CMV promoter (Fig. 15). When 293T cells, plated in 10cm dishes, reached 70% of confluence, they were transfected with 6.5µg of Oct4-EGFP or CMV-EGFP plasmid, 5µg of pLP1 (carrying the gag/pol gene, whose expression was made rev-dependent), 2.5µg of pLP2 (coding for the rev protein) and 2µg of pLP/VSV-G plasmid (harbouring the envelope G glycoprotein from Vesicular Stomatitis Virus, which allows the production of a virus with a broad host range). Three days post transfection, when the supernatant containing the viral particles was collected, the cells were analysed at the fluorescence microscope in order to determine whether the transfection was successful: most CMV-EGFP transduced cells were fluorescent (Fig. 16), thus confirming the vectors penetrated into the cells. Also few Oct4-EGFP cells showed EGFP expression; this was not expected for the Oct4 promoter is supposed to be active only in ESCs (Tada, 2001; Pan, 2002; Kimura, 2004): the fluorescence

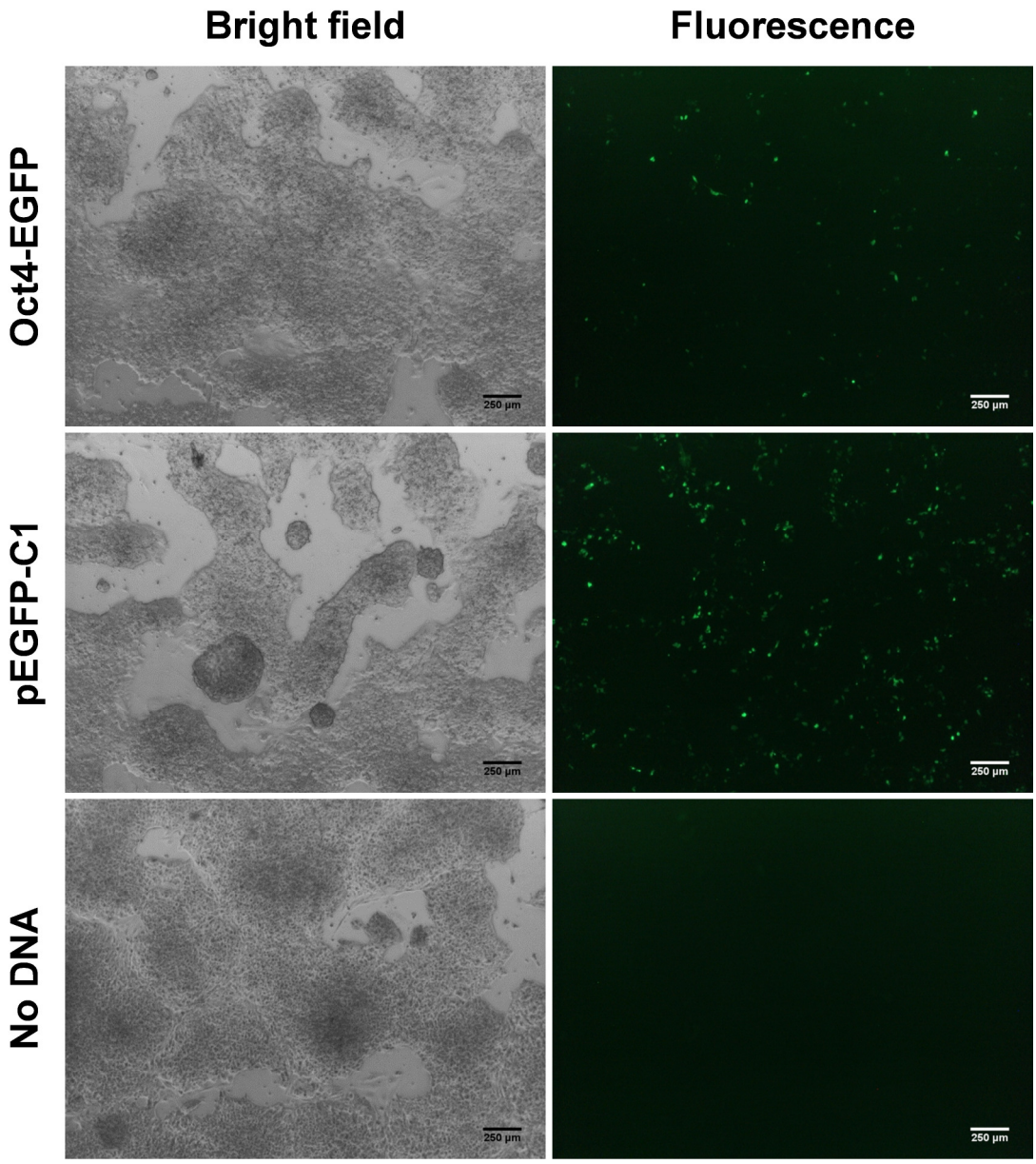


Figure 14. Bright field and fluorescence images of mouse ESCs transfected with Oct-EGFP and the positive control pEGFP-C1. The plasmid pEGFP-C1 carries an expression cassette where the EGFP gene is driver by a CMV constitutive promoter. The negative control is represented by transfection of reagents only. Scale bars: 250µm.

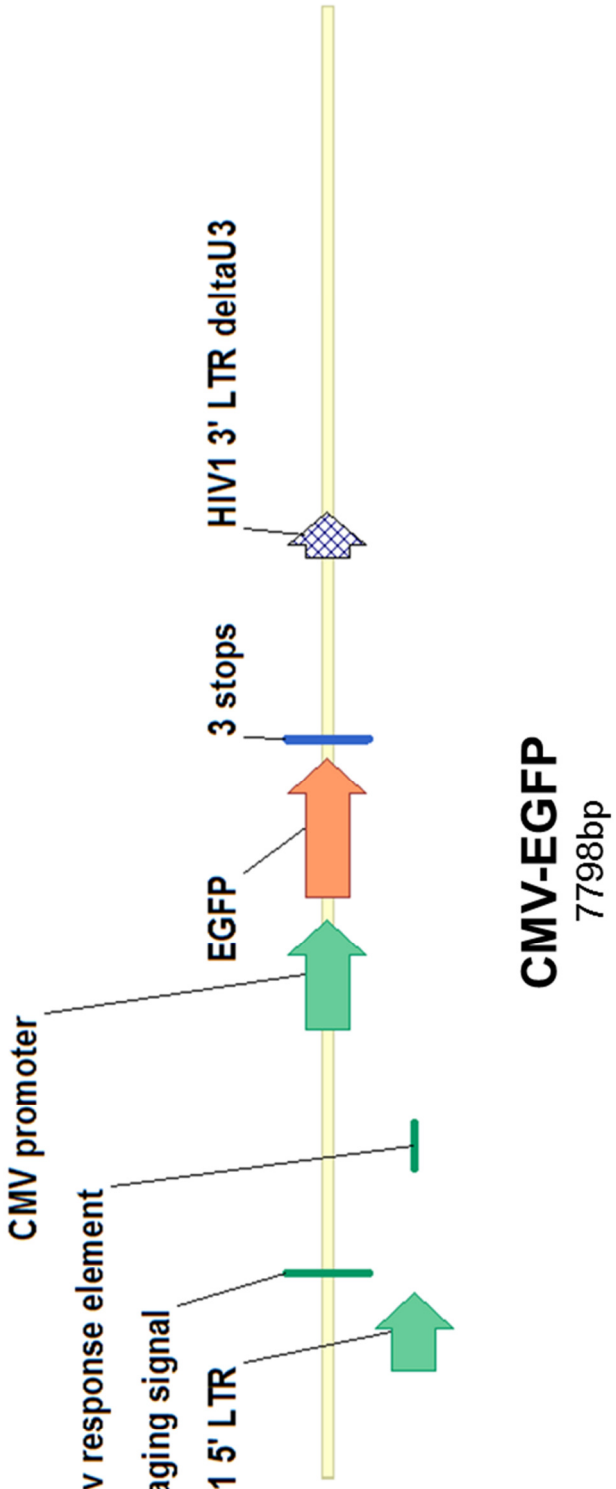


Figure 15. Map of CMV-EGFP expression plasmid: the EGFP gene is driven by the cytomegalovirus promoter. RSV/HIV1 5' LTR (Rous sarcoma virus/human immunodeficiency virus hybrid long terminal repeat) is required to drive high titres during the packaging step. HIV1 psi packaging signal is required for the encapsidation of the viral genome. HIV1 Rev responsive element: in the presence of the HIV-1 accessory protein Rev, HIV-1 mRNAs that contain the HIV1 Rev responsive element can be exported from the nucleus to the cytoplasm for translation. The 3 stop codons prevent the formation of viral mRNAs. HIV1 3' LTR deltaU3: the 3' LTR does not carry the enhancer in U3, so that, once integrated, if the retroviral sequence is translated it will lack a functional promoter in the 5'LTR.

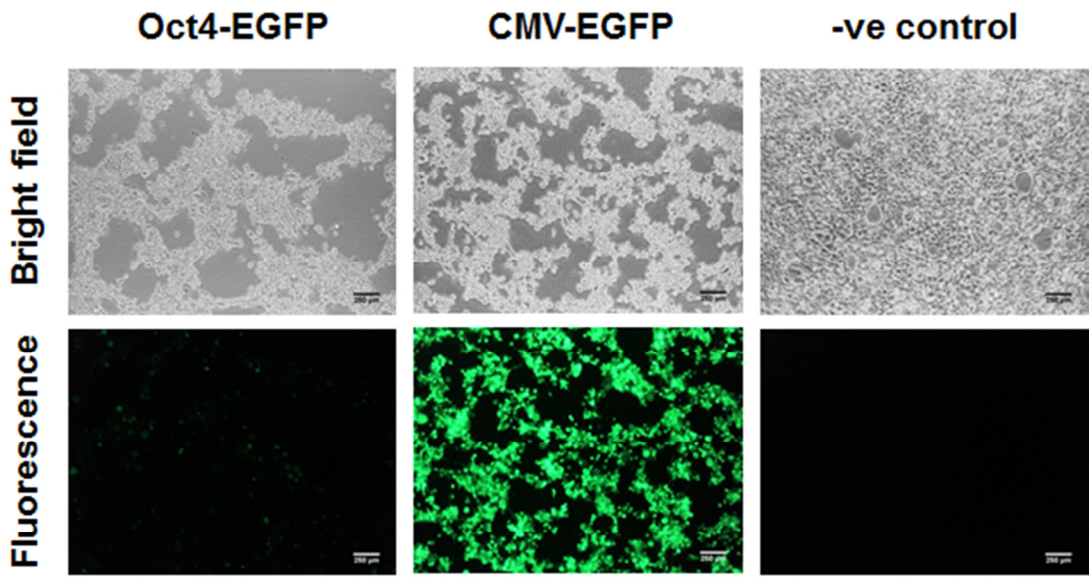


Figure 16. Oct4-EGFP packaging: 293T cells were monitored three days after transfection to ensure the protocol was successful. CMV-EGFP virus was packaged and monitored at the same time as a control of the reaction. The negative control is represented by the packaging protocol of a virus that carried no reporter genes. The green cells in the CMV-EGFP and Oct4-EGFP plates confirmed that the transfection had taken place and that the expression cassettes of both plasmids were functional. Scale bars: 250µm.

observed is likely due to leaky expression of the Oct4 promoter rather than to its real activation.

In order to verify whether the packaging protocol was successful, the CMV-EGFP virus was titred in 293T cells. Since the two lentiviruses are built on the same backbone and had been packaged at the same time, this could give a preliminary figure of the titre of Oct4-EGFP virus.

Cells were plated at low density ($7x10^4$ cells/well in a 24 well plate) and, 24h later, were transduced in duplicate with serial dilutions (ranging between 10^{-1} and 10^{-4}) of viral particles. Two days afterward the cells were monitored, but no fluorescence was visible, indicating that either the virus was not viable or the EGFP expression was too low to be detected. The packaging was repeated two more times employing fresh expression and packaging plasmids and the titration was performed supplementing the virus with 4μg/ml polybrene, which improves the transduction by neutralizing the charge repulsion between viral membrane and cell surface (Davis, 2004). Again, no green fluorescing cells were observed. This was unexpected and never resolved; it is indeed worth mentioning that, at the time others in the lentiviral laboratory experienced similar problems with different vectors.

Due to the inability to produce efficient Oct4-EGFP viral vectors, we decided to isolate iPSCs only upon morphology criteria, supported by the success achieved by other groups following this protocol (Maherali, 2007; Meissner, 2007).

4.2.2 Transduction of mouse foetal fibroblasts

A schematic representation of the reprogramming process is shown in figure 17.

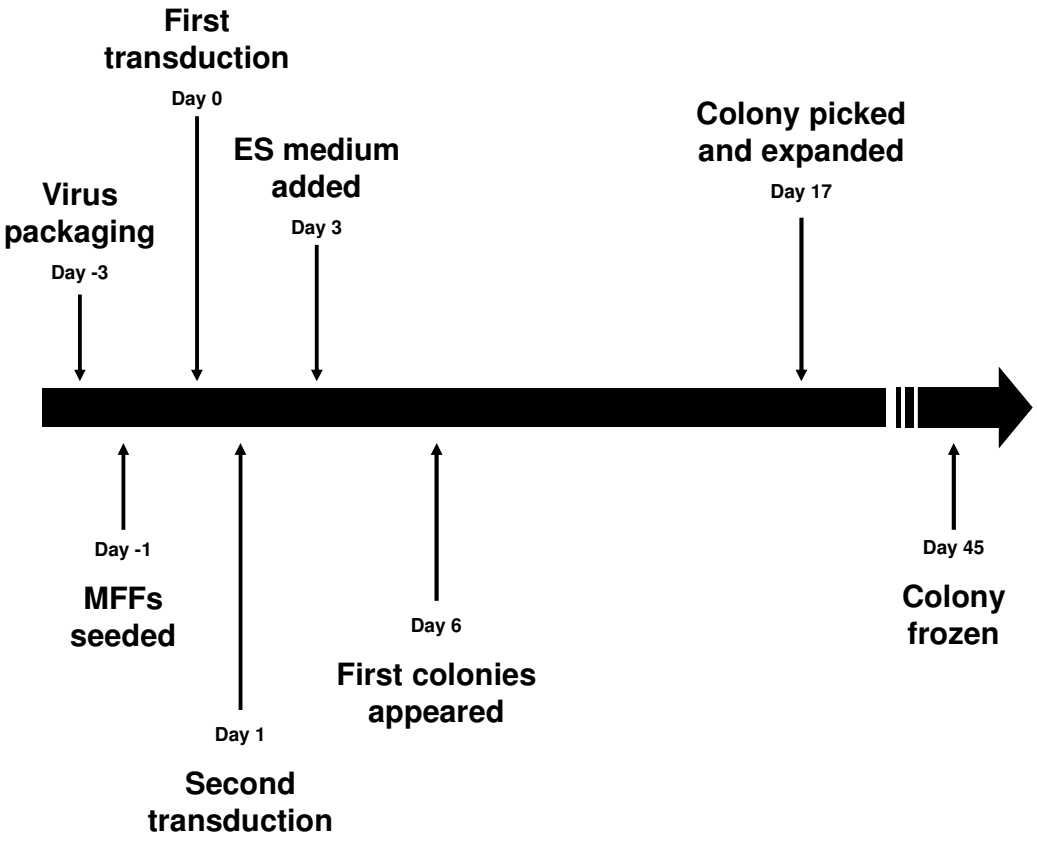


Figure 17. The diagram shows the experiment time line of the mouse iPSC reprogramming: the packaging protocol for the 4 retroviral vectors (cMyc, Klf4, Oct4 and Sox2 viral vectors) was started 3 days before transduction; 1 day before transduction MFFs were seeded in fibroblast medium. The day of the transduction (day 0), the medium was removed from the MFFs and each fresh viral vector carrying a different reprogramming gene (cMyc, Klf4, Oct4 or Sox2) was added to the MFFs, after being supplemented with 4µg/ml of polybrene. The same viral transduction was repeated the day after. At day 3 fibroblast medium was replaced with mouse ESC medium. The first colonies appeared on day 6; when big enough the colonies were picked and expanded.

___Derivation and characterization of putative mouse induced pluripotent stem cells

MFFs at passage 2 (Fig. 18a) were seeded in a 6 well plate ($1.3x10^5$ cells/well) on a feeder layer composed of $1.5x10^5$ irradiated SNL cells/well. The day after, the MFFs were transduced twice (on day 0 and day 1) with four MoML viral vectors (1ml each), each carrying one reprogramming factor (cMyc, Klf4, Oct4 or Sox2). On day 4 fibroblast medium was replaced with mouse ESC medium. In the following days the cells were examined for changes indicative of transformation events such as morphological alterations, loss of contact inhibition, increased growth rate, lack of senescence. On day 6 the transduced fibroblasts started changing morphology. Seven days after the transduction, foci of increased growth emerged and small ESC-like colonies appeared in the experimental MFFs (Fig. 18b), in contrast to the control where no aggregates of cells could be detected (Fig. 18c).

To ease the handling of the cells, I decided to follow the strategy employed by Meissner et al (Meissner, 2007) who assumed that reprogrammed cells would have outgrown the starting fibroblasts; based on this assumption this group attempted to generate iPSCs by passaging the entire plate instead of picking colonies. Many small colonies appeared every 2-3 days after passaging (Fig. 18d), but they were not allowed to become large because every 4-5 days the cells would become confluent and would need to be passaged (Fig. 18e). When the cells reached passage 6, the non-transformed MFFs around the experimental putative iPSCs became senescent and stopped proliferating allowing ESC-like colonies to grow and be picked.

A total of 211 colonies was picked at different time points (on day 17, 19, 25, 33 and 34 days post-transduction), disaggregated and seeded on SNL feeder layer ($3.5x10^3$ cells/well in 96 well plate) in mouse ESC medium. After picking, most of the

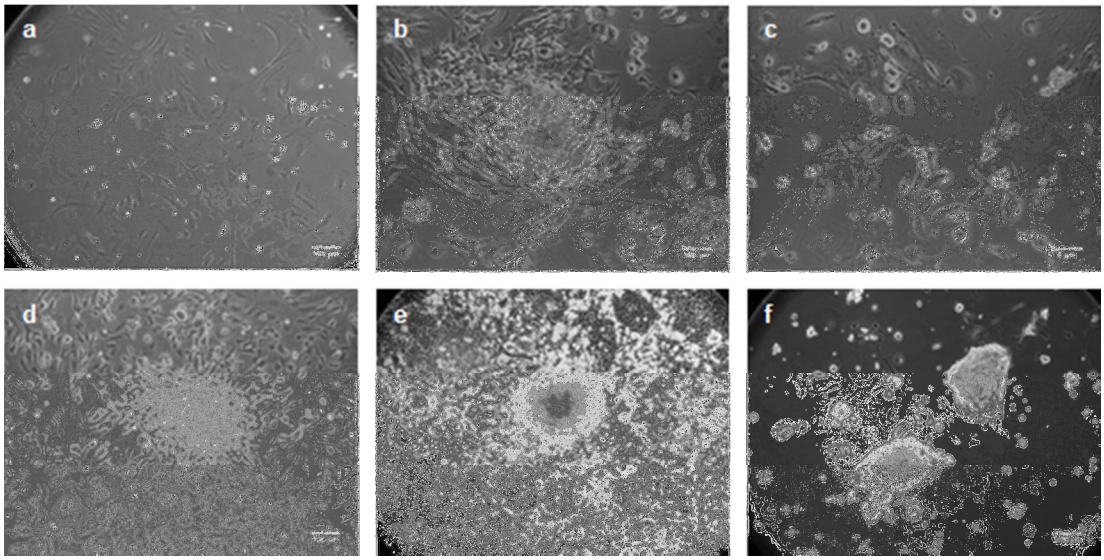


Figure 18. (a) MFFs before the transduction with the four genes. (b) 4-gene MFFs at day 7 after transduction: the first colonies started appearing among the fibroblasts. (c) Non-transduced control MFFs at day 7 after transduction: no colonies could be located. (d) Example of a small colony appearing after the cells were passaged. (e) Confluent plate of 4-genes transduced MFFs. (f) An established putative mouse iPSC line at passage 10 after transduction, displaying a morphology similar to that of ESCs. Scale bars: 100µm for (a), (b), (d) and (f); 5µm for (c) and 250µm for (e).

colonies became flat. Based on morphology, I selected and expanded 4 colonies focussing on those clones whose appearance was more similar to that of mouse ESCs, with round bodies and sharp edges (Fig. 18f).

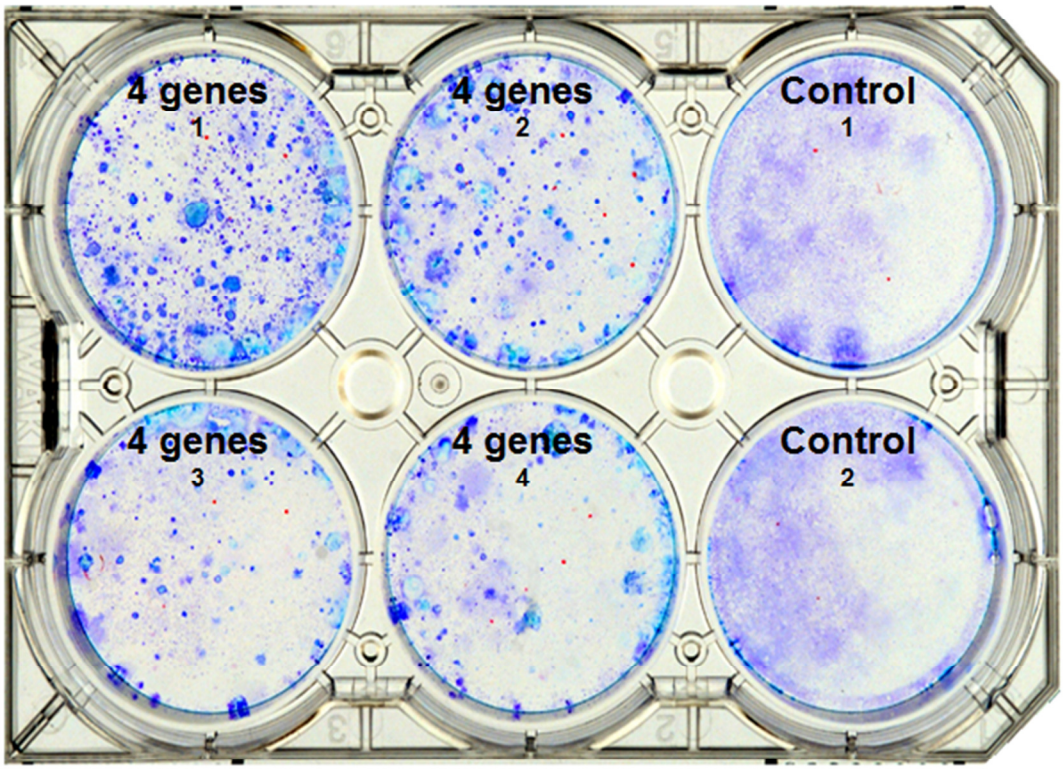
4.2.3 Colony-formation efficiency

In the experiment described above, the cells became quickly confluent and had to be passaged; consequently it was not possible to calculate the efficiency of reprogramming because it cannot be excluded that distinct iPSC lines were derived from the same reprogrammed parental cell. Another transduction was therefore carried out to assess the efficiency of reprogramming. Due to the lack of reporter genes, Giemsa staining was employed to mark the colonies so that it was possible to count theme.

The protocol employed this time was slightly different from the previous: after the transduction MFFs were plated at much lower density ($4x10^4$ cells/well in 6 well plates), so that it could be possible to maintain the cells without passaging them. Twenty days post transduction experimental and control MFFs were stained with Giemsa. While in the control MFFs no aggregates of cells were detectable, the 4-gene transduced fibroblasts gave rise to many colonies (Fig. 19a). The colonies were counted and the efficiency was expressed as the percentage of the ratio between the number of colonies and the number of seeded cells (Fig. 19b). Surprisingly, the four replicates, although performed following an identical protocol, yielded a considerably different number of colonies.

In this experiment the efficiency of colony formation was very high, reaching 13.2%

a



b

	N° colonies	N° seeded cells	Efficiency
4 genes 1	528	$4x10^4$	13.2%
4 genes 2	251	$4x10^4$	6.3%
4 genes 3	125	$4x10^4$	3.1%
4 genes 4	111	$4x10^4$	2.8%
Control 1	0	$4x10^4$	0%
Control 2	0	$4x10^4$	0%

Figure 19. (a) Giemsa staining of 4-gene transduced MFFs 20 days after transduction. (b) The table shows the efficiency of the reprogramming process: the colonies generated by the transduction of the four reprogramming genes were counted and divided by the total number of cells seeded; the assumption is that each colony comes from a single reprogrammed cell.

in one of the four replicates. During reprogramming, however, only a partial number of the total colonies appearing during the early phases will develop in authentic iPSCs (Hotta and Ellis, 2008). Since Giemsa does not have any specificity for pluripotent cells, part of the stained colonies might represent only partially reprogrammed cells. As a result, it is not possible to compare the efficiency calculated in this experiment with that estimated in other reports, where more rigorous parameters based on the ratio of authentic iPSCs derived or on the activation of endogenous pluripotency genes have been employed (Meissner, 2007; Huangfu, 2008b).

4.2.4 Pluripotency of dedifferentiated mouse foetal fibroblasts

In order to assess whether the isolated cells presented ESC-like features, mouse putative iPSCs were characterised according to the procedures utilised for ESCs (Wobus, 1984; Hoffman and Carpenter, 2005). We initially focussed on the expression of the pluripotency marker Nanog (Chambers, 2003; Mitsui, 2003). Four clones were stained alongside a positive (mouse ESCs) and a negative (MFFs) control: the clones were seeded into 2 wells of a 24 well plate (splitting ratio 1:2) and when at around 80% confluence (usually about one or two days later) they were fixed in 4% PFA and stained for Nanog. All clones were Nanog-positive, with a brighter signal in 2D10 clone (Fig. 20); however while in the ESCs the staining was clearly restricted to the nucleus, in the putative iPSCs the fluorescence appeared to be spread in the nucleus as well as in the cytoplasm.

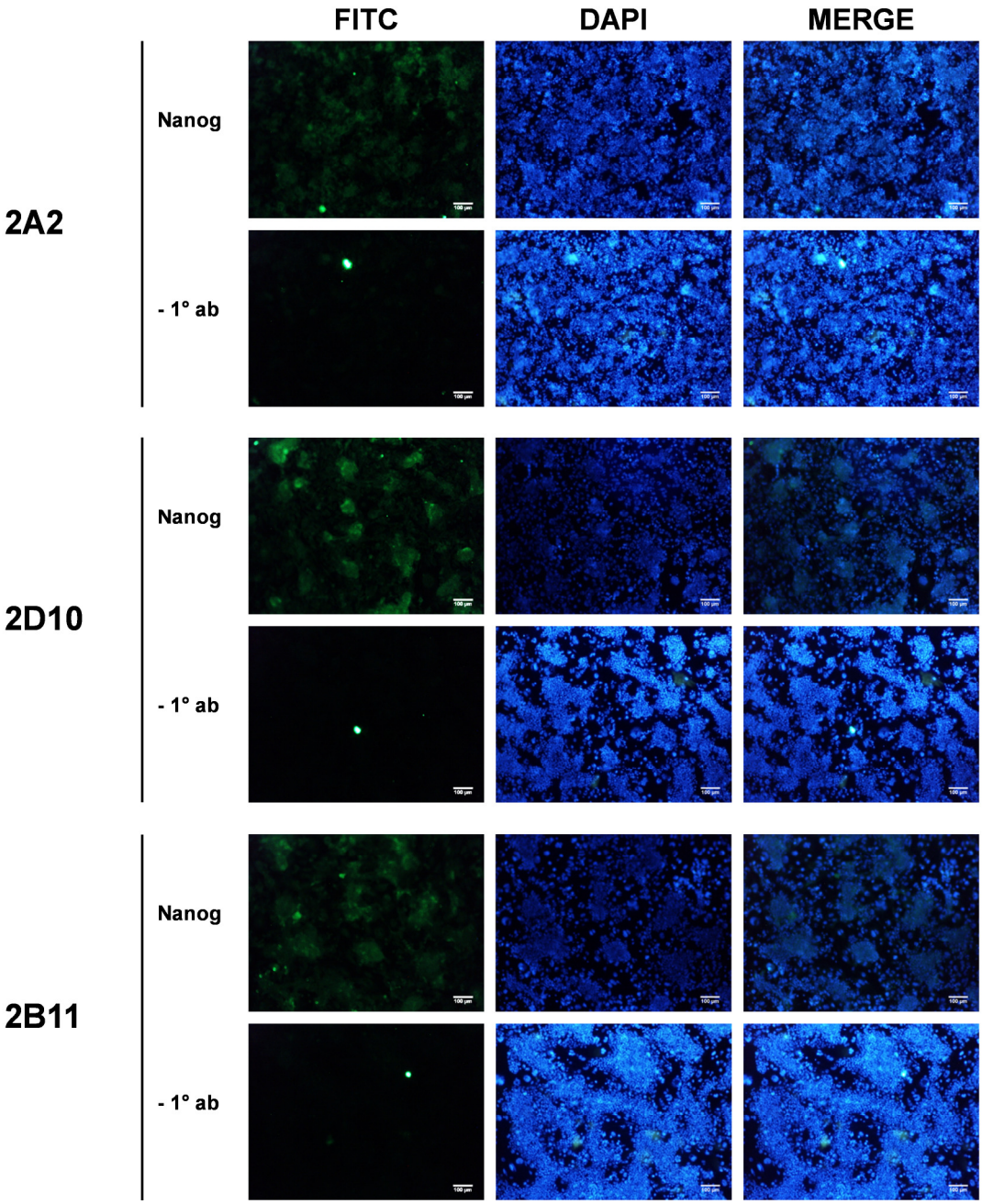


Figure 20 (legend in the next page).

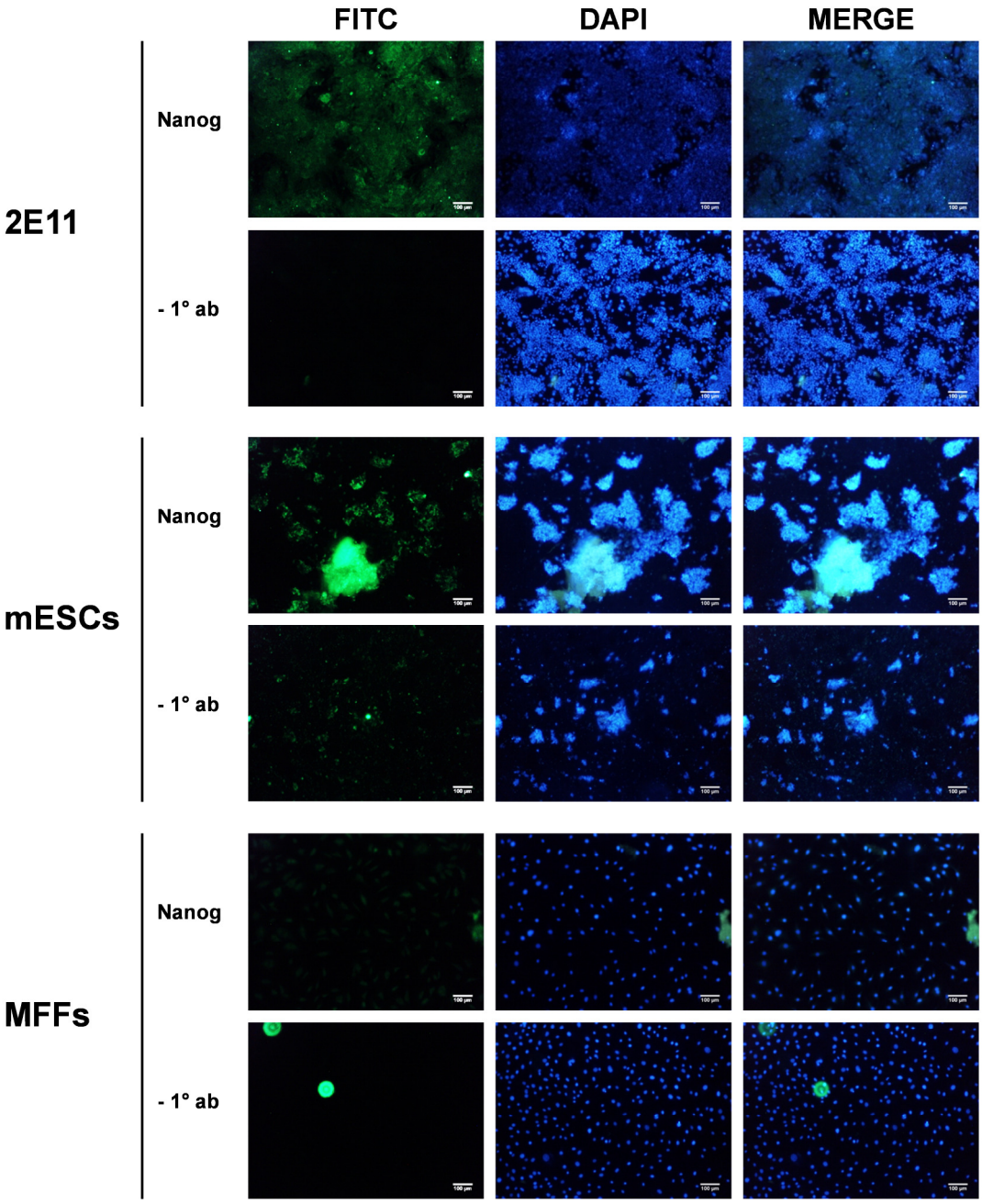


Figure 20. Nanog immunocytochemistry on the putative mouse iPSC lines obtained after expansion from single colonies. Mouse ESCs (mESCs) were employed as a positive control, whereas the parental MFFs were utilised as a negative control. For each cell line an immunocytochemical reaction was performed without adding the primary antibody in order to exclude the presence of any signal coming from unspecific binding of the secondary antibody. Images were taken at 10x magnification. Scale bars: 100µm.

4.2.5 *In vitro* differentiation and immunocytochemistry

In order to assess the differentiation ability of putative mouse iPSCs *in vitro*, the 2D10 cells (Fig. 21a), that displayed the more ESC-like morphology and the greater Nanog staining, were mildly trypsinized from a T25cm^2 flask, separated from the feeder layer and seeded in a 9cm Petri dish (low attachment plate) in mouse differentiation medium (mouse ESC medium with no LIF). The differentiation was also performed on mouse ESCs (Fig. 21b) as a positive control for the experiment. After a few days in suspension culture, both 2D10 cell line and the mouse ESC positive control formed round structures (Fig. 21c, d) which were later plated in gelatin-coated wells. The cells were then allowed to attach to the bottom of the plate and spread. During this period of time the 2D10 clone and the positive control grew and expanded in a similar way. Among both cell lines it was possible to recognize signs of differentiation: from round and small cells with, many cells changed their shape enlarging their cytoplasm and acquiring different morphologies (Fig. 21e, f); in both cell lines it was also possible to detect beating cardiomyocytes. The outgrowths were eventually fixed and stained against markers of differentiation.

At this stage of the characterisation I wanted to identify at least one cell type for each germ layer. Induced pluripotent stem cells were examined by immunocytochemistry and were found positive for βIII-tubulin, an ectodermal marker (Fig. 22), and troponin T (Fig. 23), a mesodermal marker, were identified. The morphology and the position of the stained cells, together with the florescence, concurred in the identification of the differentiated cells. Neurons, which are detected with anti βIII-tubulin antibodies, are characterised by small nuclei and long,

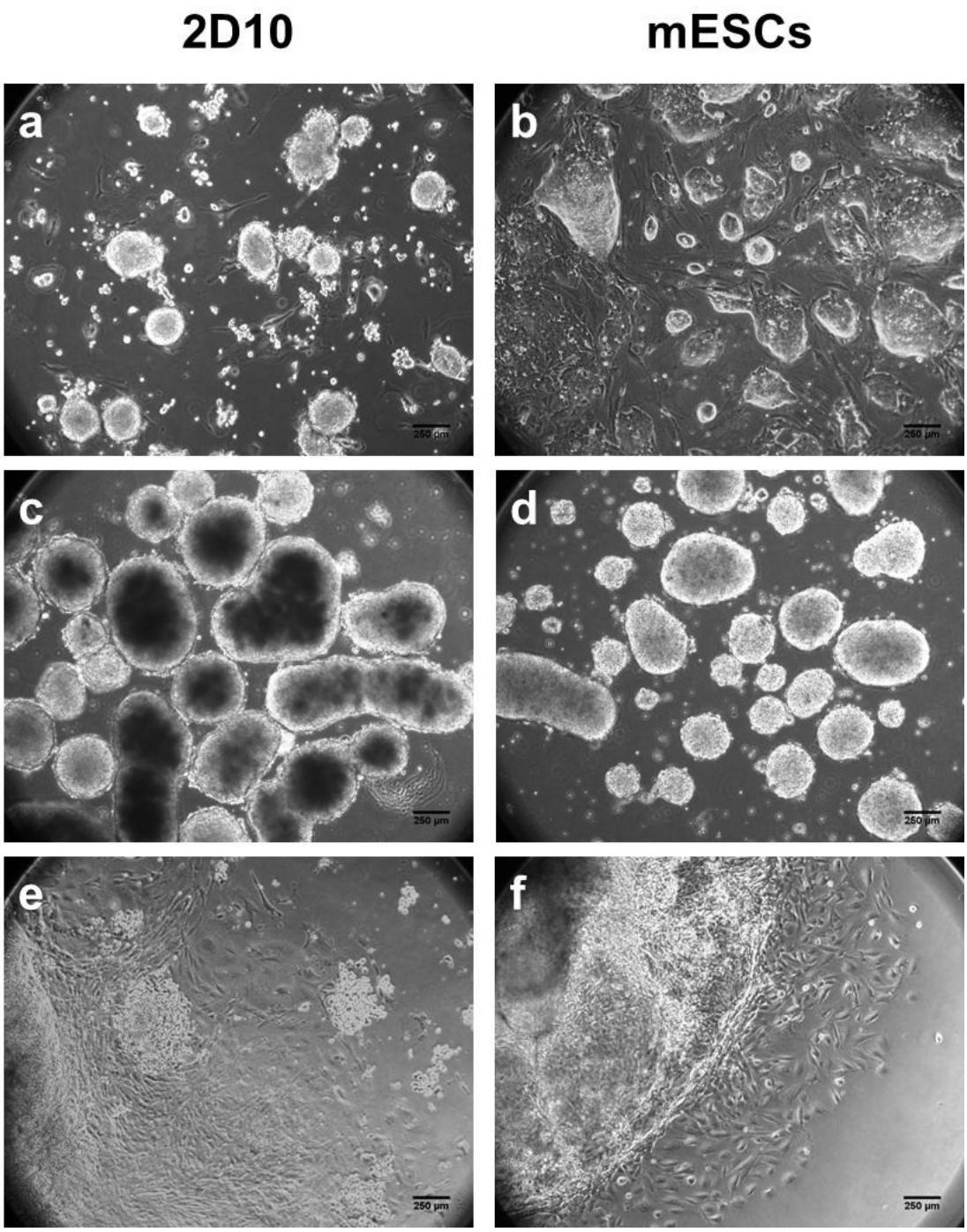


Figure 21. The figure shows the differentiation process of putative mouse iPSCs (a, c, e) and mouse ESCs (b, d, f). (a, b) Cells before differentiation. (c, d) In suspension culture, both cell types form round and smooth embryoid bodies. (e, f) When the embryoid bodies are seeded on gelatin-coated plates, they spread giving rise to an outgrowth composed of distinct differentiated cell types. Scale bars: 250µm.

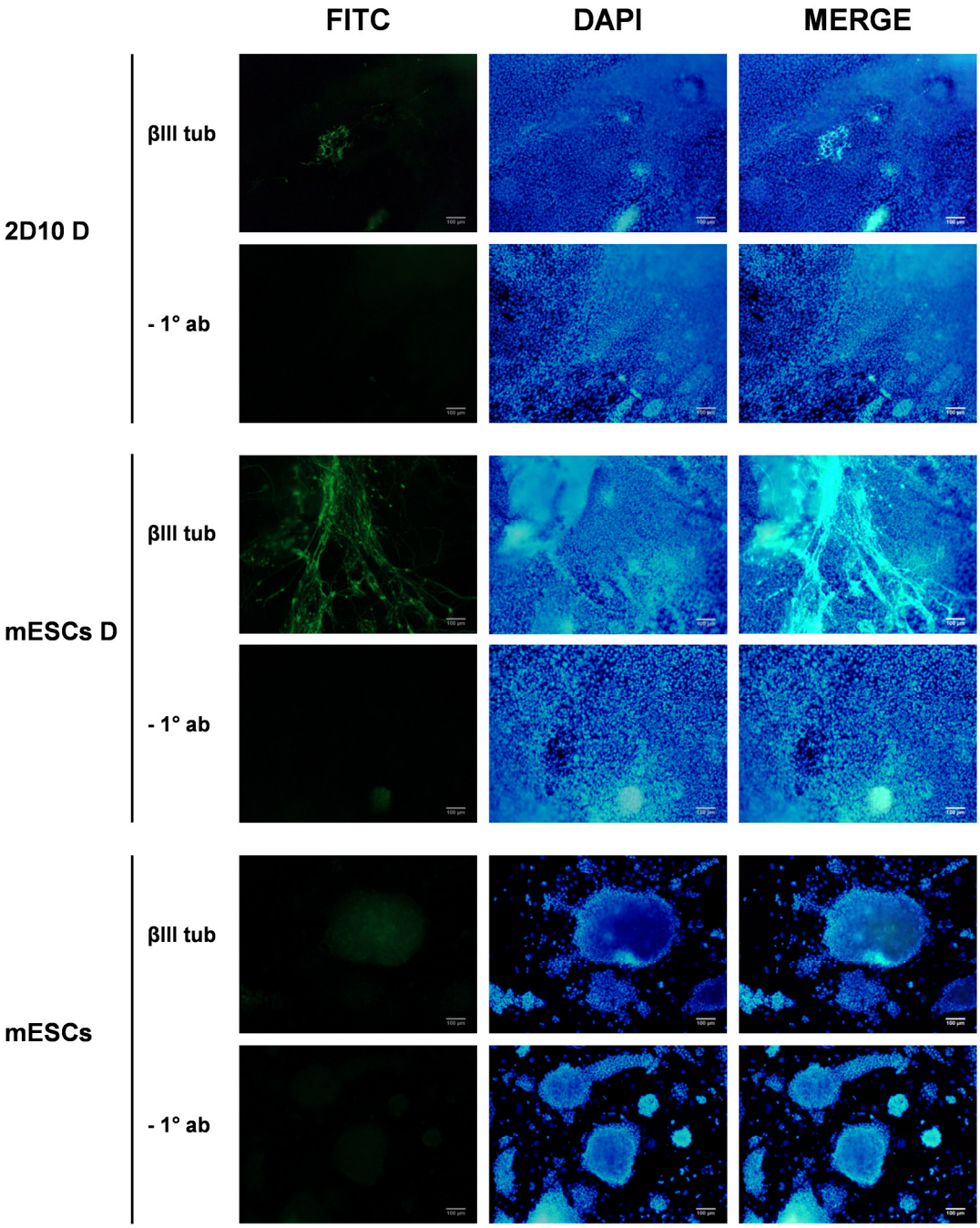


Figure 22. Differentiated 2D10 cell line gives rise to cells belonging to the ectodermal germ layer. Differentiated mouse ESCs are the positive control. Undifferentiated mouse ESCs are the negative control. A minus primary antibody control was performed alongside the βIII tubulin staining. Scale bars: 100µm.

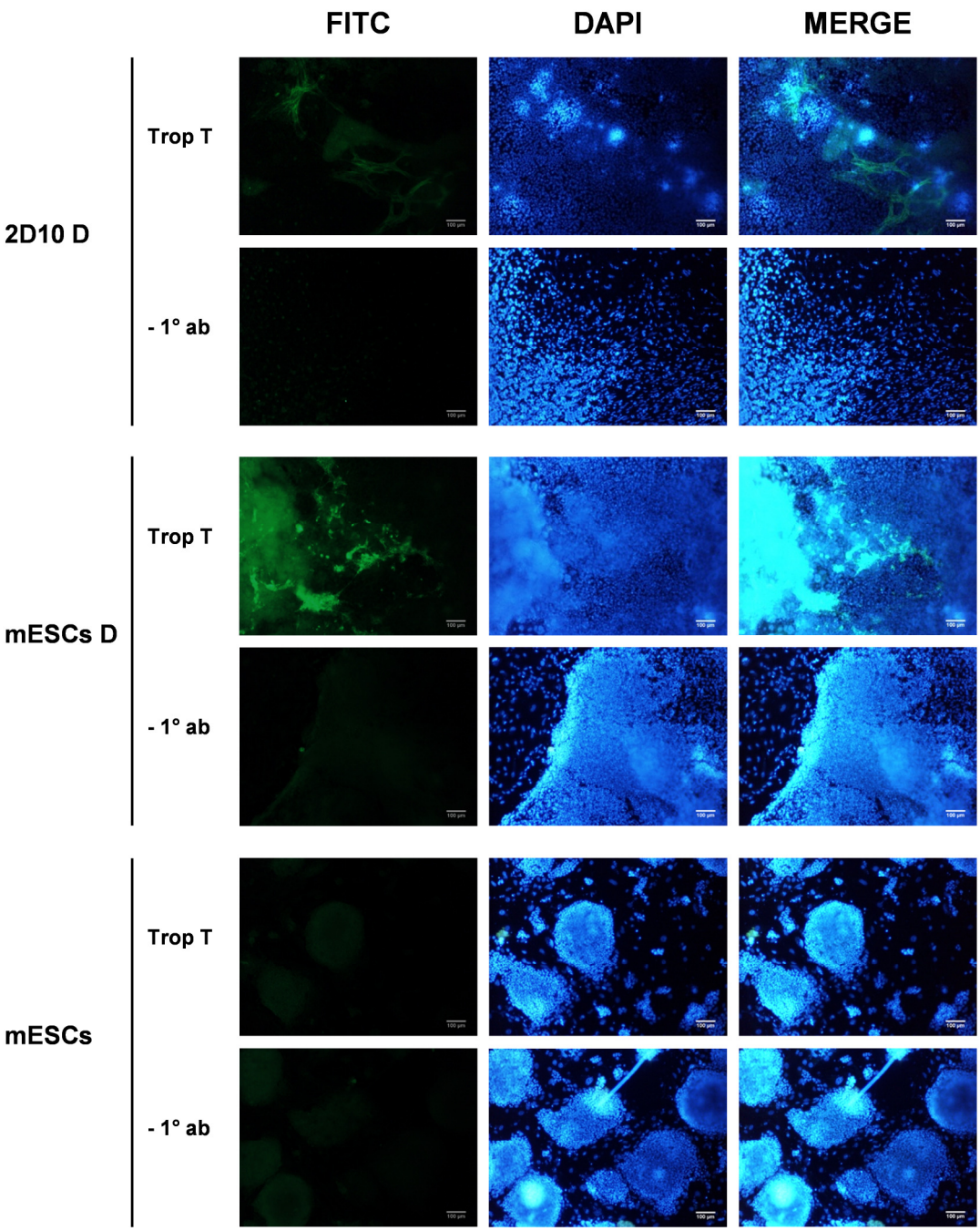


Figure 23. Troponin T staining was carried out on differentiated 2D10 cells, confirming their ability to give rise to mesoderm. Differentiated mouse ESCs are the positive control. Undifferentiated mouse ESCs are the negative control. A minus primary antibody control was performed alongside the troponin T staining. Scale bars: 100µm.

thin dendrites, as evident in the 2D10 staining and even more in the mouse ESC staining. Troponin T positive cells represent muscle cells and the fluorescent signal often appeared where beating cells had been previously recognised.

In order to detect the endodermal lineage, an anti α-fetoprotein antibody was selected; yet the optimization of the immunocytochemistry protocol for this antibody was complicated and was achieved only when the sheep experiment had already started. The mouse experiment was not addressed towards the isolation of fully reprogrammed cells, its main aim was the validation of the transduction protocol, therefore the α-fetoprotein staining on mouse iPSCs was not continued.

4.3 Discussion

I have illustrated the isolation of mouse ESC-like cells obtained from MFFs utilising the 4-gene transduction protocol described in the original iPSC report. This work was performed primarily to establish the methodology and the strategy for subsequent sheep studies.

In comparison to the Takahashi and Yamanaka method, only a few aspects were modified. The main difference between the published technique and that utilised here regarded the procedure aimed at selecting the reprogrammed colonies: while Takahashi and Yamanaka applied antibiotic in order to eliminate the non-reprogrammed cells, later reports stated that the use of selectable markers could even undermine the derivation of iPSCs when applied with the wrong timing (Maherali, 2007). My initial strategy, the production of a lentivirus carrying a reporter gene under the control of the Oct4 promoter, proved not possible. Given the inability to pursue this strategy and sustained by the evidence that pluripotent cells

could be isolated without the need for any selection (Meissner, 2007), I based the identification of the reprogrammed cells upon morphological criteria. In their study, Takahashi and Yamanaka let the cells grow in the same wells until colonies appeared and could be picked, while I followed Meissner's strategy (Meissner, 2007) and passaged the cells with the expectation that eventually iPSCs would have outgrown the non-reprogrammed fibroblasts.

While many colonies were generated as a consequence of the transduction, the technique could be improved in many ways, which I will discuss below.

First, the transduction was carried out utilising MFFs plated on feeder layer. This can reduce the efficiency of the transduction since the irradiated SNL cells can compete with the MFFs for the virus. In the following reprogramming experiments, in order to avoid the competition between the feeders and the OFFs the transduction will be performed in the absence of irradiated SNL cells.

Second, the reprogramming protocol requires fresh virus for the transduction. In order to verify that each new viral preparation is viable it would be desirable to build an expression vector similar to the pMXs plasmids but constitutively expressing GFP. While performing a reprogramming experiment, control parental fibroblasts could be transduced with the GFP virus and the success of the packaging could be confirmed by the appearance of green cells.

The iPSC isolation might have benefited from the culture in 3i or 2i media instead of serum and LIF (Ying, 2008). The 3i and 2i culture conditions are a defined growth media where no cytokines are needed to maintain the cells in a pluripotent state: these media are supplemented with inhibitors which block differentiation pathways maintaining the population of cells in a pluripotent state and leading differentiated or

differentiating cells to death (Ying, 2008). These media may provide a more suitable environment for iPSCs, since they might represent a selection able to discern between differentiated and reprogrammed cells.

Last, the amount of feeder layer was too low: SNL cells should be nicely spread with little gaps in between, while in this first experiment they were very sparse.

Although the transduction protocol was successful and many of the generated colonies displayed morphology similar to that of ESCs, I elected to only partially confirm the derivation of authentic mouse iPSCs. The characterization of ESCs is normally achieved by staining them against markers of pluripotency such as Nanog, Oct4, Sox2, AP, SSEA1, Rex1 (Koestenbauer, 2006). I utilised only one marker, Nanog, and the immunocytochemistry showed that Nanog localization performed in the putative mouse iPSCs I generated is both nuclear and cytoplasmic; this is in contrast with what observed in in ESCs, where Nanog is strictly nuclear (Do, 2007). The cytoplasmic localization of this pluripotency marker has been found in ASCs (adipose-derived stem cells and mesenchymal stem cells, (Zuk, 2009; Carlson, 2011), primitive germ cells (Goel, 2008) and is also often correlated with tumours (Ezeh, 2005; Goel, 2008; Ye, 2008). This leads to many hypotheses: the insertion of the four transcription factors, in particular cMyc, might have directed the MFFs towards a cancerous fate instead of driving them into a pluripotent state; another possibility is that the putative mouse iPSCs might be only partially reprogrammed, behaving more like ASCs than ESCs. Alternatively when Nanog staining was performed the cells were still in the process of reprogramming: the immunocytochemistry was performed at passage 7 after transduction, but

___Derivation and characterization of putative mouse induced pluripotent stem cells reprogramming is a long process and the cells might have been still in an intermediate state. Nanog staining should have been repeated later, in order to verify whether the expression pattern of this pluripotency marker had changed. Furthermore, while Oct4 and Sox2 were used as reprogramming factors and immunochemistry against them would have not been able to discern between the viral and the cellular protein, other markers should have been employed for a better characterization.

In order to prove the pluripotency of the generated putative iPSCs, further assays should have been performed. As already discussed above, showing the expression of multiple markers of pluripotency would have given a more robust evidence of the generation of true iPSCs. In addition, an RT-PCR should have been done with the purpose to verify the silencing of the four transgenes without loss of pluripotency, which is considered one of the main evidences of full reprogramming (Hotta and Ellis, 2008). Again, in case of female cells, the re-activation of the X chromosome is another distinctive trait of true iPSCs. Moreover, in order to assess whether the reprogramming had led to chromosomal alterations, which are very common in fast cycling cells (Hanson and Caisander, 2005; Mantel, 2007), a metaphase spread should have been carried out.

The *in vitro* differentiation potential was not completely investigated either. I showed the putative mouse iPSC ability to differentiate into mesoderm and ectoderm, but omitted the endoderm due to the long optimization required for the α-fetoprotein antibody. In order to identify endodermal markers or to provide further evidences of the other two lineages, immunocytochemistry might have been substituted or associated with RT-PCR. A complete characterization of the cells should also have

___Derivation and characterization of putative mouse induced pluripotent stem cells included *in vivo* analyses such as teratoma formation, chimera formation and germline transmission.

Although the positive and negative controls employed for βIII-tubulin and troponin T stainings were appropriate, a better negative control would have been represented by the undifferentiated putative iPSC lines in place of the parental MFFs. This would have shown whether the original cells were effectively in a pluripotent state, demonstrating that the cell types we observed were indeed the result of the differentiation protocol.

While aware that the above characterization results do not lead to strong conclusions and that many other important assays should be performed in order to prove that authentic mouse iPSCs have been generated, it is nevertheless true that our main aim, establishing and validating the transduction protocol, was achieved. The technique could therefore be exploited for the generation of ovine iPSCs.

As described next, I was able to generate iPSC-like cells from sheep fibroblasts and their characterisation quickly became my focus preventing me form continuing with the characterisation for the putative mouse iPSCs.

CHAPTER 5

Derivation and *in vitro* characterization of ovine induced pluripotent stem cells

5.1 Introduction

In the previous chapter I have described the reprogramming protocol in mouse. After repeating the original Takahashi and Yamanaka's work and proving its effectiveness in my hands, I moved to the main aim of the project: the derivation of iPSCs from sheep.

The ovine species was chosen for it represents a distinctive platform in medicine and agriculture. The sheep is a recognised model for human disease, because its size, anatomy and physiology are closer to human if compared with other animal models such as the mouse and the rat (Harris, 1997; Abraham, 2008). The derivation of ovine pluripotent cells would offer significant opportunities to perform genetic modifications for the study of pluripotency in large animals, for the creation of disease specific mutants with significant human implications and for agricultural purposes (Harris, 1997; Abraham, 2008). On the other hand, ovine iPSCs would facilitate the understanding of developmental biology in this species. Additionally sheep pluripotent cells could be exploited in medicine and toxicology for the screening of drugs and other compounds (Telugu, 2010).

In order to achieve the generation of ovine iPSCs, OFFs were transduced with an amphotropic MoML viral vectors carrying the four original reprogramming genes (the mouse orthologs of cMyc, Klf4, Oct4 and Sox2). The cells were then grown in different culture conditions and analysed both *in vitro* and *in vivo* to evaluate the acquisition of pluripotency.

The data presented in this chapter shows the isolation of ovine iPSCs and the *in vitro* aspects of their characterization, consisting of:

- analysis of integration and silencing of the reprogramming transgenes,
- assessment of the expression of pluripotency markers,
- *in vitro* differentiation assay,
- karyotyping,
- calculation of the population doubling time.

5.2 Results

5.2.1 Transduction of ovine foetal fibroblasts

For the generation of ovine iPSCs, a protocol similar to that already employed for murine cells was utilised (Fig. 17), with only few modifications: OFFs (Fig. 24a) were seeded in gelatin-coated wells of 6 well plate ($1.3x10^5$ cells/well) in 2ml DMEM, 10% FCS and transduced the day after (day 0) with 1ml of each MoML viral vector. The transduction was repeated on day 1. In parallel, a control transduction performed employing a MoML viral vector expressing GFP indicated that the virus penetrated in the OFFs (Fig. 24b, c). MFFs were also transduced as a positive control for the reprogramming protocol.

At day 2 both ovine and murine cells were passaged onto γ-irradiated SNL cells and at day 4 fibroblast medium was replaced. Mouse ESC medium was added into the plates containing transduced MFFs, while two different culture conditions were applied to the sheep cells: mouse ESC medium and human ESC medium, the former

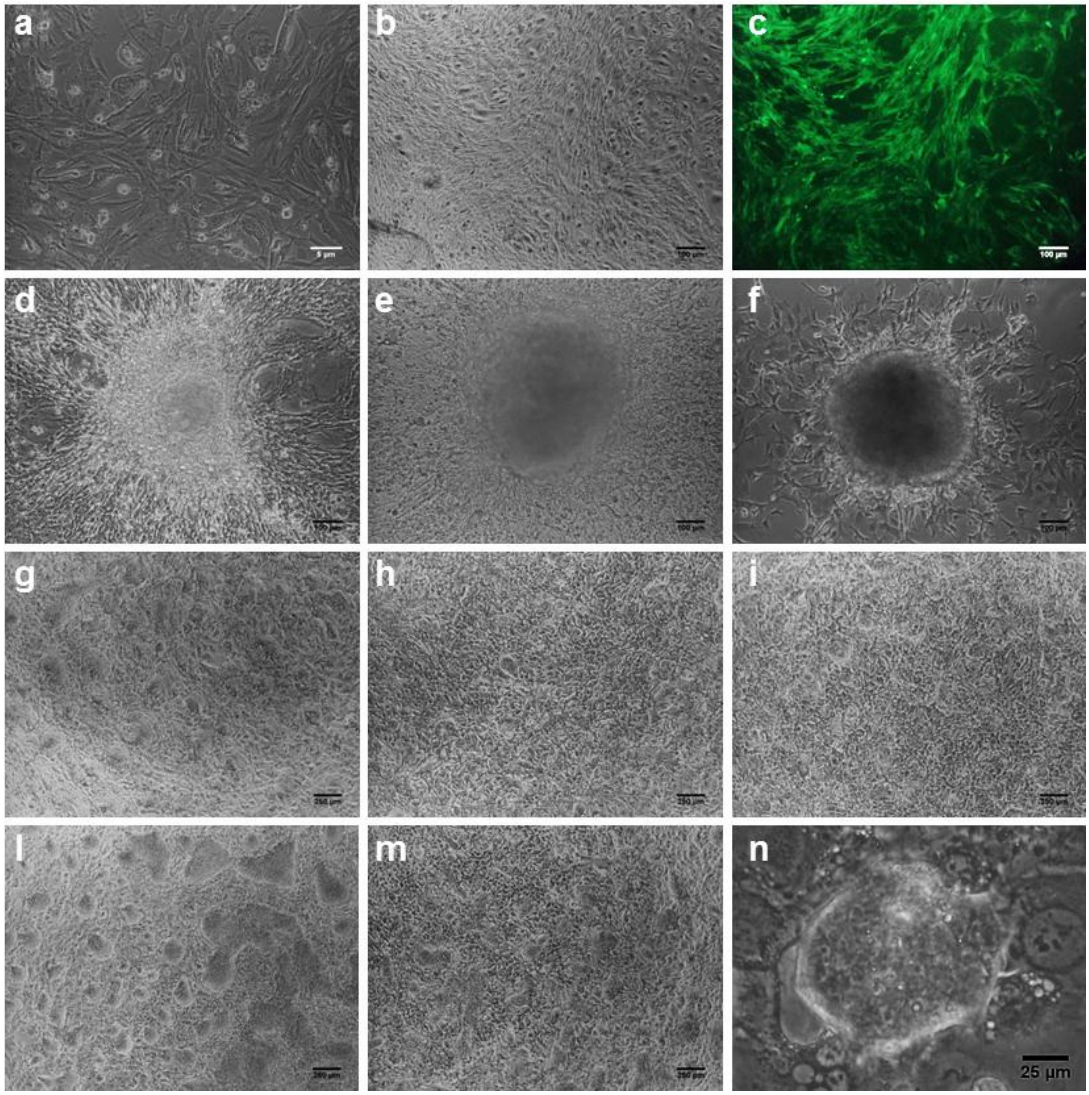


Figure 24. (a) OFFs at passage 6 before the transduction. Bright field (b) and fluorescence (c) images of GFP-transduced OFFs at day 8 post transduction; the cells are green, meaning that the viral vectors are functional. (d) Four-gene transduced OFFs grown in mouse ESC medium conditions display an irregular surface and indistinct borders. (e, f) Four-gene transduced OFFs grown in human ESC medium conditions have smoother domes and a morphology more similar to that of ESCs. (g-m) Different ovine iPSC lines isolated and expanded: (g) C2 clone, (h) C3 clone, (i) D1 clone, (l) F2 clone, (m) G2 clone. (n) High magnification (20x) image of G2 clone; the cells show ESC-like characteristics, with high nucleus to cytoplasm ratio, and have a morphology different from the parental OFFs. Scale bars: 100µm for (b), (c), (d), (e), (f); 5µm for (a); 250µm for (g), (h), (i), (l), (m); 25µm for (n).

containing FCS and mouse LIF, the latter including serum replacement and human bFGF instead. In the following 2-3 days the cells started changing morphology, becoming rounder, smaller and with larger nuclei. At day 7 the first aggregates of cells could be observed but at this stage it was difficult to discern between real ESC-like colonies and clusters of fibroblasts. Eight days after transduction, the ovine cells, but not the MFFs, became confluent. In the previous mouse experiment we had observed that MFFs had been plated at too high density, therefore in the sheep experiment the amount of seeded cells had been reduced. However, while the mouse cells were still quite sparse, OFFs although plated at the same density as MFFs, were confluent. This underlines a different behaviour between the embryonic fibroblasts belonging to the two species, with OFFs growing much faster than MFFs. For the transduction protocol ovine cells should be seeded at even lower density (i.e. 5 $x10^4$ cells/well in 6 well plate).

Due to the high confluence of OFFs I faced two possibilities: maintaining the cells too confluent for too long could possibly have affected the reprogramming process and eventually led to cell death; on the other hand, passaging the cells would have meant being unable to calculate the reprogramming efficiency. We decided to follow both strategies, passaging half of the wells, while maintaining the others at high confluence.

At day 13 large ESC-like colonies were visible in the transduced OFFs which had been passaged but not in the ones maintained at high confluence, albeit the cells were not dying, since they stayed attached to the bottom of the plate. Morphologically different clusters of cells were noticeable in the two medium conditions. In mouse ESC medium the colonies looked granulated and without a precise segregation from

the layer of parental fibroblasts (Fig. 24d), suggesting that the reprogramming was not complete. Conversely, in human ESC medium the colonies showed a more ESC-like morphology, with sharp edges and smooth domes (Fig. 24e, f).

At day 13, colonies were picked from the human ESC condition group and expanded on SNL feeder layer in human ESC medium. At first, different populations of cells growing together were clearly recognizable. Some non-reprogrammed OFFs, involuntarily carried along, continued proliferating alongside the iPSCs, but after 4-5 passages the senescence set in and the clones became more homogeneous.

Different cell lines were derived which displayed variegated morphology (Fig. 24g-m) and growth characteristics: some stopped growing, some started differentiating, others flattened and acquired a very high proliferation rate, probably becoming tumour cell lines. Only few clones maintained the typical traits of ESCs, displaying compact colonies with defined borders and an even surface, characterised by small, round cells with a high nuclear/cytoplasmic ratio (Fig. 24n). Most of the clones with morphology similar to that of ESCs were characterised by a fast growth rate if compared to the parental fibroblasts and they required to be sub-cultured every 2-4 days at a ratio between 1:3 and 1:5.

5.2.2 Ovine identity of the colonies

In the derivation protocol we have utilised mouse feeders, therefore we performed a sheep specific PCR assay on the gDNA of 8 clones in order to confirm the ovine identity of the iPSCs. Primers were selected specific for the endogenous Jaagsiekte sheep retrovirus (enJSRV), which is a provirus naturally integrated in the *Ovis aries*

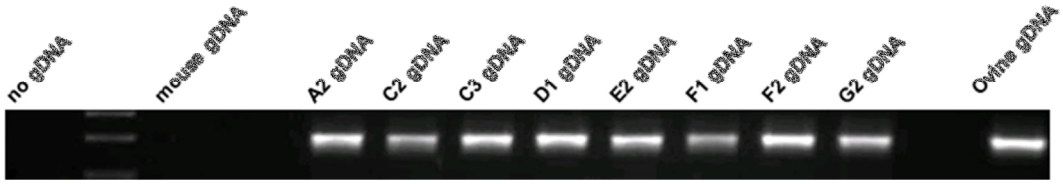


Figure 25. PCR reaction confirming the ovine origin of the iPSC clones. The PCR primers are specific for a virus integrated only in the ovine genome. The positive control was represented by gDNA extracted from ovine kidney. Two negative controls were included: mouse gDNA from SNL cells was employed to verify that the primers recognise only the sheep genome and an additional control, a PCR reaction without template, was utilised to detect contaminations in the reaction.

genome (Dunlap, 2006). As shown in figure 25, this PCR confirmed that all the clones analysed were indeed of sheep origin.

5.2.3 Silencing of the retroviral vectors carrying the reprogramming genes

As widely recognised, the silencing of the MoML viral vectors employed to deliver the transgenes is one of the most important elements that allow to discern between partially and fully reprogrammed iPSCs in mouse (Hotta and Ellis, 2008). Initially, the exogenous genes are active and drive the cells towards dedifferentiation, activating many transcription factors and pluripotency genes, including the same four endogenous reprogramming factors. Only once complete dedifferentiation has been achieved, with the viral genes silenced and the appropriate cellular genes active, is pluripotency maintained (Okita, 2007; Jaenisch and Young, 2008). Although mouse iPSC lines still expressing the transgenes had been found positive for markers of pluripotency, when analysed for *in vivo* differentiation these clones eventually failed to give rise to teratomas (Brambrink, 2008).

The first step for the evaluation of silencing in the ovine iPSCs was to confirm the integration of all four transgenes in the clones. To achieve this, gDNA was extracted from the cell lines and a PCR reaction with primers specific for the four genes was performed. In order to discriminate between transgenic and endogenous transcripts, a common forward primer was designed in the retroviral vector, while single reverse primers were designed to be specific to each of the four mouse genes. The negative control was represented by the parental cells, while the positive control was obtained adding the four pMXs reprogramming plasmids, each carrying one of the transgenes, into the negative control.

As figure 26a shows, cMyc, Klf4 and Oct4 constructs are integrated in all the clones, while Sox2 is integrated only in six cell lines, but not in clone A2 and in clone C3. Interestingly, this reduced presence of Sox2 is in accordance with other studies (personal communication; Dr Amandine Bretton, University of Edinburgh, and Prof Scott Fahrenkrug, University of Minnesota).

After proving the integration of the transgenes, in order to assess whether the ovine iPSC clones still expressed the four transduced factors, we carried out a RT-PCR on mRNA extracted from the clones at passage 7 and using the same primers employed for the integration assay (Fig. 26b). Again, the parental cells were used as negative control, while the positive control MFFs transduced with the four genes. As expected from the integration results, lines A2 and C3 did not show any Sox2 mRNA. Most clones still expressed the integrated genes (A2, C2, E2 and F1 cell lines). Of the remaining clones, C3 and F2 achieved to silence only Klf4, while G2 and D1 cell lines succeeded to silence three out of four genes (cMyc, Klf4 and Sox2), with G2 clone retaining only a very low expression of Oct4 if compared to the positive control. Nevertheless this analysis does not allow to state whether all cells display very low Oct4 transcripts or only few cells retain a strong Oct4 expression.

5.2.4 Markers of pluripotency

The ovine iPSC clones were examined for their ability to express markers characteristic of ESCs. Time-wise, the first important marker appearing during reprogramming is AP (Brambrink, 2008). AP is a hydrolase enzyme most active at

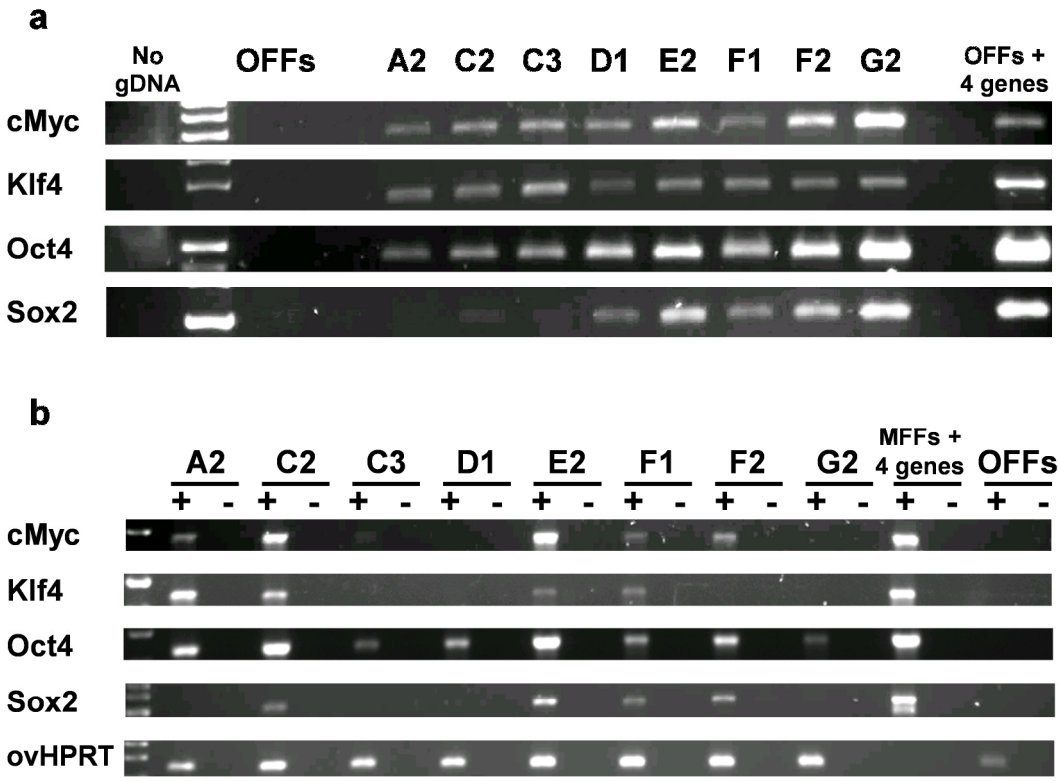


Figure 26. Silencing of the reprogramming genes: (a) PCR on the ovine iPSC gDNA to assess whether the reprogramming factors are indeed integrated. Negative controls: gDNA from OFFs, no DNA control; positive control: pMXs reprogramming plasmids mixed with OFF gDNA. (b) RT-PCR to verify the expression profile of the transgenes: most of the clones retained the transgene expression, while only D1 and G2 achieved to silence them, maintaining only a little expression of Oct4. Non-transduced OFFs represent the negative control; 4-gene transduced MFFs represent the positive control.

basic pH (Thomson and Marshall, 1998), responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins and alkaloids. Elevated expression of this enzyme is associated with ESCs, embryonic germ cells and embryonal carcinoma cells (Shamblott, 1998; Thomson and Marshall, 1998) and its decrease is an evidence of differentiation.

In order to evaluate whether the ovine iPSCs expressed AP, an activity assay was performed on clones at passage 7 (Fig. 27). Cells were seeded (1:3 ratio) on feeder layer in wells of 24 well plate and fixed one or two days post plating for 20min in 4% PFA. In this assay, mouse ESCs were employed as a positive control and the parental OFFs as a negative control. Since the ovine iPSCs were plated on feeder layer, irradiated SNL cells were included in the analysis in order to exclude that any positive results in the samples could be attributed to the feeder layer. The negative control and the SNL cells, as well as A2, C2, C3 and F1 clones resulted negative for AP activity, while D1 and E2 cells displayed only a faint staining. Two clones (F2 and G2) showed a very strong staining, which is comparable with that of the positive control.

Interestingly, the clones positive for AP activity were the ones which displayed a morphology more similar to that of ESCs and, in the case of D1 and G2, also the better transgene silencing profile. F2, the other clone that together with G2 showed the strongest AP signal, silenced only one transgene; this might be explained by the fact that AP, although being an ESC marker, is not strictly specific to ESCs, being expressed also in tumour and germ cell lines. On the other hand, the faint staining of D1 and E2 cells could be explained as a partial reprogramming, with the cells

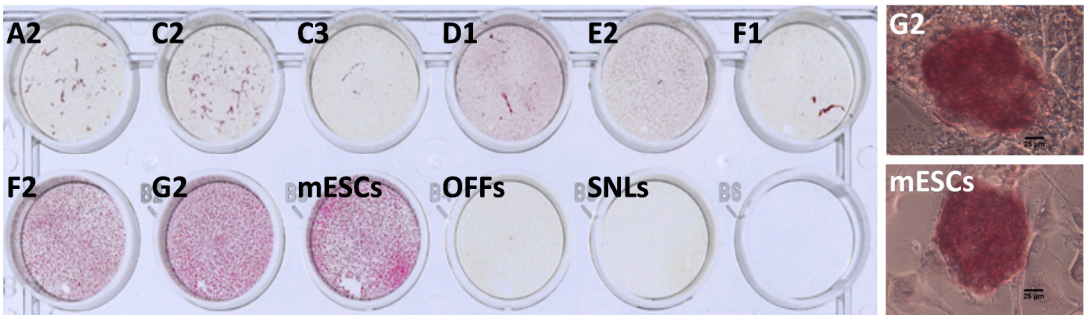


Figure 27. AP staining of isolated iPSC clones: on the left the figure shows the AP staining in the wells of a 24 well plate containing different cell types; on the right, 20x magnification images of G2 clone compared with mouse ESCs. Scale bars: 25µm.

trapped in a pre-iPSC state. An alternative hypothesis could be that these cells are just slower in their dedifferentiation process, as suggested by their slower growth rate compared with F2 and G2 clones. While AP expression marks the initial phases of reprogramming, Nanog and Oct4, two transcription factors, are later markers of iPSC induction (Brambrink, 2008) but more stringent for the assessment of the pluripotency (Koestenbauer, 2006). In our ovine iPSC clones Oct4 expression was not determined: as shown in the above silencing assay (Fig. 26) this protein is still expressed by the mouse transgene making it challenging to discern between exogenous and endogenous signal, either employing immunohistochemistry (because of the absence of a species specific antibody) or RT-PCR (because of the lack of the ovine Oct4 sequence).

Ovine iPSCs at passage 7 were passaged at 1:3 ratio in a 24 well plate on feeder layer and fixed after one or two days in 4% PFA for 20min. They were stained against Nanog (Fig. 28), with the positive control represented by mouse ESCs and the negative by OFFs. A further control, the same staining but without primary antibody, was carried out on samples and controls in order to exclude a non-specific binding of the secondary antibody to the cells. Four clones (A2, C3, E2 and F1) showed no signal for Nanog, whereas the others were positive for it, with the signal restricted to the nucleus, which is Nanog characteristic localization (Do, 2007).

SSEA1 and SSEA4 surface proteins are markers of pluripotency which are differentially expressed in mouse and human ESCs: in human SSEA4 is expressed in the pluripotent state, while SSEA1 is found only upon differentiation (Draper and Fox, 2003). Conversely, in mouse SSEA1 is expressed in ESCs, whereas SSEA4 is

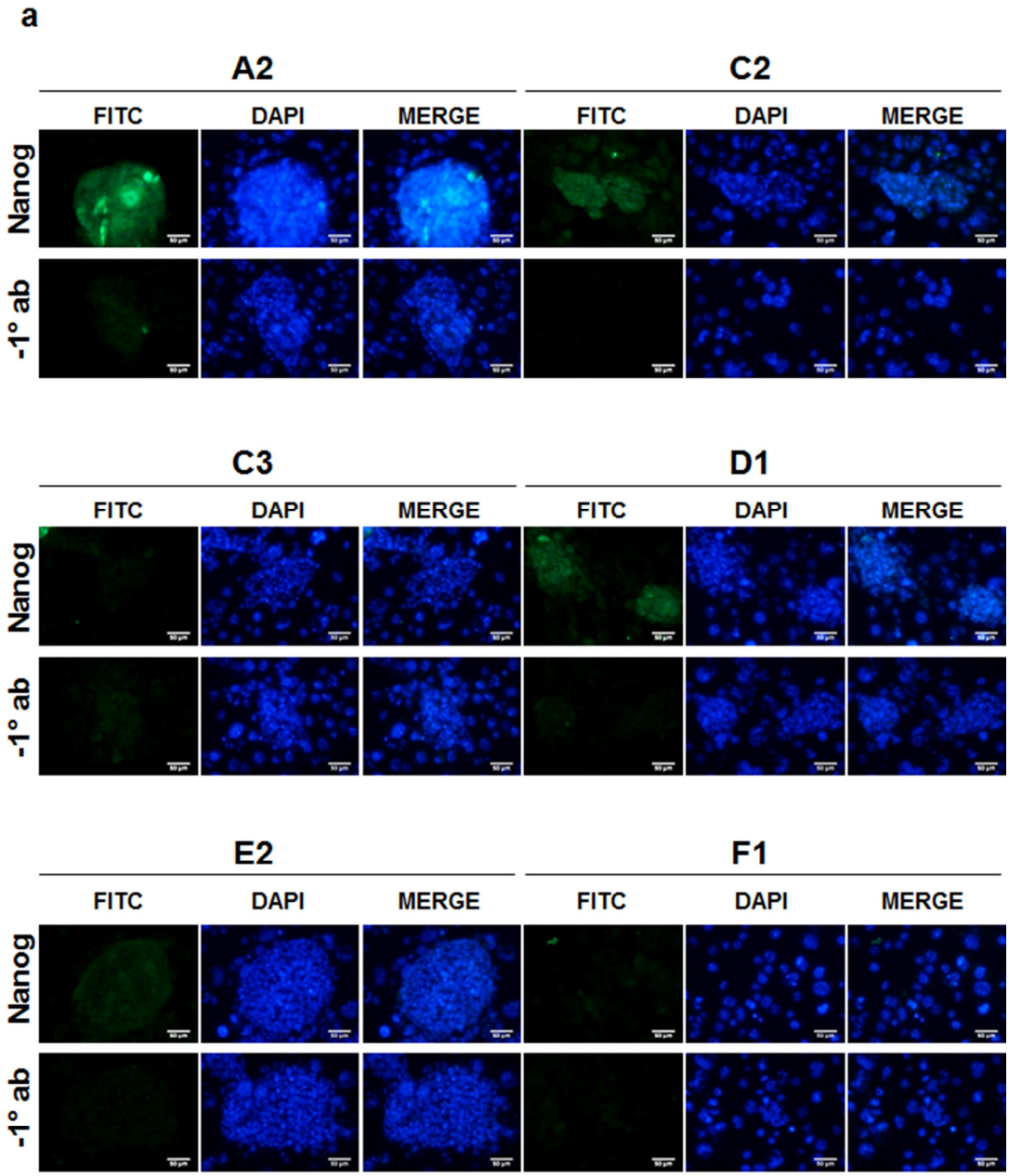


Figure 28 (legend in the next page).

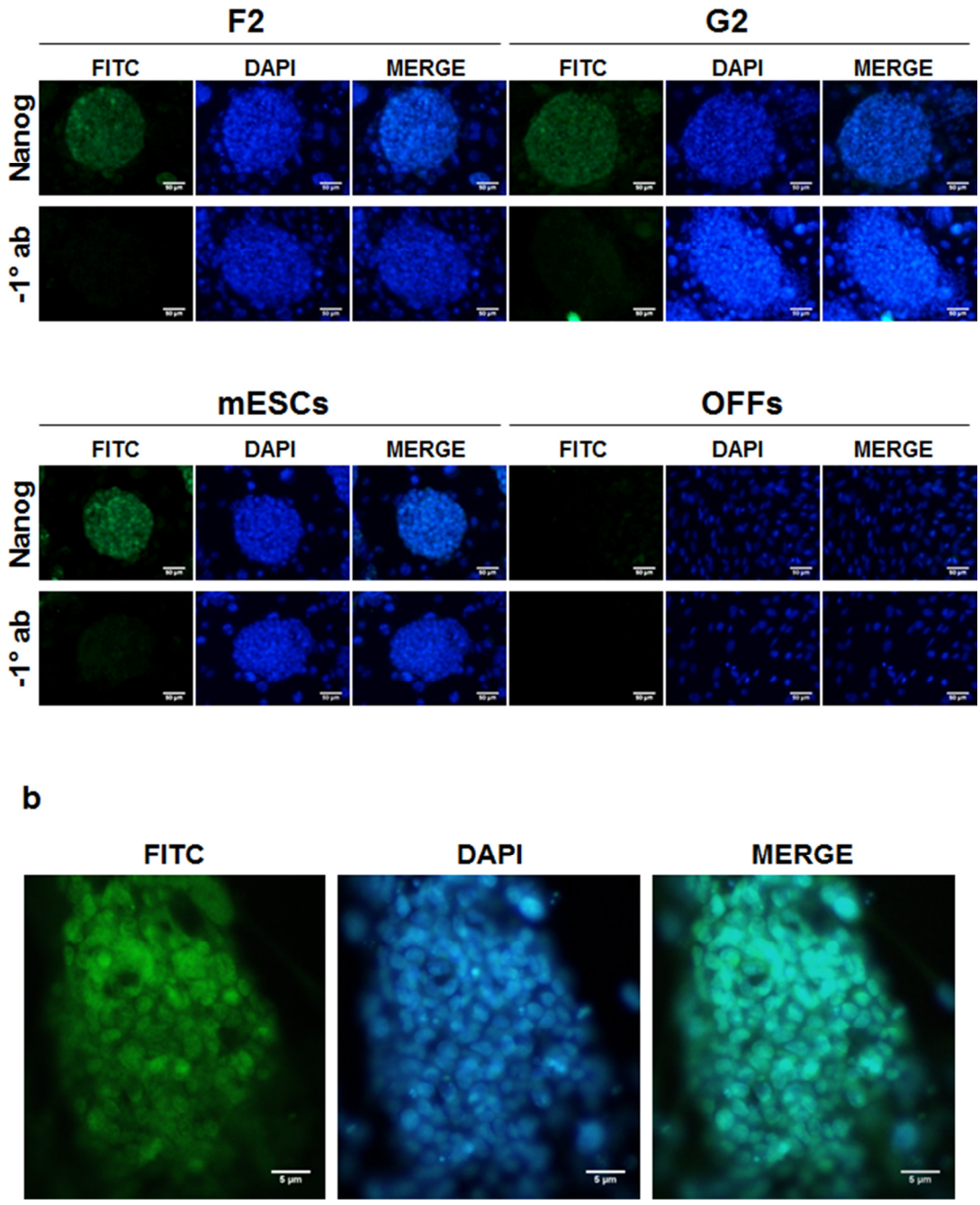
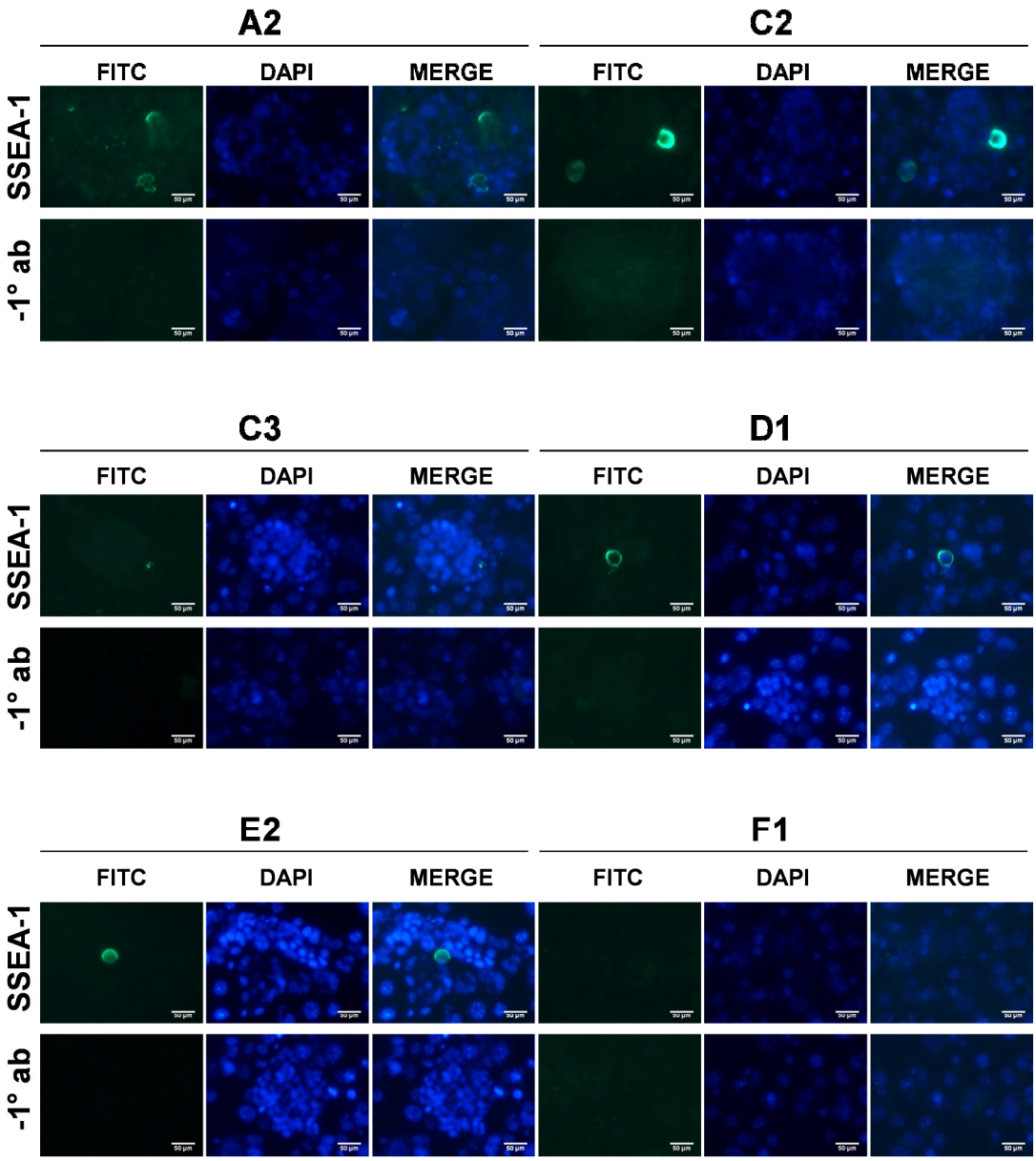


Figure 28. (a) Nanog immunocytochemistry of the ovine iPSC clones. Mouse ESCs are the positive control, OFFs are the negative control. A control without the primary antibody was performed for each clone. (b) Higher magnification images of G2 clone show that Nanog staining is restricted in the nucleus. Scale bars: 50µm for (a), 5µm for (b).



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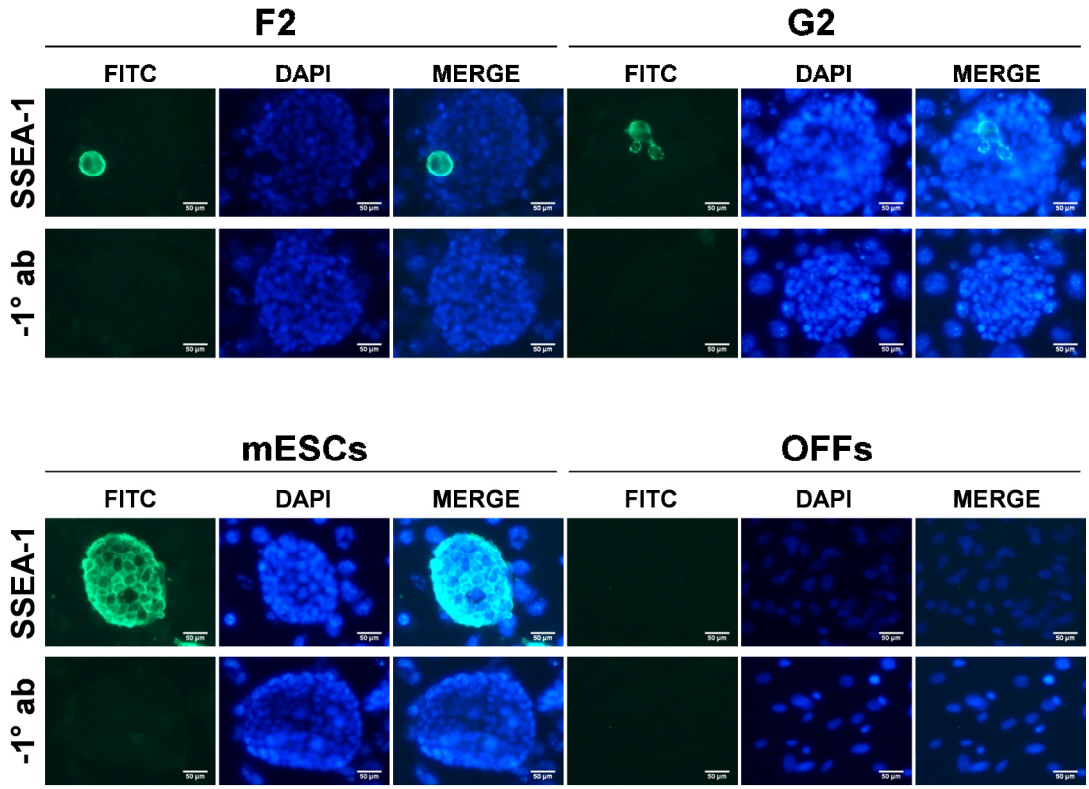
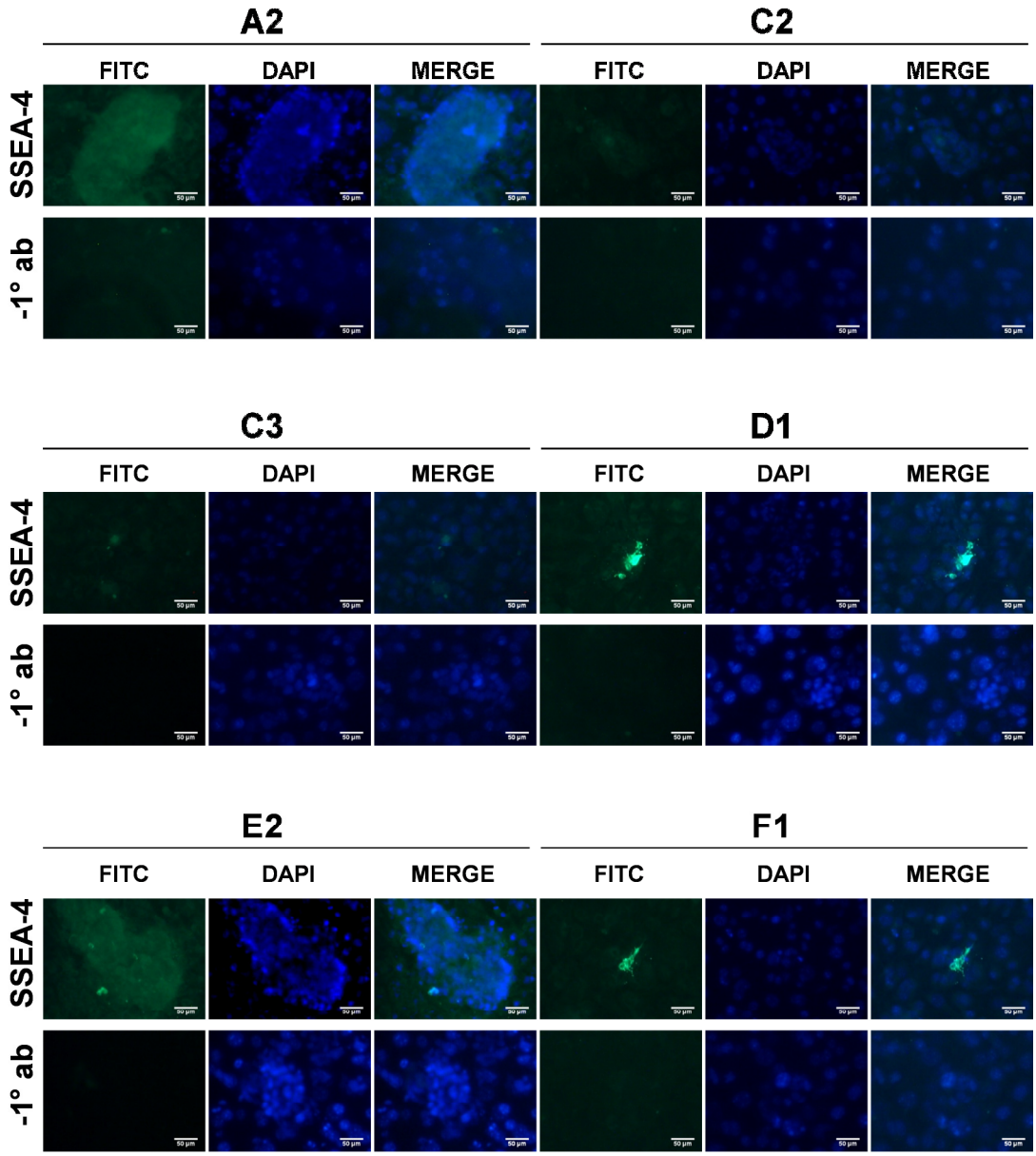


Figure 29. SSEA1 staining on the isolated iPSC lines: only a very small amount of cells in some clones were positive. Positive control: mouse ESCs; negative control: OFFs. Scale bars: 50µm.



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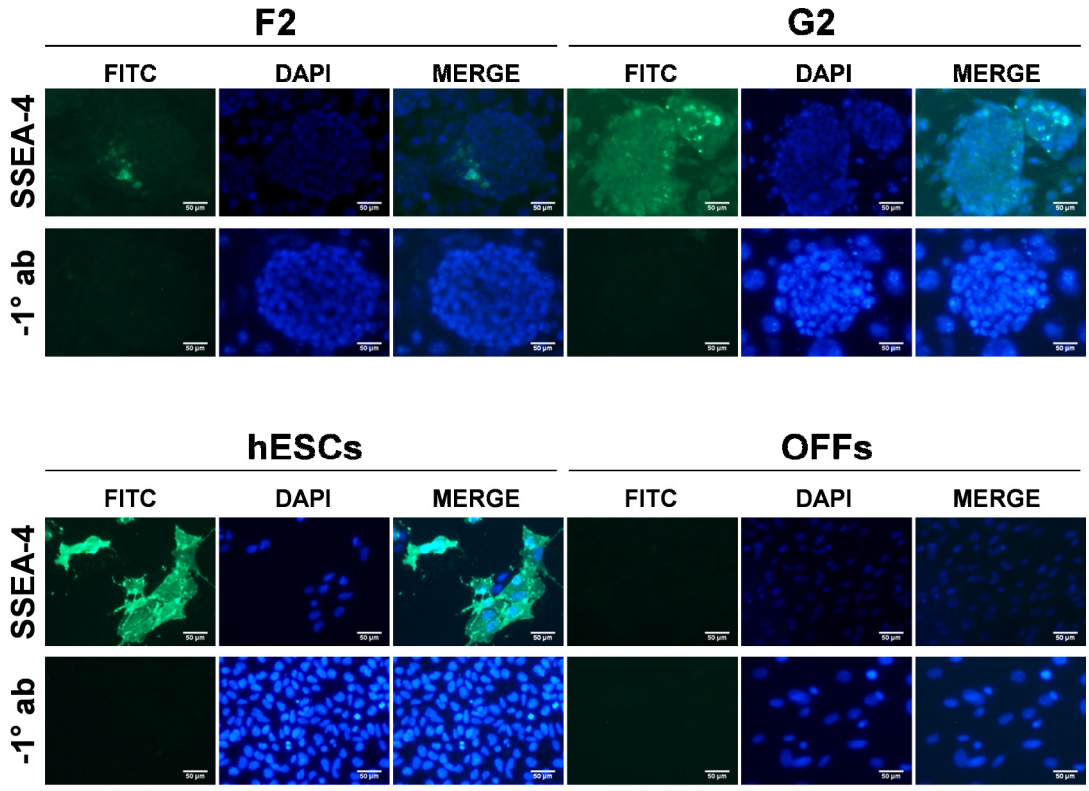


Figure 30. SSEA4 immunocytochemistry: the ovine iPSC clones have a SSEA4 expression pattern similar to that obtained for SSEA1: only few cells were positive. The positive control is represented by human ESCs, whereas the negative control is represented by the parental fibroblasts. Scale bars: 50µm.

absent (Koestenbauer, 2006). All the derived clones were tested at passage 7 for these two markers and found mostly negative, with a very small number of cells in only few clones displaying some staining (Fig. 29, 30).

5.2.5 *In vitro* differentiation of ovine induced pluripotent stem cells

In the light of the previous analyses (Table 7), we graded the cells and decided to perform the *in vitro* differentiation on three clones:

- G2, which shares many characteristics of ESCs;
- C3, which does not display any pluripotency feature;
- D1, which lies in the middle as it shows an ESC-like morphology and a positive Nanog staining but it is slower in growth and presents a low AP activity when compared with ESCs.

Since currently nothing is known about ovine pluripotency, the decision to carry out differentiation of these clones was made in order to compare whether there is a correlation between classical mouse pluripotency features and differentiation potential in ovine iPSCs.

Spontaneous *in vitro* differentiation was performed as follows: cells at around 70% of confluence were trypsinized leaving clumps of cells, separated from the feeder layer and grown in differentiation medium for 4 to 7 days in low attachment plates (typically from 1 T25cm2 flask cells were plated into 2 6cm diameter bacterial Petri dishes). As shown in figure 31:

- G2 cell line formed smooth, spherical, floating EBs, which are typical of ESC differentiation (Fig. 31e);

CLONE	GROWTH RATE	MORPHOLOGY	OVINE ORIGIN	INTEGRATION				EXPRESSION				AP STAINING	NANOG STAINING	SSEA1 STAINING	SSEA4 STAINING
				M	K	O	S	M	K	O	S				
A1	Very slow	/	/	/	/	/	/	/	/	/	/	/	/	/	/
A2	Fast	Granulated	Yes	+	+	+	-	+	+	+	-	None	Faint	Few cells	None
C1	Slow	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C2	Fast	Granulated	Yes	+	+	+	+	+	+	+	+	None	Strong	Few cells	None
C3	Slow	Differentiated	Yes	+	+	+	-	+	-	+	-	None	None	None	None
D1	Fast	ES-like	Yes	+	+	+	+	-	-	+	-	Faint	Strong	Few cells	Few cells
D3	Slow	/	/	/	/	/	/	/	/	/	/	/	/	/	/
E2	Fast	ES-like	Yes	+	+	+	+	+	+	+	+	Faint	Faint	Few cells	None
E3	Slow	/	/	/	/	/	/	/	/	/	/	/	/	/	/
F1	Slow	Flat	Yes	+	+	+	+	+	+	+	+	None	None	None	Few cells
F2	Very Fast	ES-like	Yes	+	+	+	+	+	-	+	+	Strong	Strong	Few cells	Few cells
G2	Very Fast	ES-like	Yes	+	+	+	+	-	-	+	-	Strong	Strong	Few cells	Few cells
H3	Very slow	/	/	/	/	/	/	/	/	/	/	/	/	/	/

Table 7. Table summarizing the characteristics of the different ovine iPSC clones. Some clones (A1, C1, D3, E3 and H3) stopped proliferating few passages after being picked and no analyses could be performed on them. The clone showing characteristics more similar to ESCs was G2 clone; the evidence of its reprogramming was further supported by the silencing profile of the reprogramming genes.

- D1 clone also generated floating EBs, but their shape was more irregular and the edges were rough; moreover some cells attached to the bottom (Fig. 31h);
- C3, the clone with characteristics more dissimilar from ESCs, did not form any floating clusters, but proliferated spreading at the bottom of the low attachment plate (Fig. 31m).

Next mouse ESC EBs, G2 EBs and D1 EBs were seeded to cell culture plates (usually 1-5 EBs were plated in a single well of 24 well plate) where they attached and spontaneously differentiated for 2 to 4 weeks. Initially, in order to have better images of the differentiation markers, EBs were plated on glass chambers coated with gelatin, poly-L-lysine or laminin, but the cells either did not adhere to the plates or, if they did, did not succeed in spreading and differentiating. Following this, EBs were eventually plated into gelatin-coated plastic plates.

During the differentiation process, cells with different shape and size could be recognized in the outgrowths, suggesting that the clones were differentiating towards many cell types (Fig. 31c, f, i). In the mouse control beating cardiomyocytes could be observed; in contrast these types of cells did not appear in the ovine cells, implying a restricted differentiation potential or sub-optimal differentiation conditions.

To assess whether the differentiated cells were derived from all three germ layers, after 2 to 4 weeks the outgrowths were fixed in 4% PFA for 20min and stained for five differentiation markers: βIII-tubulin (ectoderm), vimentin (mesoderm), Troponin T (mesoderm), α-fetoprotein (endoderm) and cytokeratin-18 (endoderm).

The differentiated G2 (G2 D) cells gave rise to cells belonging to all three germ layers. This cell line was positive to βIII-tubulin (Fig. 32), α-fetoprotein (Fig. 33),

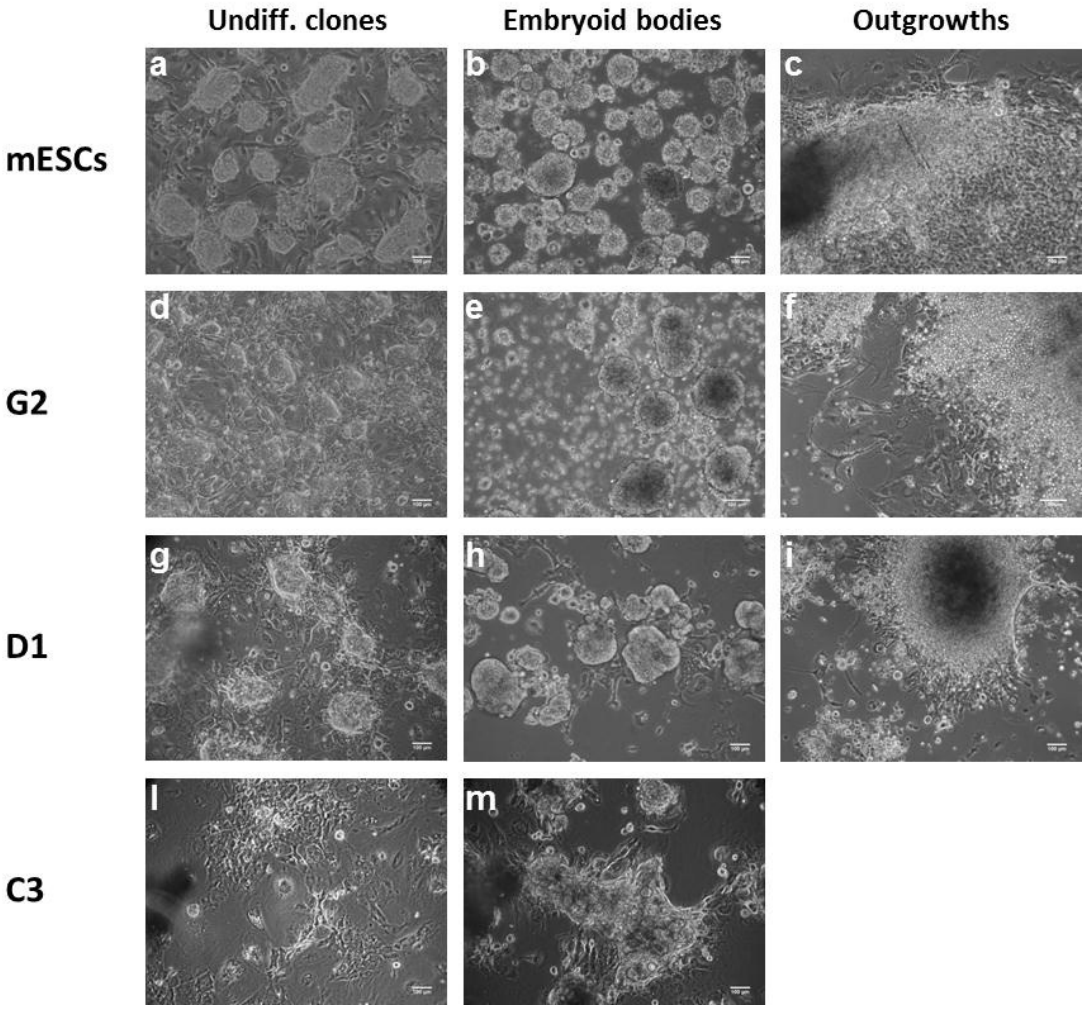


Figure 31. Differentiation course of different quality clones of ovine iPSCs: a spontaneous differentiation assay was carried out on G2 (d-f), D1 (g-i) and C3 (l, m) clones. (a-c) Positive control: mouse ESCs. (a, d, g, l) Undifferentiated cells. (b, e, h, m) Embryoid bodies. (c, f, i) Outgrows. C3 clone did not form EBs, consequently it was not possible to obtain any outgrows. Scale bars: 100µm.

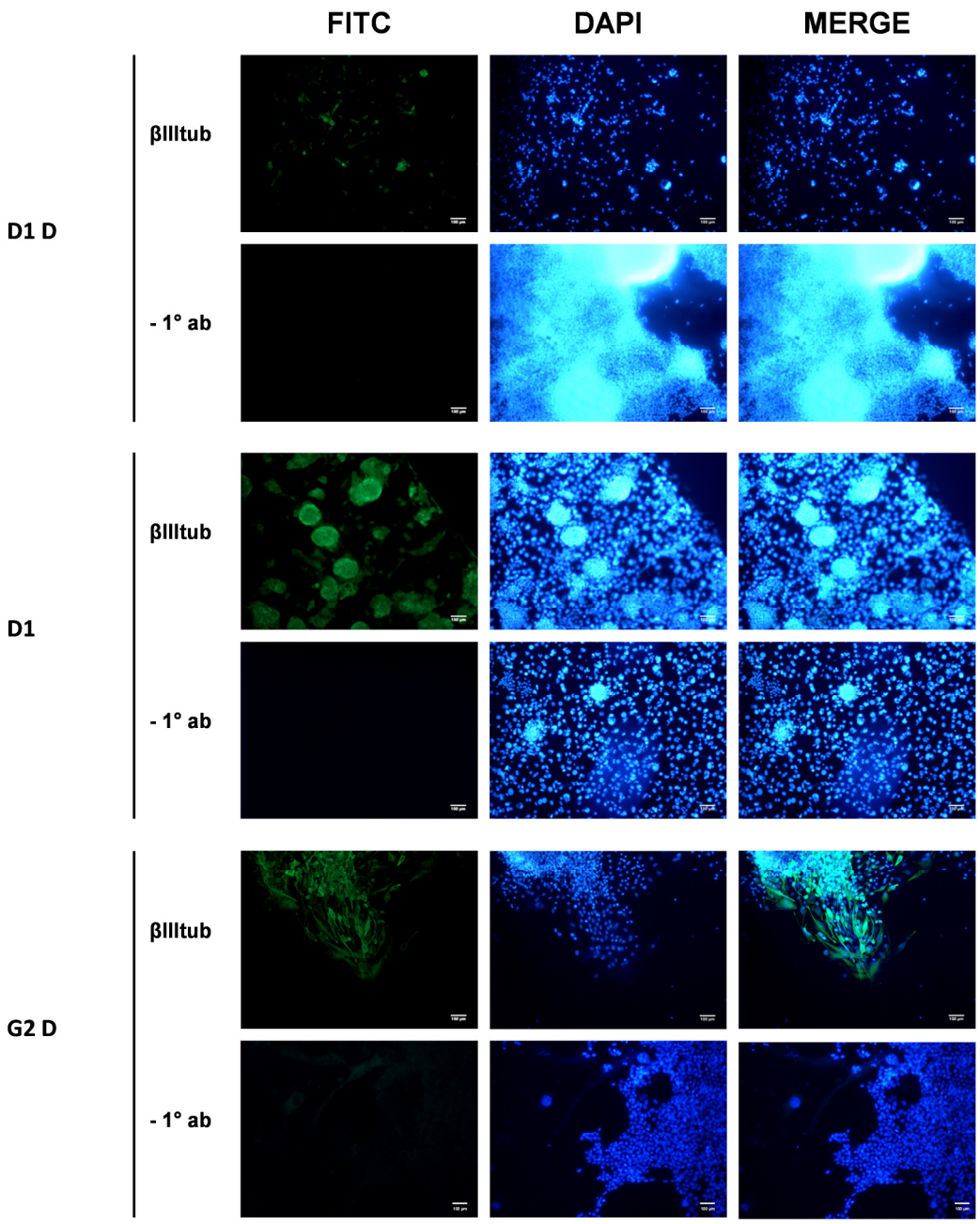


Figure 32 (legend in the next page).

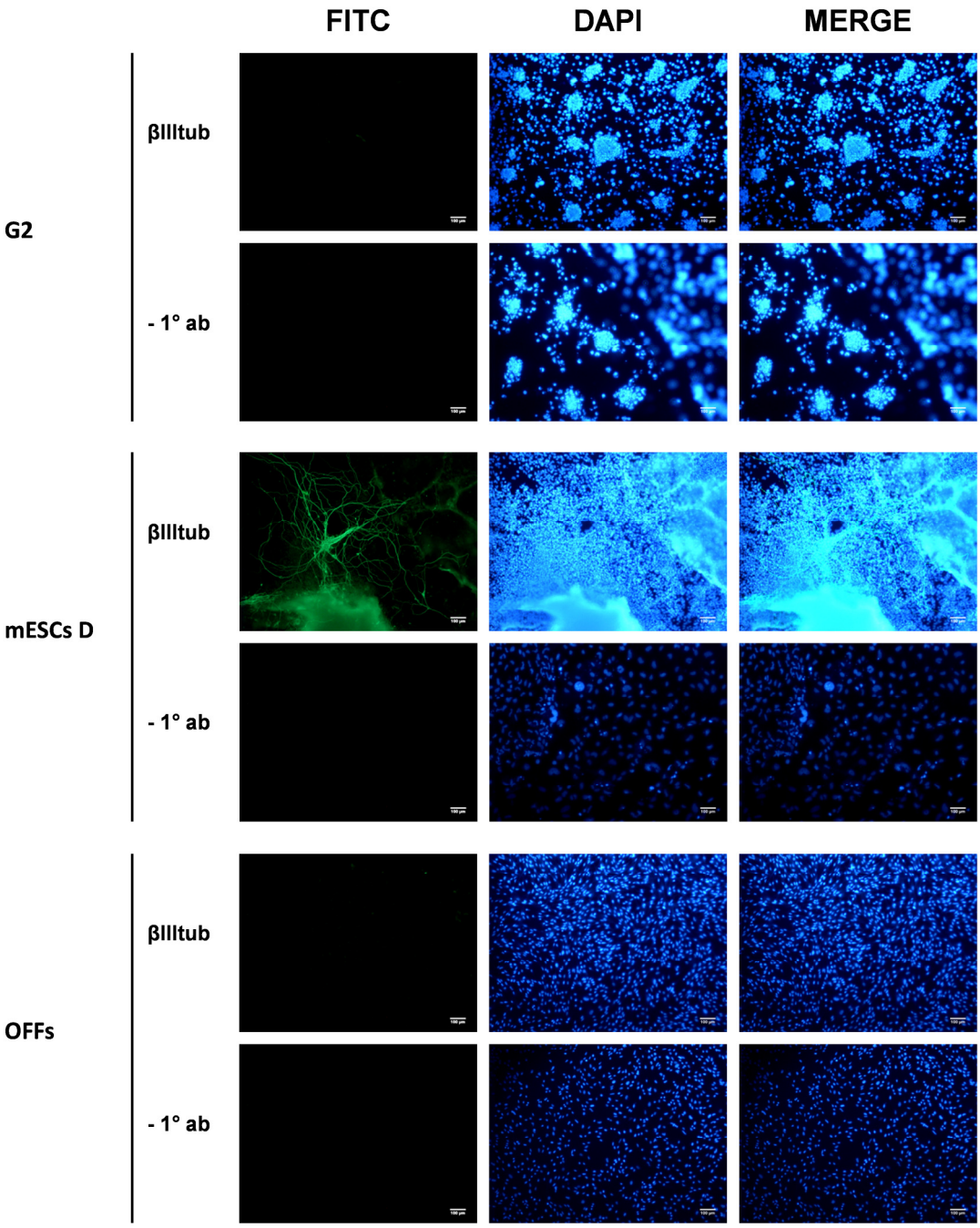
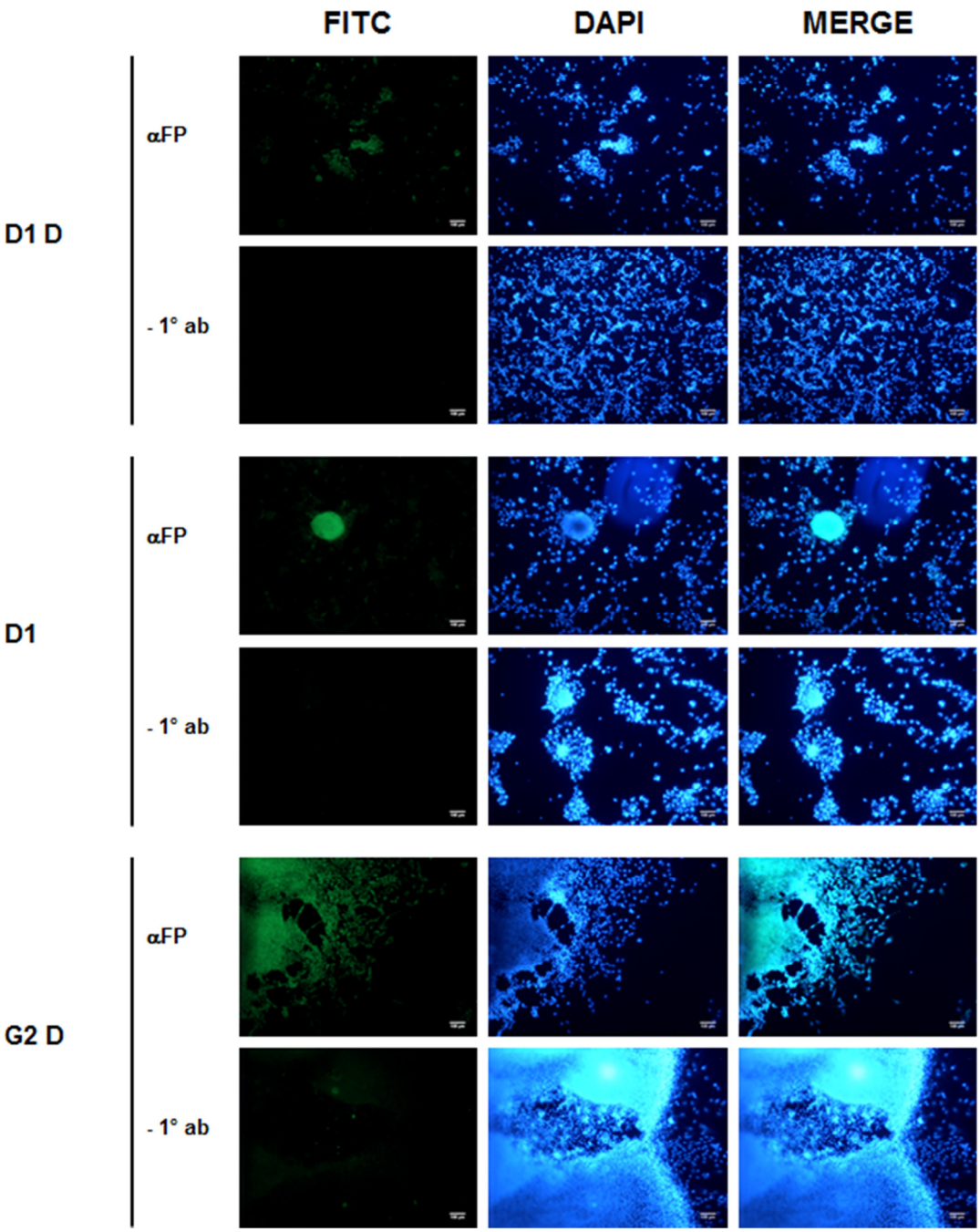


Figure 32. Immunostaining of differentiated ovine iPSC clones against βIII tubulin. Differentiated mouse ESCs were employed as positive control; the parental fibroblasts were utilised as negative control. As additional negative control for each single clone, the undifferentiated cell lines were stained too. The undifferentiated G2 clone is negative to βIII tubulin, confirming its pluripotency profile, while the D1 iPSC clone stained positive, indicating it tends to differentiate even in ESC culture conditions. Scale bars: 100µm.



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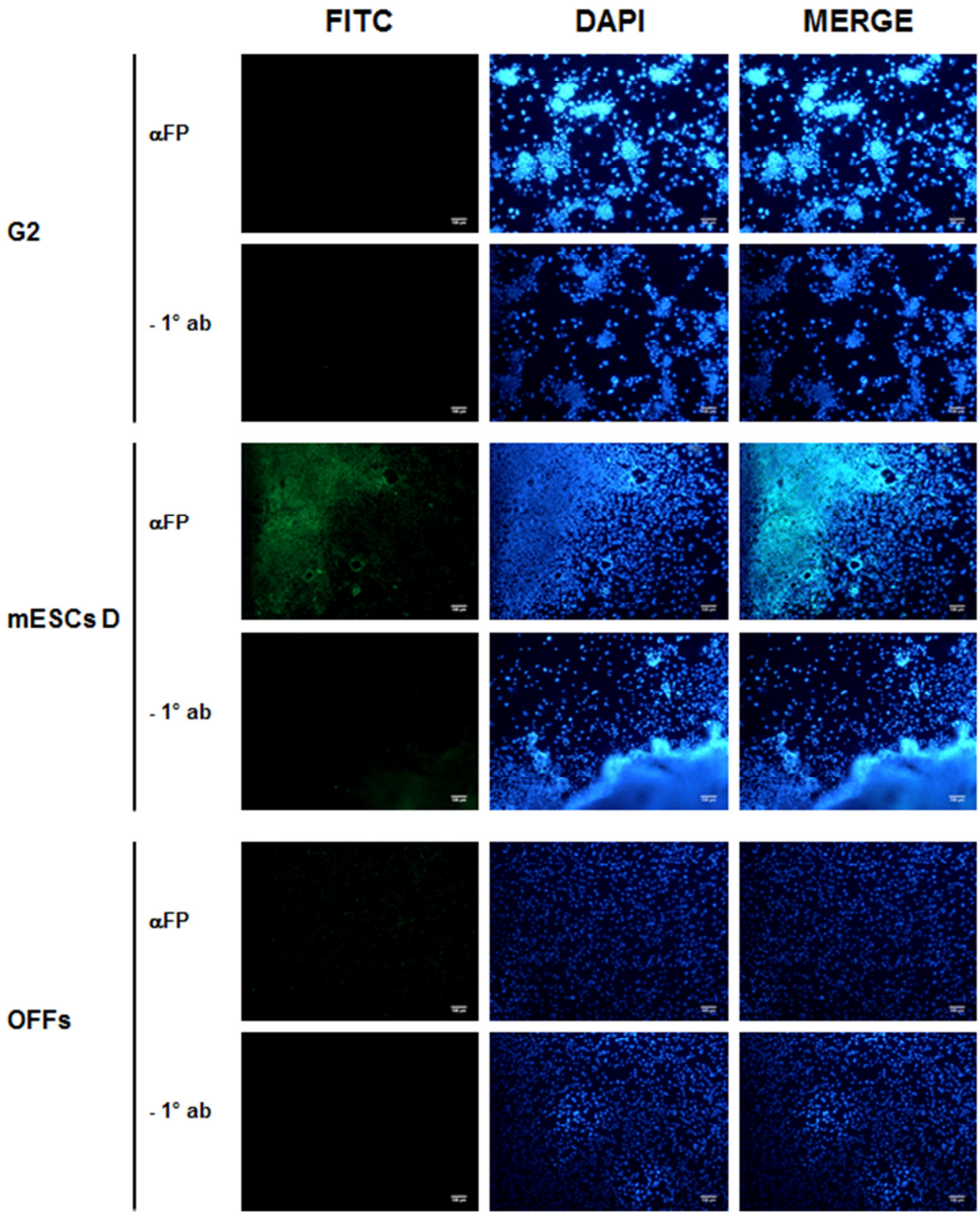


Figure 33. Anti α-fetoprotein immunocytochemistry: the eight differentiated ovine clones were analysed alongside mouse ESCs as a positive control and OFFs and the undifferentiated iPSCs as a negative control. As in the anti βIII tubulin staining, the undifferentiated G2 iPSC clone is negative, while the undifferentiated D1 is positive. Scale bars: 100μm.

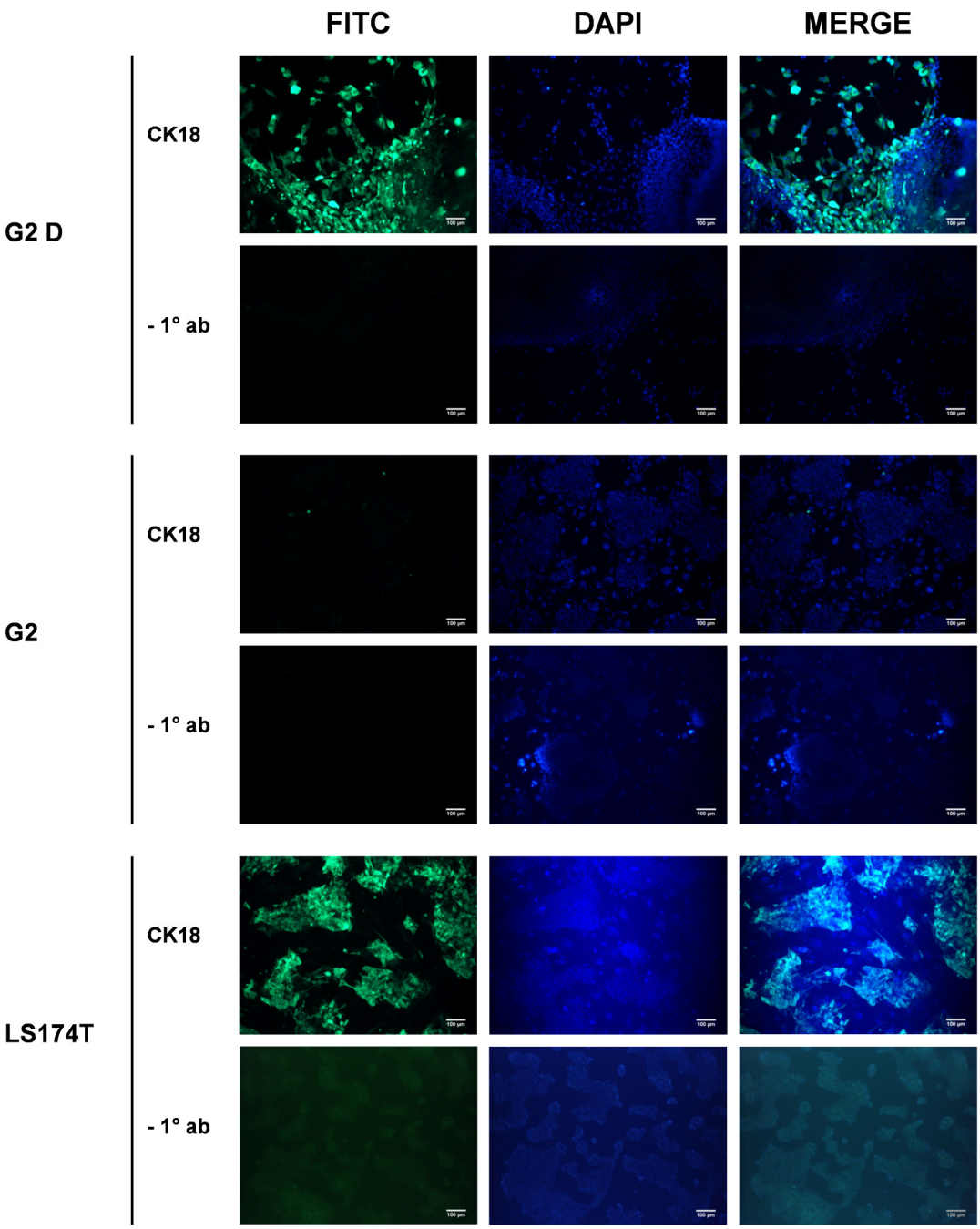


Figure 34. Immunocytochemistry against cytokeratin-18: the differentiated G2 cells were strongly positive for this endodermal marker. The negative control, undifferentiated G2 iPSCs, was negative, while the positive control, LS174T colon carcinoma cells, was positive. A control reaction performed without the supplementation of the primary antibody was included. Scale bars: 100µm.

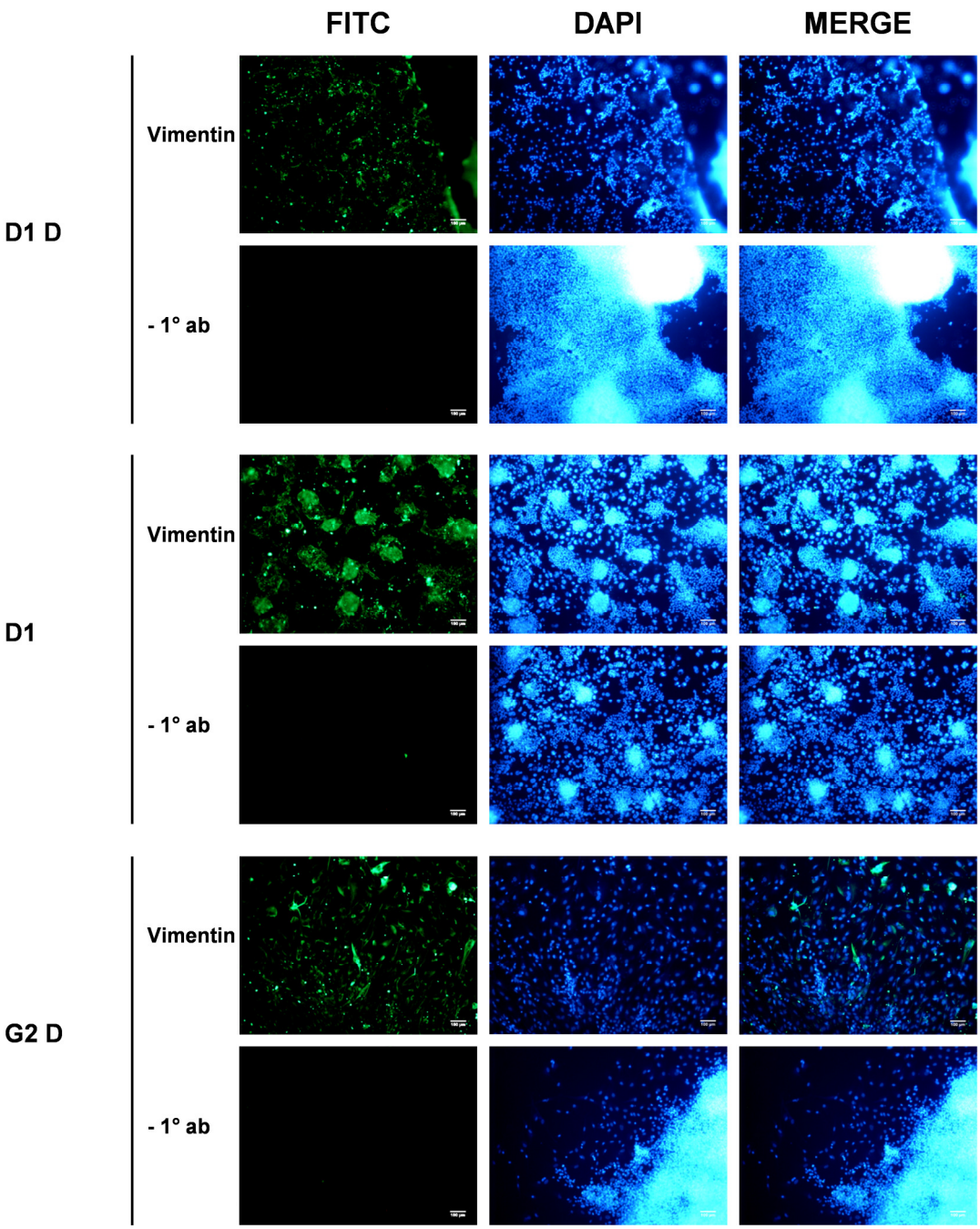


Figure 35 (legend in the next page).

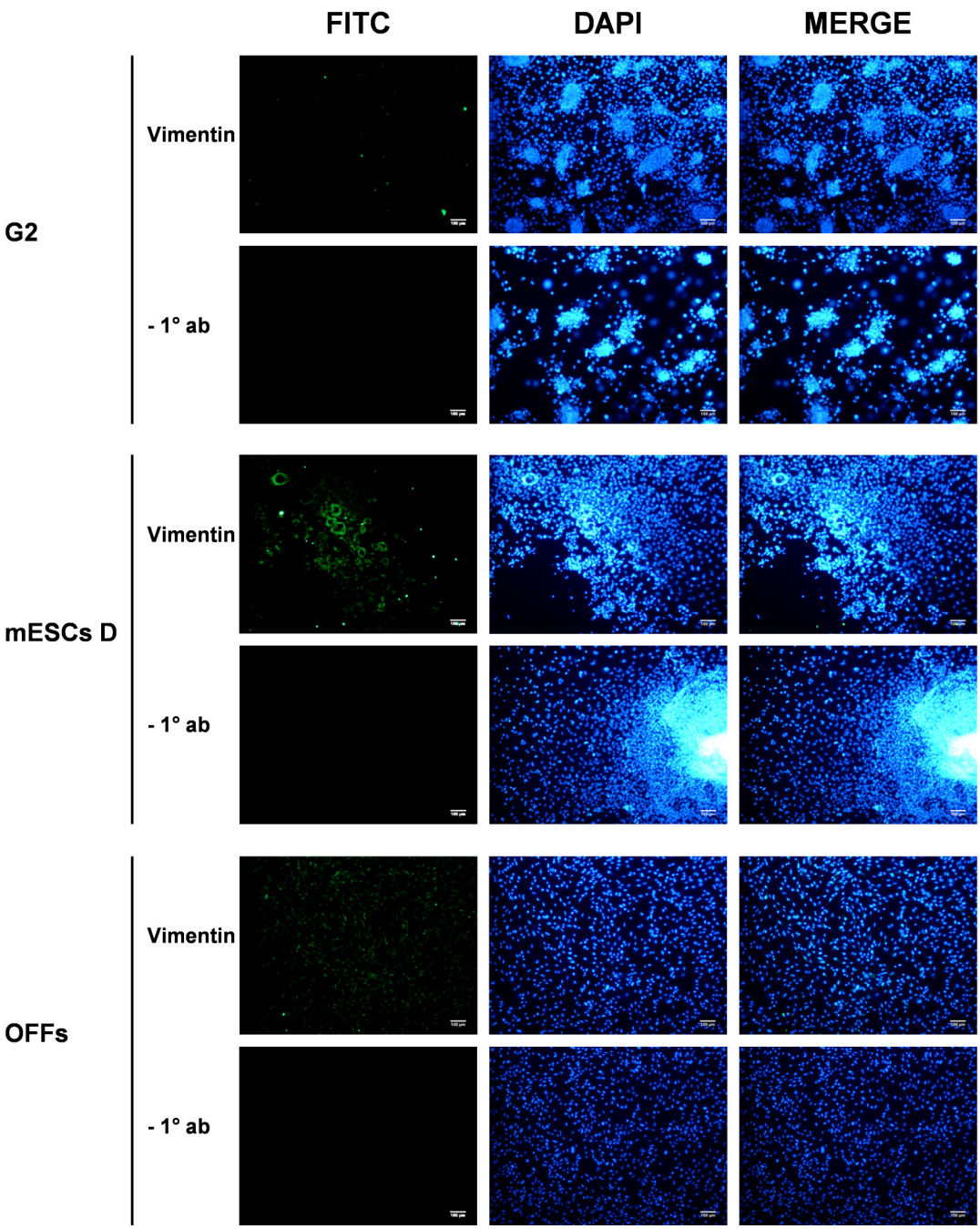
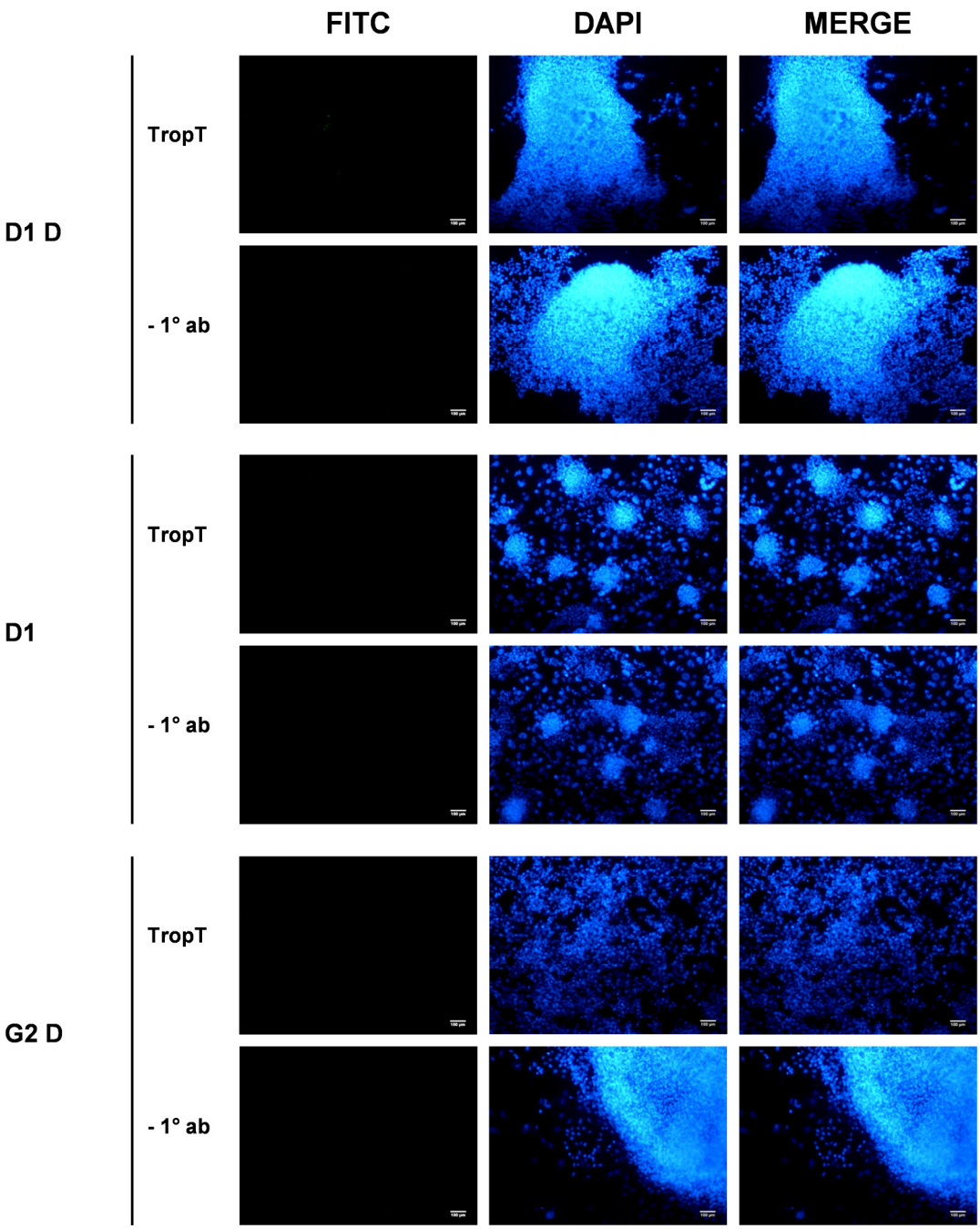


Figure 35. Anti-vimentin staining on ovine G2 and D1 cell lines: both differentiated clones were positive. The undifferentiated G2 cells were negative, while the undifferentiated D1 cells were positive. This confirmed the previous results acquired with anti βIII tubulin and anti α-fetoprotein, which indicated that G2 iPSCs are pluripotent, while D1 iPSCs cannot be maintained in a completely undifferentiated state. Scale bars: 100µm.



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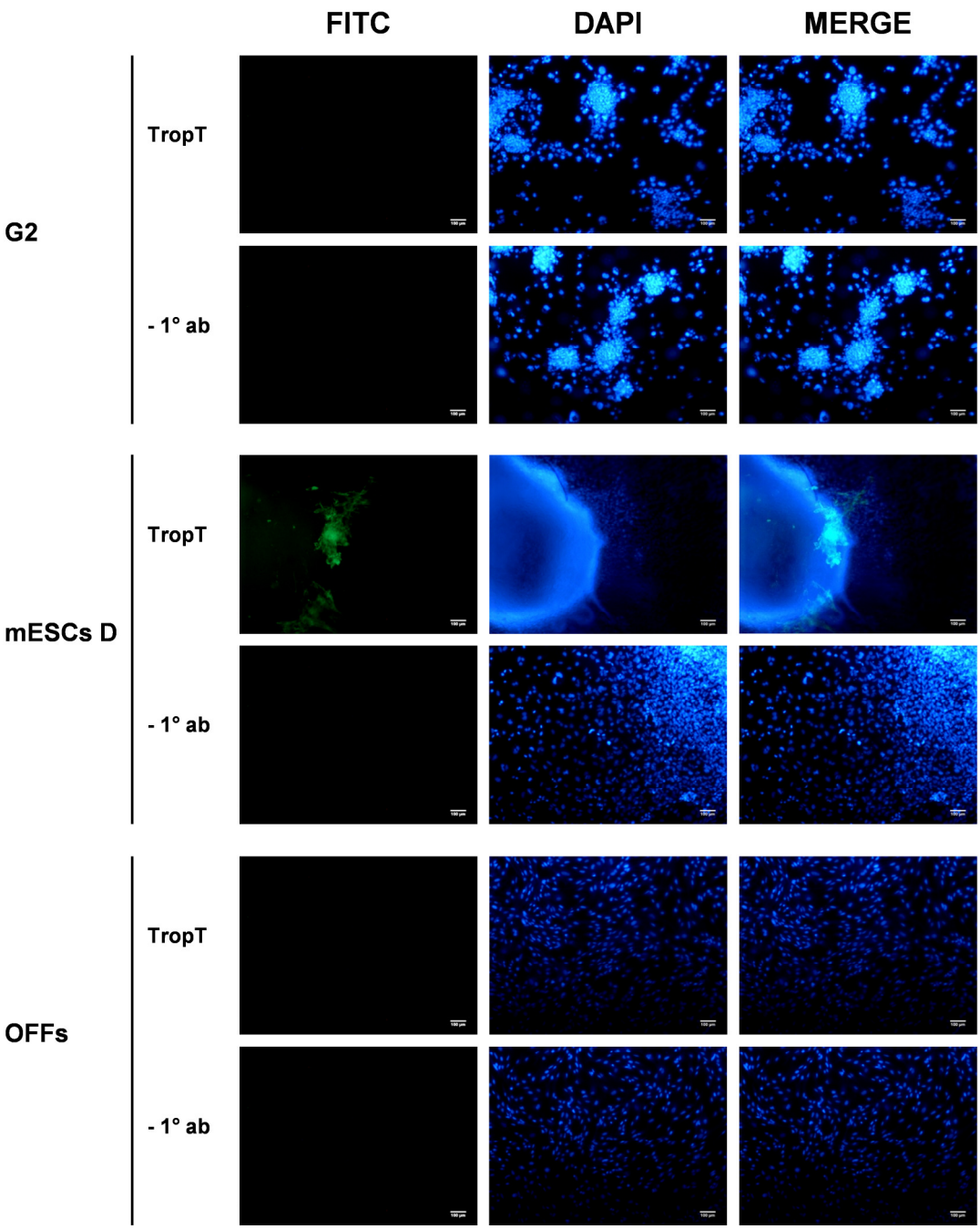


Figure 36. Staining against troponin T mesodermal marker: the ovine clones are negative in both differentiated and undifferentiated state. The positive control, differentiated mouse ESCs, is positive to the immunocytochemistry, with the staining co-localising where beating cells had appeared. Scale bars: 100µm.

cytokeratin-18 (Fig. 34) and vimentin (Fig. 35), while troponin T immunocytochemistry was negative (Fig. 36). Undifferentiated G2 cells were stained against the same five markers and found negative, thus leading to an additional evidence of the pluripotency status of this clone (Fig. 32-36).

The differentiated D1 (D1 D) cells also stained positive to βIII-tubulin (Fig. 32), α-fetoprotein (Fig. 33) and vimentin (Fig. 35), but also the undifferentiated D1 cells were positive to these markers (Fig. 32, 33, 35). This means that D1 clone is not able to maintain its pluripotency even in ESC conditions, thus it is likely that this cell line had undergone to only partial reprogramming.

Results of immunocytofluorescence analyses for markers of differentiation are summarised in table 8.

The absence of troponin T in the ovine iPSCs was consistent with the lack of beating cells observed during the differentiation process. In contrast, differentiated mouse ESCs displayed a strong troponin T signal, which was often colocalized with the beating cells we observed during the differentiation process.

OFFs were employed as a negative control in the staining against βIII-tubulin, α-fetoprotein, cytokeratin-18 and troponin T. They were included also in the vimentin immunocytochemistry, but as a positive control. Vimentin is a marker of mesodermal cells and embryonic fibroblasts derive from mesoderm. The negative control for the vimentin reaction was represented by the undifferentiated iPSCs.

In all the stainings a control reaction without the primary antibody was carried out to ensure that the secondary antibody did not produce any unspecific signal.

CLONE	EB FORMATION	EB MORPHOLOGY	STAININGS FOR MARKERS OF DIFFERENTIATION				
			βIII TUBULIN	α FETOPROTEIN	CYTOKERATIN 18	VIMENTIN	TROPONIN T
C3	**-**	/	Undiff: / Diff: /	Undiff: / Diff: /	Undiff: / Diff: /	Undiff: / Diff: /	Undiff: / Diff: /
D1	**+**	Rough, irregular	Undiff: **+** Diff: **+**	Undiff: **+** Diff: **+**	Undiff: / Diff: /	Undiff: **+** Diff: **+**	Undiff: - Diff: -
G2	**+**	Smooth, spherical	Undiff: **-** Diff: **+**	Undiff: **-** Diff: **+**	Undiff: **-** Diff: **+**	Undiff: **-** Diff: **+**	Undiff: **-** Diff: -

Table 8. Table summarizing the characteristics of three ovine iPSC clones upon differentiation. C3 was not able to generate EBs, while D1 and G2 formed EBs that proliferated when plated again in cell culture dishes. The outgrowths (Diff) were then stained for markers of pluripotency, alongside the undifferentiated clones (Undiff). Both G2 D and D1 D gave rise to cells belonging to the three germ layers, but for D1 the undifferentiated counterpart also stained positive for these markers, suggesting the clone is not truly pluripotent; on the other hand, undifferentiated G2 cells were not positive for any of the markers.

5.2.6 Growth curve

After analysing all clones for the main features linked to pluripotency and differentiation, we chose the cell line with characteristics more similar to those of ESCs to carry on further characterizations.

An important trait of pluripotent cells is their very fast cell cycle. Although the high growth rate is not solely restricted to ESCs, it represents a significant mark in the identification of true iPSCs: the reprogramming process starts from differentiated cells whose growth is often slow. As a consequence of the dedifferentiation, these cells experience changes that accelerate their proliferation rate and make it similar to that of ESCs. The calculation of the doubling time of the ovine iPSC population and the comparison with that of the parental fibroblasts and ESCs could give further indication of the reprogramming of the generated cells.

Based on these considerations, a growth curve was obtained for G2 cells, OFFs and mouse ESCs. For each cell line $3.55x10^4$ cells were plated at day 0 in 24 wells of a 48 well plate in the appropriate conditions. Three wells for each cell line were counted every 12h for 96h. The graph in figure 37 shows that ovine iPSCs have a proliferation rate much higher than that of the parental fibroblasts and similar to that of mouse ESCs. In order to have a more precise indication of the proliferation, the population doubling time (pdt) was calculated. The pdt is the period of time required for the cells to double their number. When the cells undergo exponential growth they have a constant pdt, which can be calculated directly from the growth curve: a linear regression was performed on the exponential phase of each cell type and the pdt was calculated as the slope of the equations obtained with the regression. The statistical results confirmed what we earlier predicted from the graph: during the

reprogramming process the doubling time had decreased from 63.01h (confidence interval: 41.07h – 135.28h) of the OFFs to the 17.46h (confidence interval: 16.35h – 18.72h) of the ovine iPSCs; additionally, the pdt of the ovine iPSCs was similar to that of mouse ESCs (13.78h, confidence interval: 12.31h – 15.64h).

5.2.7 Self-renewal

Somatic cells give rise to primary cell cultures, which go towards senescence and death after a certain amount of passages (the Hayflick limit). Only after immortalization, a process often involving the mutation of genes responsible for the proliferation, somatic cells are able to be maintained in culture for an indefinite time (Sherr and DePinho, 2000; Wright and Shay, 2002). Conversely, pluripotent cells are characterised by their ability to indefinitely proliferate in culture without loss of phenotype (Suda, 1987; Smith, 2001a; Zeng, 2007). Partially reprogrammed iPSCs with time undergo to changes of morphology which are the early signal of loss of pluripotency (Okada, 2010).

With the purpose of evaluating their self-renewal potential, our ovine iPSCs G2 were grown for more than 25 passages and then evaluated. As shown in figure 38, G2 clone achieved to maintain an ESC-like morphology even after 25 passages.

5.2.8 Metaphase spread

One of the potential risks associated with fast growing cells, such as pluripotent cells, in long term cultures is the acquisition of karyotypic abnormalities (Robertson, 1987; Brown, 1992). The isolation of stem cells with chromosome aberrations would hamper future applications, restricting the possible uses of these cells.

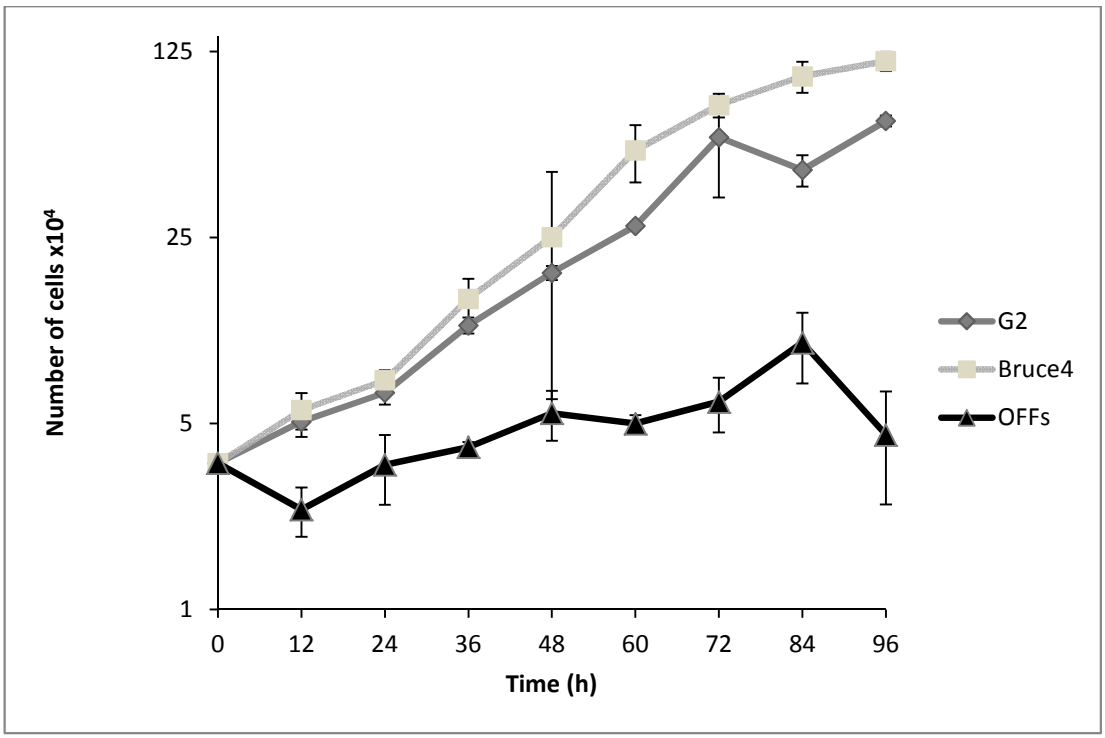


Figure 37. Growth curve of G2 iPSCs, mouse ESCs and OFFs. The growth rates of the three cell lines were compared. Mouse ESCs also represented a positive control for the reaction; in other reports it is stated that their pdt ranges between 10h and 16h (Berrill, 2004), which is comparable with the results we obtained.

In order to examine whether our G2 cells were karyotypically stable, a metaphase spread of the cells at passage 24 was analysed and the chromosomes were counted. Out of 100, we counted 83 cells with a normal number of chromosomes (a representative sample in shown in figure 39a), which in sheep is 54. The other 17 metaphase spreads had fewer chromosomes (ranging between 44 and 53), while no cells exhibited polyploidy (Fig. 39b).

In mouse ESCs a karyotype is considered normal when more than 70%-80% of the cells show the correct number of chromosomes (Nagy, 2003); however in rat ESCs this threshold has not been reached and nonetheless cells were able to give rise to chimeric animals (Buehr, 2008).

In our ovine iPSCs, we found 83% of normal cells; hence we can consider our cells to have a normal karyotype.

5.3 Discussion

In this chapter I have illustrated the derivation of ovine iPSCs that express markers of pluripotency and can give rise to cells characteristic of all three germ layers.

After the first report in mouse (Takahashi and Yamanaka, 2006), iPSCs have been derived from many different species, including pig (Esteban, 2009; Ezashi, 2009; Wu, 2009) and recently reports of ovine iPSCs have been published (Bao, 2011; Li, 2011). In ungulates the isolation of iPSCs has been achieved through a variety of different reprogramming genes and culture conditions, but always involving retro or lentiviral transduction. In mouse, iPSCs are considered fully reprogrammed when they are able to maintain the pluripotency without the continuative expression of the reprogramming factors (Hotta and Ellis, 2008). This has not been accomplished in ovine, nor in porcine species.

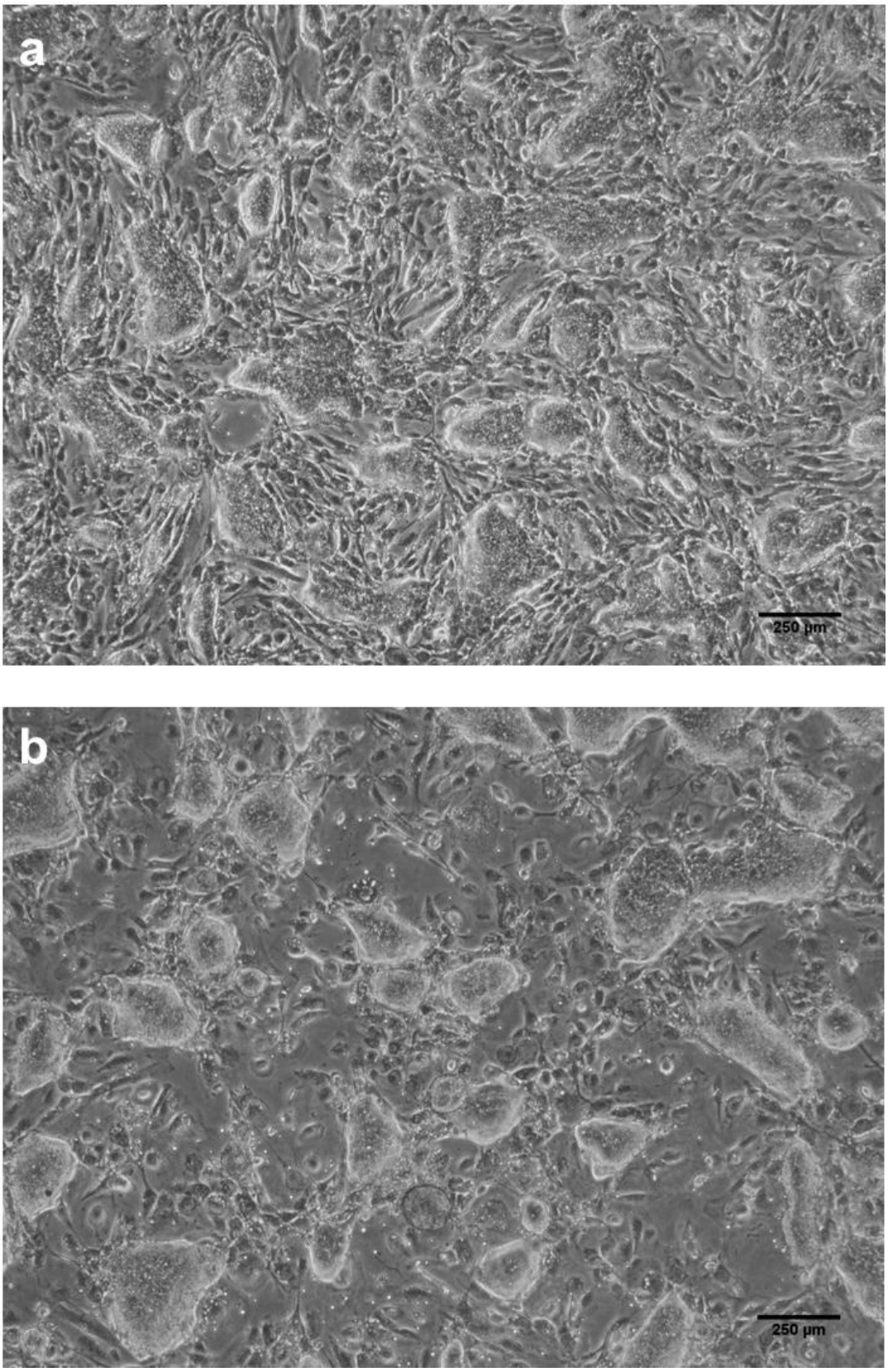
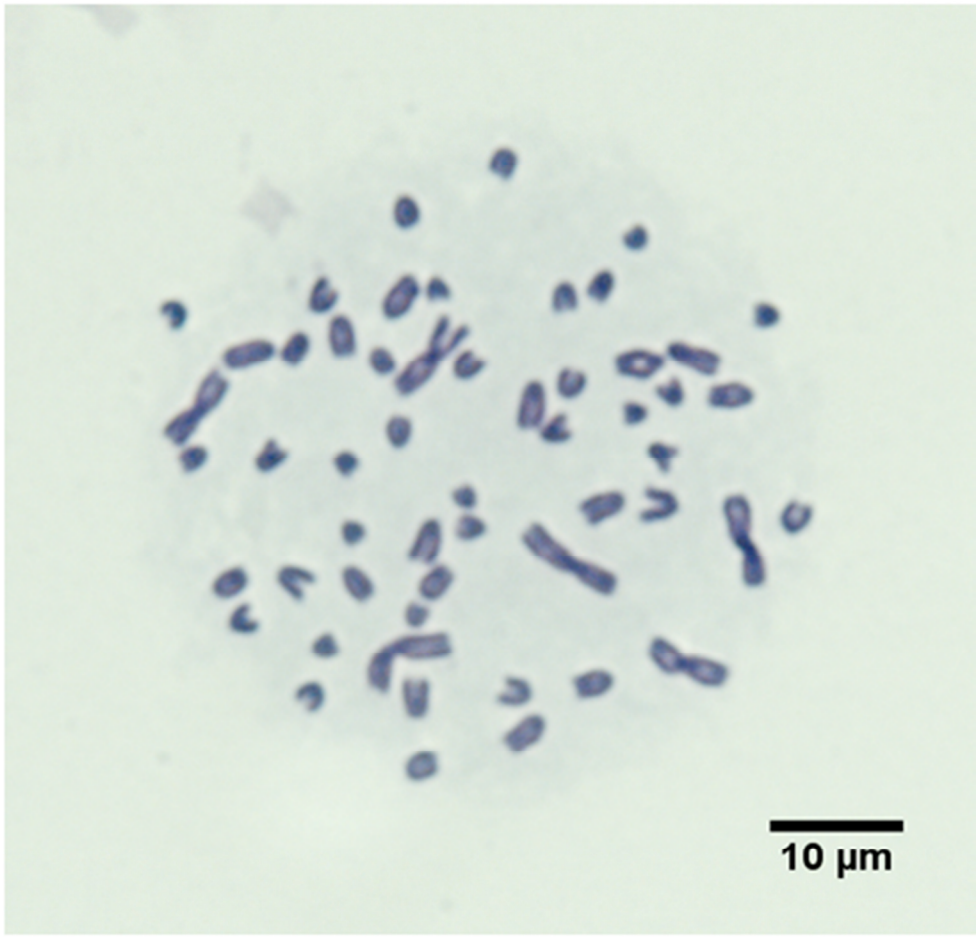


Figure 38. Comparison between (a) early (p10) and (b) late (p24) passage G2 ovine iPSCs: the morphology has not changed, suggesting this clone is able to maintain its undifferentiated state over time. Scale bars: 250µm.

a



b

	Chromosome number		
	<54	54	> 54
% of cells	17%	83%	0%

Figure 39. (a) A representative sample of the metaphase spread of G2 iPSCs (63x magnification). (b) Table displaying the percentage of cells with a normal karyotype consisting of 54 chromosomes. Scale bar: 10µm.

In our hands we were able to derive ovine iPSCs whose pluripotency was maintained after the silencing of the reprogramming genes; these cells exhibited many characteristics of iPSCs, a normal karyotype and displayed markers of the three lineages after differentiation from embryoid bodies *in vitro*.

The derivation of pluripotent cells in sheep has always been a challenge: the isolation of ESC-like cells from ovine blastocysts has been described (Dattena, 2006), but these cells could not be preserved in an undifferentiated state. Ovine iPSCs have now been obtained (Bao, 2011; Li, 2011) but many differences exist among our results and those published by Li et al (Li, 2011) and Bao et al (Bao, 2011), in particular related to both the protocols employed and the distinct expression profiles of the ovine iPSCs generated.

First iPSCs were initially isolated in mouse using only 4 genes: cMyc, Klf4, Oct4 and Sox2 (Takahashi and Yamanaka, 2006). Subsequently these four reprogramming factors have been employed for the successful derivation of iPSCs from other species (Esteban, 2009; Li, 2009; Honda, 2010). Although reported by Bao and colleagues that this strategy was not sufficient for the derivation of ovine iPSCs (Bao, 2011), our data support those of Li and colleagues in showing that ovine cells displaying iPSC characteristics can be derived using the original 4 genes (Li, 2011).

Second, our first colonies appeared 8 days after the transduction, only two days later than in the mouse control experiment but substantially quicker than the timing described by Li et al (14 days) and Bao et al (20 days). This may be the result of the choice of the vectors (MoML retrovirus versus dox-inducible lentivirus) or

differences in culture conditions. In mouse and human the growth conditions for pluripotent cells have been optimized. This has not been possible in other species due to the lack of ESCs. Consequently the challenge of deriving iPSCs in sheep is hampered by the need to identify the right conditions for the maintenance of the cells in an undifferentiated state after reprogramming. In accord with a previous report (Bai, 2008), we observed a better morphology when the ovine pluripotent cells were generated in SR rather than in FCS. When Li et al compared the two medium conditions they described a higher proportion of AP-positive cells in FCS, concluding the presence of serum had improved the quality of the iPSCs. In contrast to this was the observation that the morphology of their iPSC colonies was not characterised by the defined edge typical of ESCs, suggesting that FCS might not represent the optimal supplement to support the growth of ovine pluripotent cells. AP, which is the indicator utilised by Li et al to identify ovine iPSC colonies, although being a marker for ESCs, is not strictly specific to pluripotent cells. As such, the higher proportion of AP-positive cells in FCS might be due to this component being a more permissive culture condition than SR for the development of partially reprogrammed cells. This could be consistent with the observation that, in pig, transducing cells with only cMyc and growing them in presence of either FCS or SR, a higher proportion of AP-positive colonies appeared in the FCS conditions (personal communication, Dr Alison Thomson, University of Edinburgh).

Another consideration is the expression status of the induced genes. When working with retroviral vectors, if the cells become pluripotent the reprogramming genes are silenced through a mechanism reflected in DNA methylation (Hotta and Ellis, 2008).

In mouse the silencing of the retroviral vectors is considered a critical indicator of full reprogramming (Hotta and Ellis, 2008) and after the exogenous factors cease to be expressed, a true iPSC clone will continue to self-renew given the appropriate culture environment (Okada, 2010). In contrast, the inability to silence the reprogramming factors results in cells that have considerably impaired differentiation capacity (Brambrink, 2008). In order to control the expression time frame of the transduced genes, lentiviral vectors with transgenes driven by inducible promoters such as that based on the TET system (Wernig, 2008a) have been engineered. Lentiviral vectors are not able to be silenced in pluripotent cells (Pfeifer, 2002), but with the inducible promoters it is possible to control the expression of exogenous factors in the cells. Again when the block of the reprogramming protein expression is followed by loss of self-renewal, there is an evidence that only partial reprogramming have occurred (Hotta and Ellis, 2008).

In our iPSCs, we observe nearly complete silencing of the induced genes with maintenance of markers of pluripotency in contrast to the previous reports on ovine iPSCs (Bao, 2011; Li, 2011) where, employing a dox-inducible system, the withdrawn of doxycycline was shortly followed by differentiation of the cells.

Pluripotency markers retain a great significance in the characterization of stem cells and represent the first step in the identification of real iPSCs.

Although largely similar, differences between species (mouse and human) exist, for example SSEA1 is expressed in mouse and SSEA4 in human (Koestenbauer, 2006). The ovine iPSCs generated in this experiment expressed Nanog, but not SSEA1 or SSEA4, while Oct4 was not analysed due to the impossibility to discern between

viral and exogenous protein or transcript. This is in contrast with the other reports about ovine pluripotent stem cells, where the cells expressed either SSEA1 (Bao, 2011) or SSEA4 (Li, 2011) or both (Dattena, 2006). Given the differences in expression profile obtained, it is premature to draw conclusions with respect to the ovine pluripotent cells marker profile and to how this compares to that established for mouse and human.

In summary, I have explained a method for the reprogramming of OFFs into iPSCs. While the cells show many ESC-like characteristics, such as the expression of markers of pluripotency and fast growth, their differentiation potential is not complete, as suggested by the observation that the cells cannot give rise to beating cardiomyocyte. Whether this is a consequence of a sub-optimal culture condition or of an inappropriate differentiation protocol needs to be investigated. Further studies are required to obtain high quality ovine iPSCs; in particular the culture conditions need to be optimised. Although the generation of ovine iPSCs provides an important platform for the study of the mechanism underlying pluripotency and differentiation in ungulates, its medical and agricultural applications are impeded by the presence of the viral integration in the genome. Although retroviral vectors are silenced at the moment of dedifferentiation, it has been reported that the transgenes may be reactivated in chimeric animals (Okita, 2007). This problem may be overcome with a modification of the delivery strategy: integrating vectors might be replaced with adenoviruses, plasmids or even proteins, all non-integrating systems whose effectiveness has been reported in mouse.

CHAPTER 6

In vivo characterization of ovine induced pluripotent stem cells

6.1 Introduction

In chapter 5 I have illustrated that our ovine iPSCs display many markers of pluripotency and have demonstrated their ability to differentiate *in vitro*. The next step in the analyses is now the characterisation *in vivo*, which includes a test for the differentiation of the pluripotent cells towards the three germ lineages in an immunocompromised mouse and involves a chimera formation experiment in order to evaluate whether the cells can contribute to the offspring and go germline when injected in early stage embryos (Bradley, 1984; Huang, 2008).

The contribution to chimeras and germline is considered the gold standard for pluripotent cells. Mouse and rat ESCs have passed this test (Evans, 1983; Bradley, 1984; Buehr, 2008; Li, 2008), while human ESCs will never be tested for ethical reasons. Among iPSCs, mouse and rat have demonstrated contribution to the germline (Okita, 2007; Hamanaka, 2011), whereas pig iPSCs have so far shown their ability to give rise to chimeras (West, 2010).

The ability of pluripotent cells to contribute to an adult animal and to colonize the germ cell population allows for the birth of animals derived from only the pluripotent cells. This can be exploited for research and commercial applications: since ESCs can give rise to a whole animal, the production of genetically modified ESCs can lead to the generation of genetically modified offspring, which can be employed for the creation of disease models, for the production of specific proteins, for the generation of desired traits in farm animals or for the generation of more compatible organs for human xenotransplantation (Piedrahita and Olby, 2011) without the need to go through nuclear transfer or virus-mediated gene transfer.

In this chapter I will describe how I marked our ovine iPSCs with EGFP in order to make them traceable for *in vivo* studies; I will further describe the inoculation of the ovine iPSCs into the leg of SCID mice to assess for teratoma formation and the injection of the cells into early stage embryos to verify their ability to contribute to chimeras.

6.2 Results

6.2.1 Ovine induced pluripotent stem cells expressing GFP for *in vivo* experiments

EGFP-iPSCs were created for the *in vivo* experiments in order to be able to track the ovine iPSCs post-transplant. Indeed we wanted to prove the sheep origin of the tumours generated by injection of ovine iPSCs into SCID mice and we aimed at recognising green cells in sheep foetuses and lambs after injection of the iPSCs into early stage embryos.

Since transfection efficiency in ESCs is usually quite low, I employed a transduction-mediated protocol to insert EGFP in the genome of the ovine iPSCs. Three viral vectors carrying a constitutive promoter followed by EGFP were already available in the laboratory: PGK-EGFP, CAG-EGFP and Ubi-EGFP (Fig. 40).

Since iPSCs grow in colonies we wondered whether it were more efficient to transduce the cells when sub-confluent or just after passaging, when they are still at single cells. I sought to obtain the higher percentage of green cells, so that I would not need to perform cell sorting, with possible cell damage associated to the process.

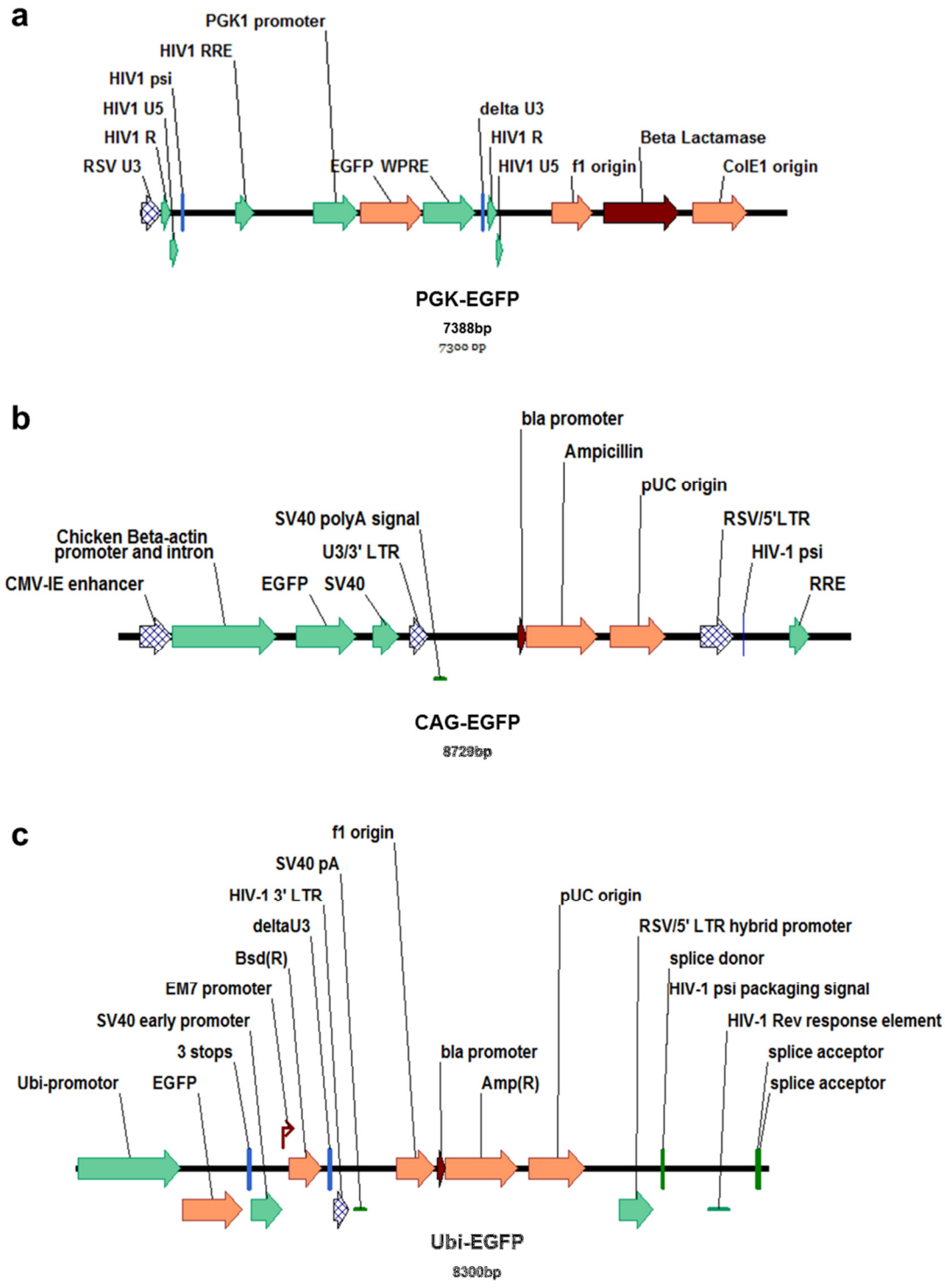


Figure 40. Lentiviral vectors employed to mark the ovine iPSCs; all carry EGFP, which is driven by different promoters: the phosphoglycerate kinase (PGK) promoter (a), the cytomegalovirus early enhancer element and chicken beta-actin (CAG) promoter (b) and the ubiquitin (Ubi) promoter (c).

I chose the PGK-EGFP viral vector to test the two different transduction protocols. Ovine iPSCs were split in two wells of 12 well plate; the first well was transduced when at 60%-70% confluence with 25µl of virus diluted in 500µl of medium; after 3h 2.5ml of medium were added and the cells were incubated with the diluted virus until the next day, when they were passaged. When the second well was confluent, cells were trypsinized, separated from the feeders and plated in a 12 well plate with 25µl of virus diluted in 500µl of medium; after 3h 2.5ml of medium were added and the cells were incubated with the diluted virus until the next day, when medium was replaced. Three days after transfection, the ovine iPSCs were quickly looked at under the fluorescence microscope and I evaluated that the transduction after passaging was the most efficient. No pictures of the experiment were taken in order to avoid exposure of expose the cells for too long to the UV light, which could cause DNA damage.

After assessing the best transduction protocol, I compared the efficiency of the EGFP vectors; the three viruses were employed in the ovine iPSCs and when passaging, few cells for each viral transduction were placed on a slide and looked at under the fluorescent microscope (Fig. 41). PGK-EGFP vector gave the brighter signal and the higher percentage of transduced cell (88%, versus 2% of CAG-EGFP and 39% of Ubi-EGFP); therefore I decided to use the PGK-EGFP transduced cells for our *in vivo* experiments.

6.2.2 Teratoma formation

In the previous chapter, I have demonstrated that the G2 clone can generate cells

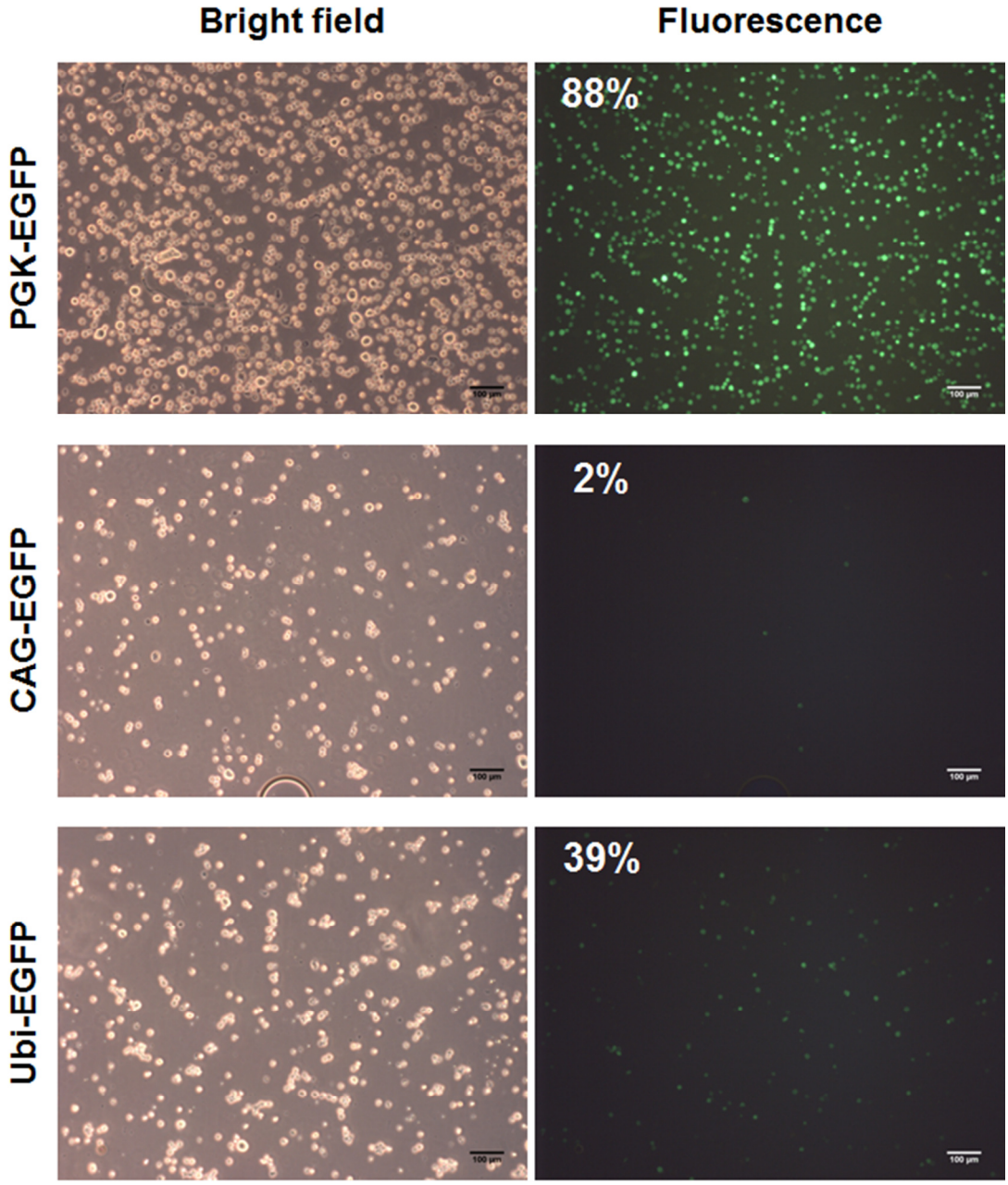


Figure 41. Ovine iPSCs were transduced with the three different EGFP vectors and the efficiency, displayed on the top right corner of each FITC image, was calculated as the ratio between the number of green cells divided by the number of total cells counted. PGK-EGFP was the viral vector which achieved to transduce the higher amount of cells (88% versus 2% of CAG-EGFP and 39% of Ubi-EGFP. Scale bars: 100µm.

_____*In vivo* characterization of ovine induced pluripotent stem cells belonging to all three germ layers *in vitro*. To further verify the differentiation potential of our ovine iPSCs, it was necessary to investigate whether these cells could form teratomas *in vivo*, after injection in immunodeficient mice.

An *in vivo* differentiation assay was performed injecting $2x10^6$ cells in the right leg of severe combined immunodeficient (SCID) mice. The ovine cells were tested alongside a mouse ESC control. Between 3 and 5 weeks after injection, all mice had developed big tumours, with diameter of about 2cm (Fig. 42), and were sacrificed. The timing of the growth of the lumps is summarized in table 9. Tumours were surgically dissected from the mice, fixed in formal saline and decalcificated in order to remove bones which could compromise the section cutting protocol. Tumours were then paraffin-embedded and sections were cut and stained with H&E staining. While mouse ESCs displayed an evident differentiation into distinct types of cells and tissues (Fig. 43a-d), the mass formed by the ovine iPSCs was more homogenous, with a very primitive organization composed of different tissues (Fig. 43e-g), but with only some cartilaginous structures that could be recognised (Fig. 43h). A lot of necrosis was observed in the ovine tumours, but not in the murine counterparts. Analysing the lumps, we soon realised that, although very likely, we did not have any proof that the tumours were originated by the iPSC-derived cells. No antibodies against the three germ layers were available that could discriminate between mouse and sheep tissues, therefore we decided to repeat the experiment employing the EGFP-iPSCs.

I injected the EGFP-G2 cell line into 6 SCID mice. Twenty two days later all mice had developed a big tumour and were sacrificed. The lumps were collected and cut in half, where one part was fixed in formal saline and paraffin embedded, while the

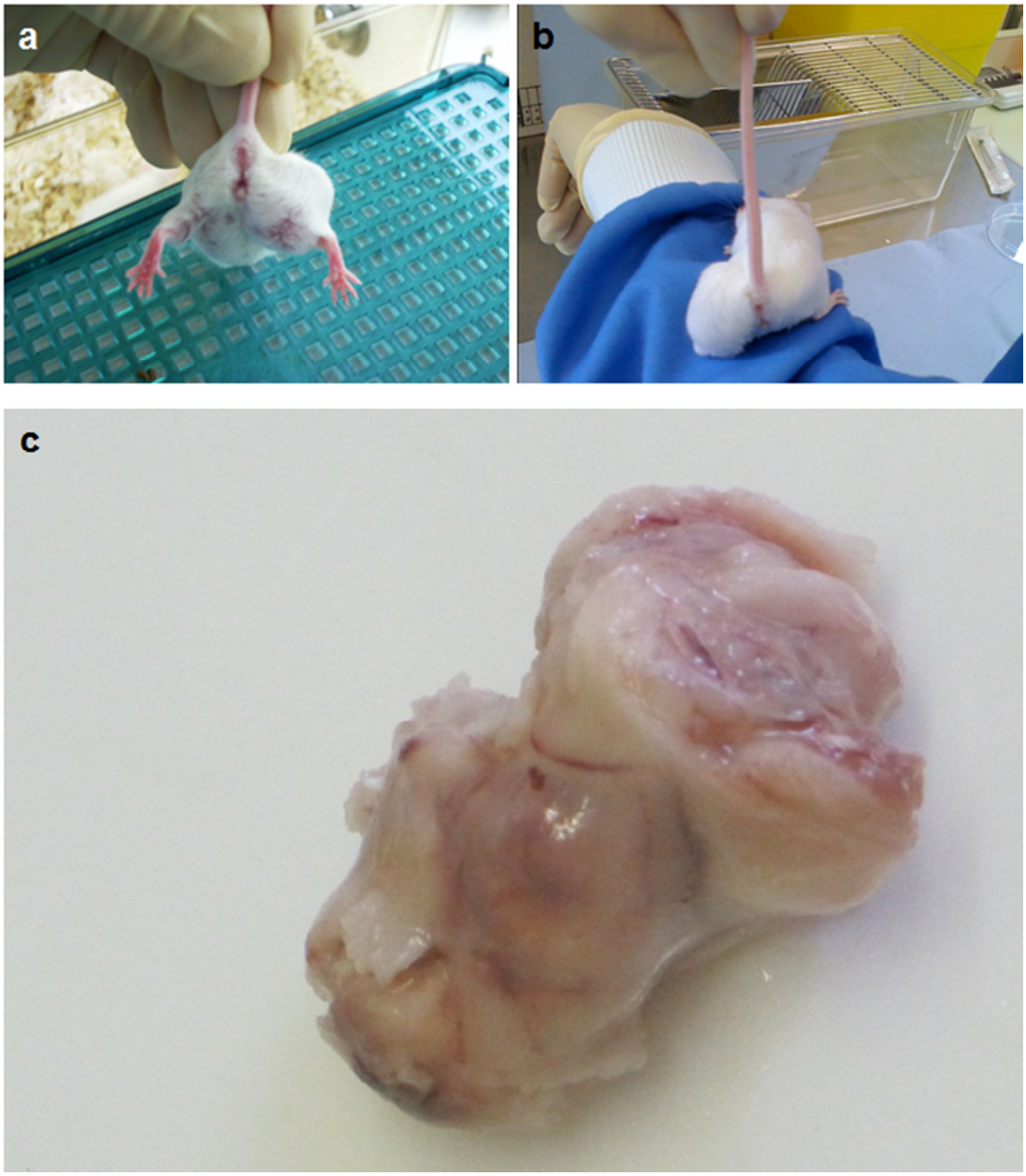


Figure 42. The tumours developed in SCID mice after injection of ovine iPSCs (a) or mouse ESCs (b) are indicated with a black arrow. The teratomas appeared and developed with similar timing for both cell types. (c) Figure of a teratoma derived by the ovine iPSCs how it appeared after excision from the mouse leg.

Cell line	Injection	Mice culled	Timing
Mouse ESCs	28/07/2009	28/08/2009	31 days
	28/07/2009	28/08/2009	31 days
	28/07/2009	21/09/2009	55 days
Ovine iPSCs E2	28/07/2009	15/09/2009	49 days
	28/07/2009	21/09/2009	55 days
	30/07/2009	15/09/2009	47 days
	30/07/2009	15/09/2009	47 days
	30/07/2009	15/09/2009	47 days
Ovine iPSCs F2	28/07/2009	04/09/2009	38 days
	28/07/2009	04/09/2009	38 days
	30/07/2009	28/08/2009	29 days
	30/07/2009	28/08/2009	29 days
	30/07/2009	04/09/2009	36 days
Ovine iPSCs G2	28/07/2009	28/08/2009	31 days
	28/07/2009	28/08/2009	31 days
	30/07/2009	28/08/2009	29 days
	30/07/2009	28/08/2009	29 days
	30/07/2009	28/08/2009	29 days

Table 9. Table summarising the inoculation of mouse ESCs and ovine iPSCs in SCID mice with the correspondent timing of tumour growth.

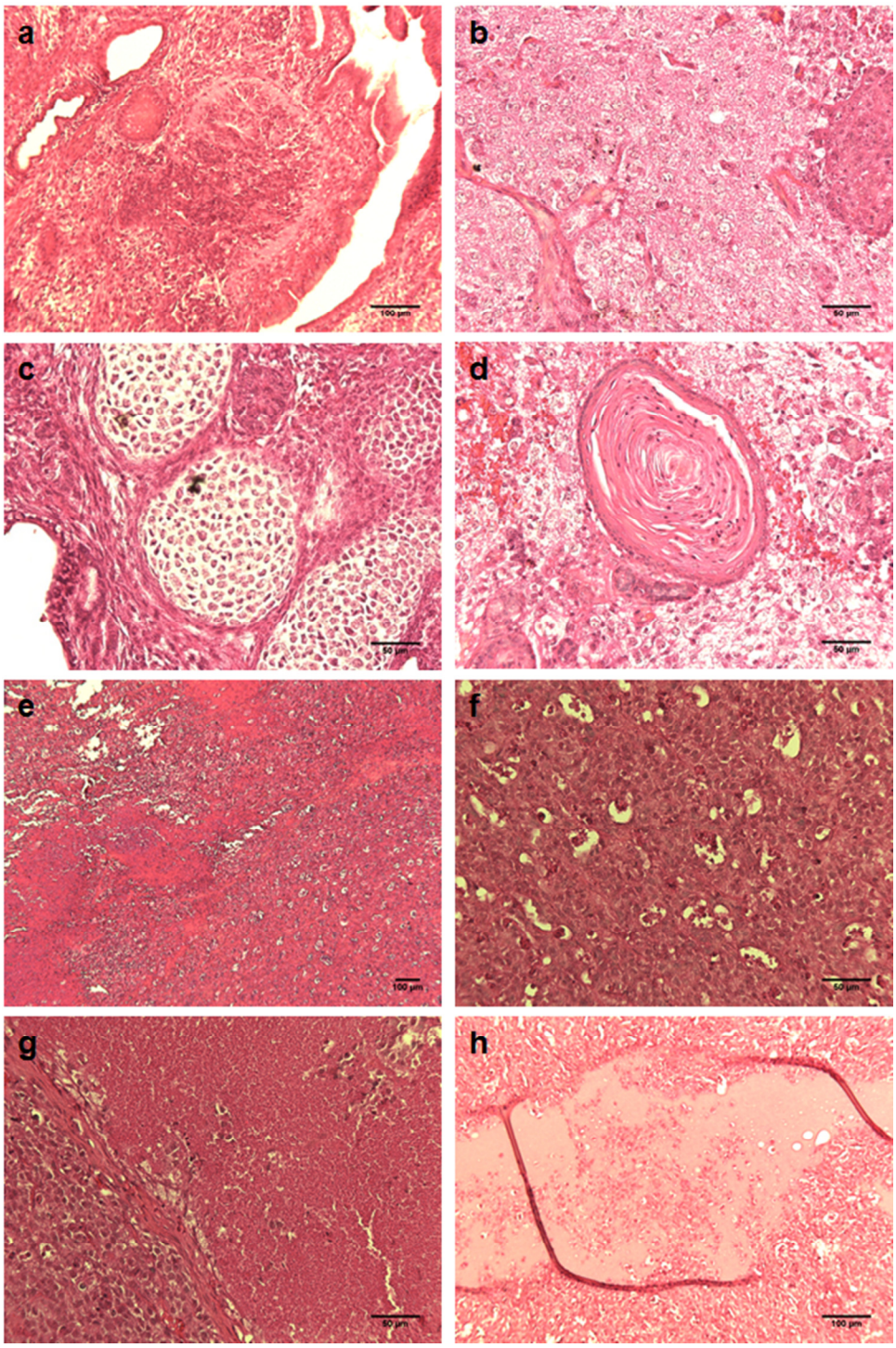


Figure 43. H&E staining of the paraffin embedded tumours derived from the mouse ESCs (a-d) or from the ovine iPSCs (e-h). Mouse ESC teratomas show that many different organised structures have developed within the tumour (a), among them we could recognise neural tissues (b), cartilage (c) and squamous epithelium (d); on the other side, ovine iPSCs gave rise to a very immature tissue (e) where it was possible to distinguish different tissue (f-h), but only few of them could be identified (h, cartilage). Scale bars: 100µm for (a), (e), (h); 50µm for (b), (c), (d), (f), (g).

other was OCT embedded and snap frozen in order to cut cryosections. The EGFP-positive sections confirmed that the tumours had been generated by the ovine iPSCs (Fig. 44a), then a H&E staining was performed, showing again mostly immature tissues with some evidence of early differentiation (Fig. 44b-e). After the examination of these and the previous tumours, I realised that the differentiation obtained with our ovine cells was not as clear as the differentiation obtained with mouse ESCs, so we proceeded with antibody stainings to be able to detect cells which, although had not given rise to defined structures yet, had started expressing proteins typical of differentiated cells. Cells were stained against markers characteristic of each of the three germ lineages: cytokeratin-18 (Fig. 44f) and α-fetoprotein (Fig. 44g) representing endoderm, vimentin (Fig. 44h) and troponin T (Fig. 44i) for mesoderm and neurofilament (Fig. 44l) and βIII tubulin (Fig. 44m) corresponding to ectoderm. Immunocytochemistry gave positive results for all markers. The stainings against cytokeratin-18, α-fetoprotein, vimentin, troponin T and βIII tubulin appeared authentic because of the morphology of the tissues: although forming some sort of structures, the positive cells were not as organised as in a healthy tissue. For the same reason we are unsure about the neurofilament results: the anti-neurofilament antibody stains in very organised areas, which do not resemble tumour tissues and might simply be nerves of the mouse around which the tumour had developed. Troponin T staining on the other hand is likely to be unspecific, since it is localised in structures that are similar to nerves more than muscle.

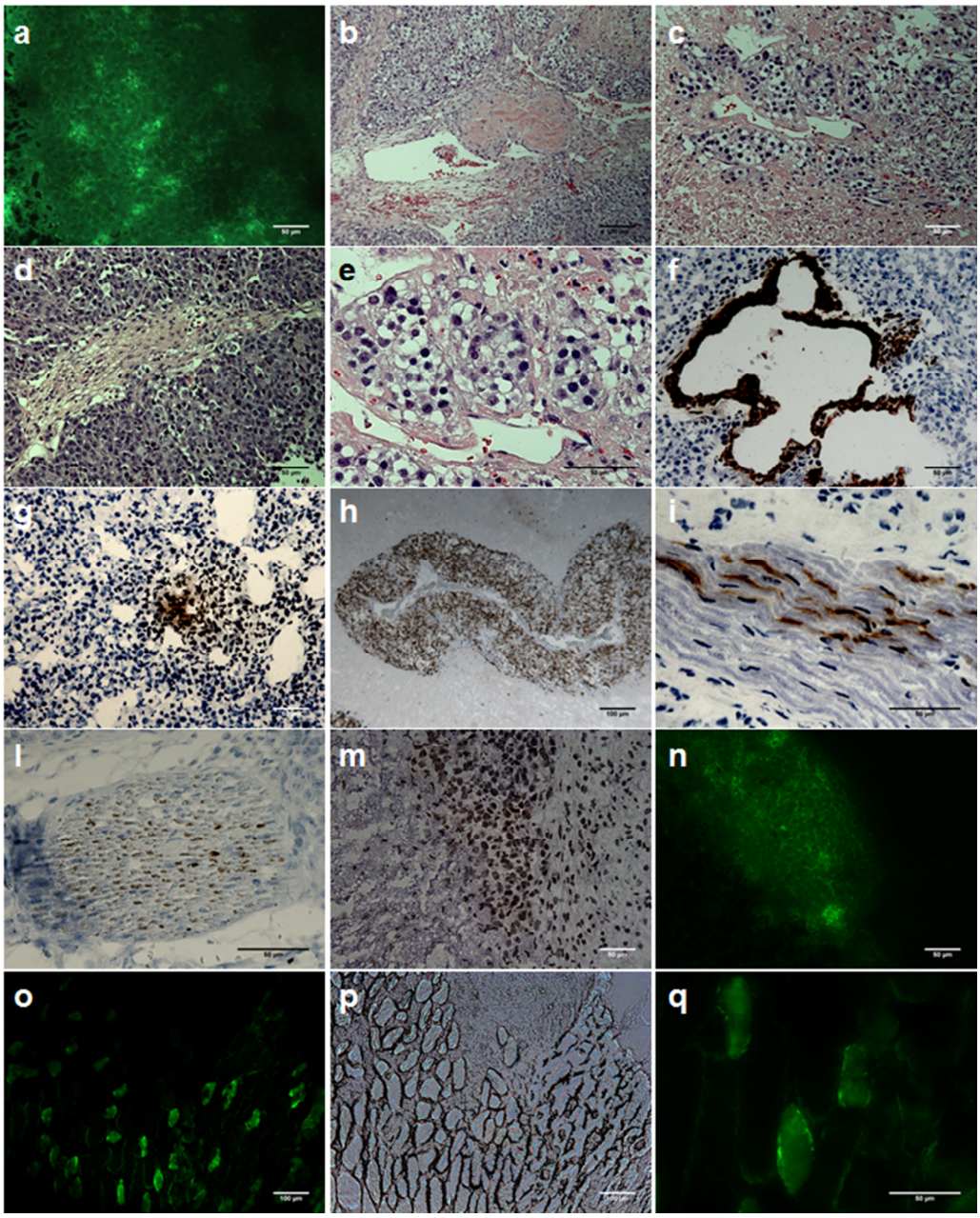


Figure 44. Teratomas generated by the inoculation of EGFP-iPSCs: (a) a section of the tumour looked at with the fluorescent microscope shows the teratoma is indeed of ovine origin; (b-e) different tissues can be observed in the H&E stained sections of the tumours; (f) anti cytokeratin-18 immunohistochemistry of a slide show a positive signal in a primitive epithelium; (g) α-fetoprotein staining, the positive cells are not correlated to any visible organization of the tissues; (h) anti-vimentin antibody marks cells which form a defined structure; (i) troponin T immunohistochemistry showing a false positive: the tissue stained appears to be nerve, not cardiac muscle; (l) neurofilament staining is localised in a nerve, however the structure is very organised and we cannot be sure it does not belong to the mouse host; (m) βIII tubulin immunohistochemistry reveals a positive tissue with a morphology resembling that of a nerve; (n) the section sequential to that of the βIII tubulin staining confirms that the neural tissue is derived from the ovine iPSCs; (o-q) EGFP-positive muscle in the teratoma sections at different magnifications (o and q, fluorescence field, p bright field). Scale bars: 50µm for (a), (c) - (g), (i) – (n), (q); 100µm for (b), (h), (o), (p).

Looking at sequential sections I identified bright green cells covering the same area of the βIII tubulin staining (Fig. 44n), which gave us a further confirmation that the differentiated cells derived from the injected ovine iPSCs.

Investigating the areas at the edge of the tumours I noticed that in 2 of the 6 tumours, among the muscle fibres of the mouse, EGFP was clearly detectable with a morphology similar to that of muscle tissues (Fig. 44o-q), indicating that our ovine iPSCs generated muscle fibres infiltrating among the tissues of the mouse. The green cells with muscle morphology may however be the result of cell fusion, instead of differentiation of the iPSCs: this event is described by Reinecke et al (Reinecke, 2004) and Nussbaum et al (Nussbaum, 2007); in both reports, however, the fusion event is very low (quantified by Reinecke et al as 0.01% likelihood), while in the teratomas derived from the iPSCs, same areas inside the muscle accounted for more than 30% EGFP positive cells, suggesting that the green cells might be authentically differentiated iPSCs. This hypothesis could match with the timing of *in vitro* differentiation of ESCs into muscle: in mouse ESCs usually a differentiation of 12 days is indeed needed to get more than 80% of cells show myogenic differentiation (Rohwedel, 1995).

6.2.3 Injection of ovine induced pluripotent stem cells in ovine embryos

Once assessed that the ovine iPSCs were able to give rise to teratomas, I moved on to the following step of characterization and tested the ability of the cells to contribute to animals.

A preliminary analysis was performed to verify what embryonic stage was more suitable for injection of the cells; classically ESCs are injected in the cavity of the

expanded blastocysts, but recently a study reported the successful generation of chimeras by injecting ESCs in the zygote or the 8 cell stage (Huang, 2008).

EGFP-iPSCs (Fig. 45) were inoculated in ovine embryos at zygote, 8 cell and blastocyst stage (Table 10). The fate of the cells was followed in order to assess whether the cells survived and were incorporated into the early embryos. When injected at zygote stage, the cells were bright green after 2h (Fig. 46a), but already after 1 day the fluorescence was weakening (Fig. 46b) and at blastocyst stage no green cells were detectable (Fig. 46c). When cells were inoculated at the morula stage, again no fluorescence was visible in the blastocysts (Fig. 46d, e). When inoculated into blastocysts and looked at 2-3 hours later, only few fluorescent cells were noticeable (Fig. 46f, g).

From 5 to 16 cells were injected in each embryo, but already few hours afterwards most of them were not visible anymore (Fig. 46a, f). The inability to detect the majority of the injected cells that were inoculated might be due to a number of possible reasons: cells may have died and burst or most of the injected cells were not EGFP transduced or simply EGFP penetrating power was not enough to pass through the layers of cells composing the embryos.

The hypothesis of the death of the ovine iPSCs is in contrast with the fact that in the zygote-injected embryos some fluorescence, albeit very weak, was detectable even the day after injection.

Although possible, the supposition that most of the injected cells were not EGFP-positive is not very likely, indeed, I calculated that after the transduction roughly 88% of the cells were green and just before the inoculation the cells were looked at again under the fluorescent microscope in order to confirm that EGFP was

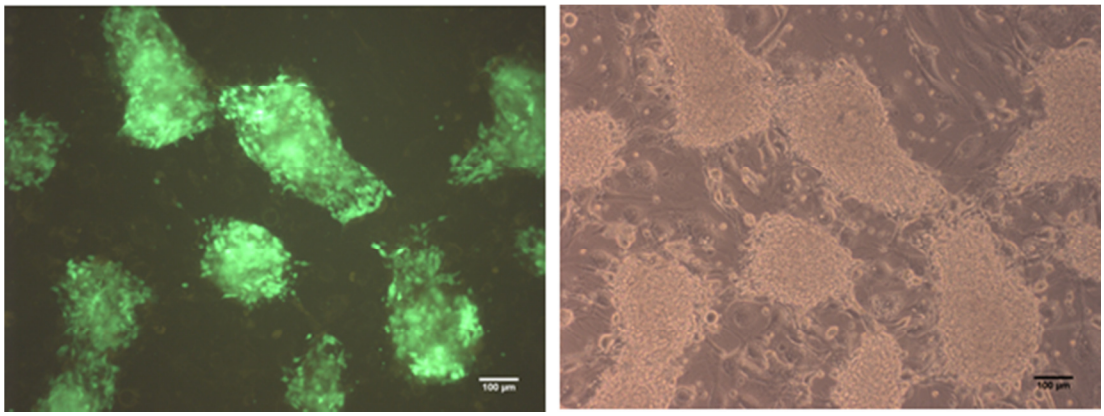


Figure 45. Fluorescence and bright field of EGFP transduced ovine iPSCs employed for the chimera experiment. Scale bars: 100µm.

Injected embryos	Stage	Injected cells	Developped blastocysts
101	Zygote	6-10	14
53	8 cell stage	6-12	4
5	d6 blaststocyst	~16	5

Table 10. Table summarising the preliminary injection of EGFP-iPSCs into embryos at different stages.

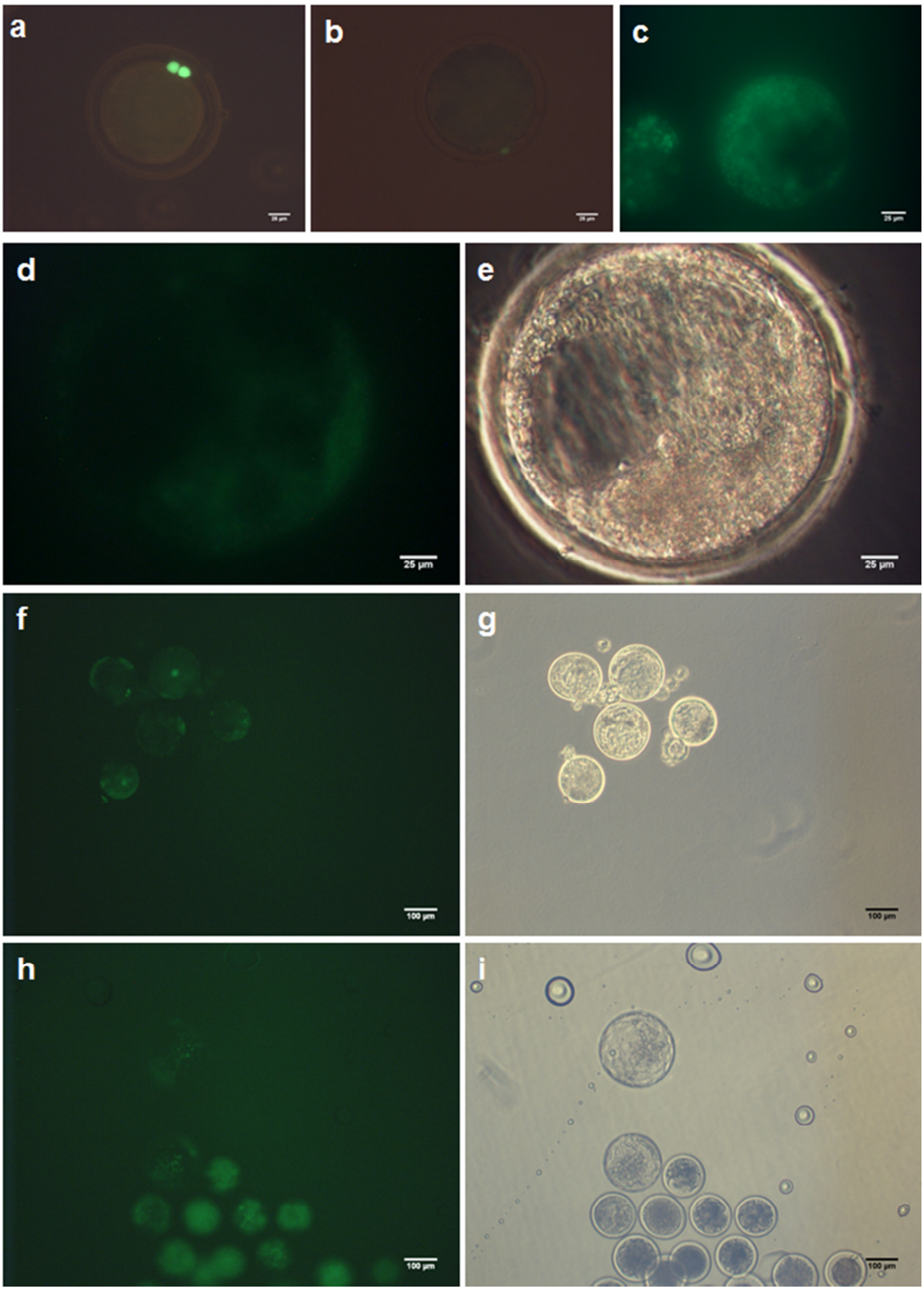


Figure 46. Outcome of the preliminary injection of EGFP-iPSCs into embryos at the zygote (a-c), 8 cell (d, e) and blastocyst (f, g) stages. (a) Zygotes 2h after inoculation, (b) 24h after inoculation and (c) at blastocyst stage. Bright (d) and fluorescent (e) fields of a blastocyst injected at 8 cell stage; bright (f) and fluorescent (g) fields of a blastocyst injected at blastocyst stage and uninjected blastocysts (h, i). Scale bars: 25µm for (a) – (e); 100µm for (f) – (i).

still expressed. It is worth to note that for the injection procedure the cells with the best morphology were chosen, so it might have happened that the non-transduced cells looked healthier and were therefore preferentially inoculated.

Regarding the third assumption, I realised that when the green cells were underneath the zygote, the fluorescence could not be seen anymore, but was visible when rotating the embryos; while the rotation could be performed on the zygote to locate all the green cells, this could not be applied to the blastocysts, since they are formed by different layers of cells; so if some green cells were not on the surface, it was possible that I could not detect their fluorescence; additionally ovine blastocysts possess auto-fluorescence in the culture conditions we employed (Fig. 46h), which could have further masked the low EGFP signal coming from hidden cells.

Since the preliminary analyses on the different stage injections did not give any clear result, we transferred the embryos inoculated at the three different stages into synchronised recipients. The ewes were culled just after 3 weeks post fertilization. Out of 19 blastocysts transferred, 9 embryos had developed (Table 11) and were recovered alongside their extra-embryonic membranes: 6 foetuses and the membranes were first looked at under the fluorescence stereoscopic microscope and then their gDNA was extracted, while the other 3 embryos were snap frozen for cryosectioning. Neither the analysis at the stereoscopic microscope nor the cryosection assay displayed any fluorescence (Fig. 47a-c). The PCR on the gDNA extracted from the foetuses and the placentas was problematic: the primers initially utilised were those already employed for the silencing and a very weak band was

Injected embryos	Stage	Injected cells	Developped blastocysts	Transferred	Pregnancies
33	Zygote	~8	9	2 sheep (3 blastocysts/sheep)	2 foetuses
19	8 cell stage	12-15	7	2 sheep (3 blastocysts/sheep)	1 foetus
7	d7 blaststocyst	17-22	10	1 sheep (7 blastocysts/sheep)	6 foetuses

Table 11. Table summarising the preliminary transfers of EGFP-iPSCs into recipients.

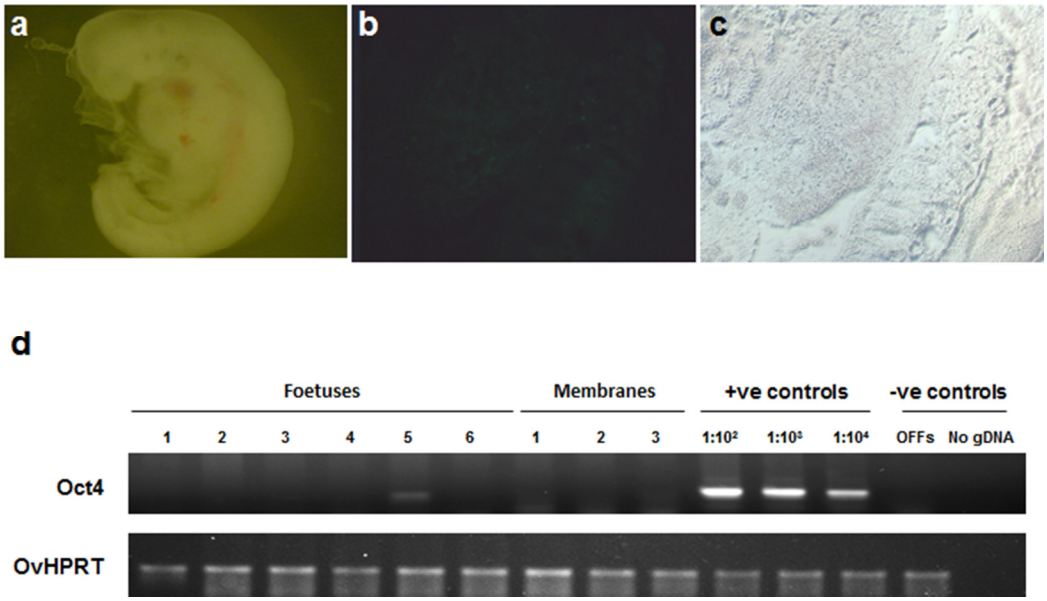


Figure 47. Analyses on the foetuses recovered from recipients carrying EGFP-iPSCs: (a-b) Image of a foetus observed at the fluorescent microscope: no EGFP signal could be detected; (b-c) cryosections of an embryo in fluorescent field (b) and bright field (c): again no fluorescence was visible; (d) PCR for viral Oct4 reveals a little contribution of the ovine iPSCs to one of the foetuses; the negative control is OFF gDNA, the positive controls consist of ovine iPSC gDNA spiked into the negative control in a ratio of $1:10^2$, $1:10^3$ and $1:10^4$. The contribution of the ovine iPSCs to the embryos appears to be lower than 1 cell in 10000.

visible in one of the samples; I then attempted to perform a nested PCR, to verify whether one of the samples was truly positive, but, although very careful, I had to deal with contamination problems. I therefore designed new primers that recognise the viral Oct4 sequence. These primers were able to detect lower concentrations of Oct4 than the previous and allowed for a weak band to be visible in one foetus (Fig. 47), corresponding to a contribution lower than 1 in 10^4 cells. In the different replicates others sample would sometimes be weakly positive and we wondered whether the results I obtained were due to a very low contribution which was at the detection limit of the PCR or whether the bands I saw were contaminations of some sort; however the latter option is unlikely because the no gDNA control was always negative.

6.2.4 Analyses on the lambs

Since no clear results were obtained from the previous analyses, we injected around 20 cells from two cell lines (G2 and D1) in day 6 blastocysts. The embryos were transferred in recipients and the ewes were left to carry the pregnancy to term. Initially we inoculated the EGFP cells, but after noticing that the fluorescence was soon switched off and was not visible in any of the foetuses, we decided to start injecting unmodified cells, assuming that fewer modifications in the genome and in the protein content of the iPSCs would increase the chances of achieving a contribution to the animals. Eighteen lambs (17 alive and 1 mummified) were born out of 99 blastocysts transferred (Table 12); at the moment of the birth the placenta samples were collected for analyses.

Cell line	Transferred blastocysts	Recipients	Pregnancies	Offspring
G2 GFP	27	9	4	6*
G2	33	11	8	8
D1	39	13	3	4
Total	99	33	15	18*

* 1 mummified foetus and 2 lambs found dead few days after birth.

Table 12. Table summarising the transfers of EGFP-iPSCs into recipients and the outcome.

Some of the ewes had a gestation term a few days longer than expected and three lambs were very big, with a weight ranging between 7.5 and 10.2kg, whereas one was very small, weighting only 2kg. The bigger lambs were likely the result of the large offspring syndrome typical of bovine and ovine embryos exposed to a variety of unusual environments prior to the blastocyst stage (Young, 1998), not a consequence of the inoculation of the ovine iPSCs.

Only one round of D1 cell injections gave rise to lambs, producing 4 lambs (OT046, OT047, OT048, OT051), suggesting that this cell line is less suitable for the growth in early embryos. Among them was one of the heavy lambs, weighting 7.5kg.

One lamb (OT029) was found dead 1 day after birth, apparently because of twisted gut; another lamb (OT027) suffered of pneumonia and was sacrificed 4 days after birth.

Ear clips and blood were collected for the live lambs, while different tissues were collected from the animals which had died and from the mummified lamb; placental samples from the mothers were gathered too. The tissues were looked at under the fluorescence microscope revealing no EGFP signal; multiple PCRs targeting the viral Oct4 were performed on the samples in order to assess whether the lambs were chimeric. Few samples were positive, even though the contribution to the animals was calculated to be lower than $1{:}10^3$ (Fig. 48).

Due to the previous results, we decided to sacrifice the lambs to investigate whether other organs and tissues may carry a higher number of iPSC-derived cells. Two animals were maintained alive: OT058 lamb, whose ear clip was positive to the Oct4 PCR and OT059 as a negative control in case other analyses needed to be done.

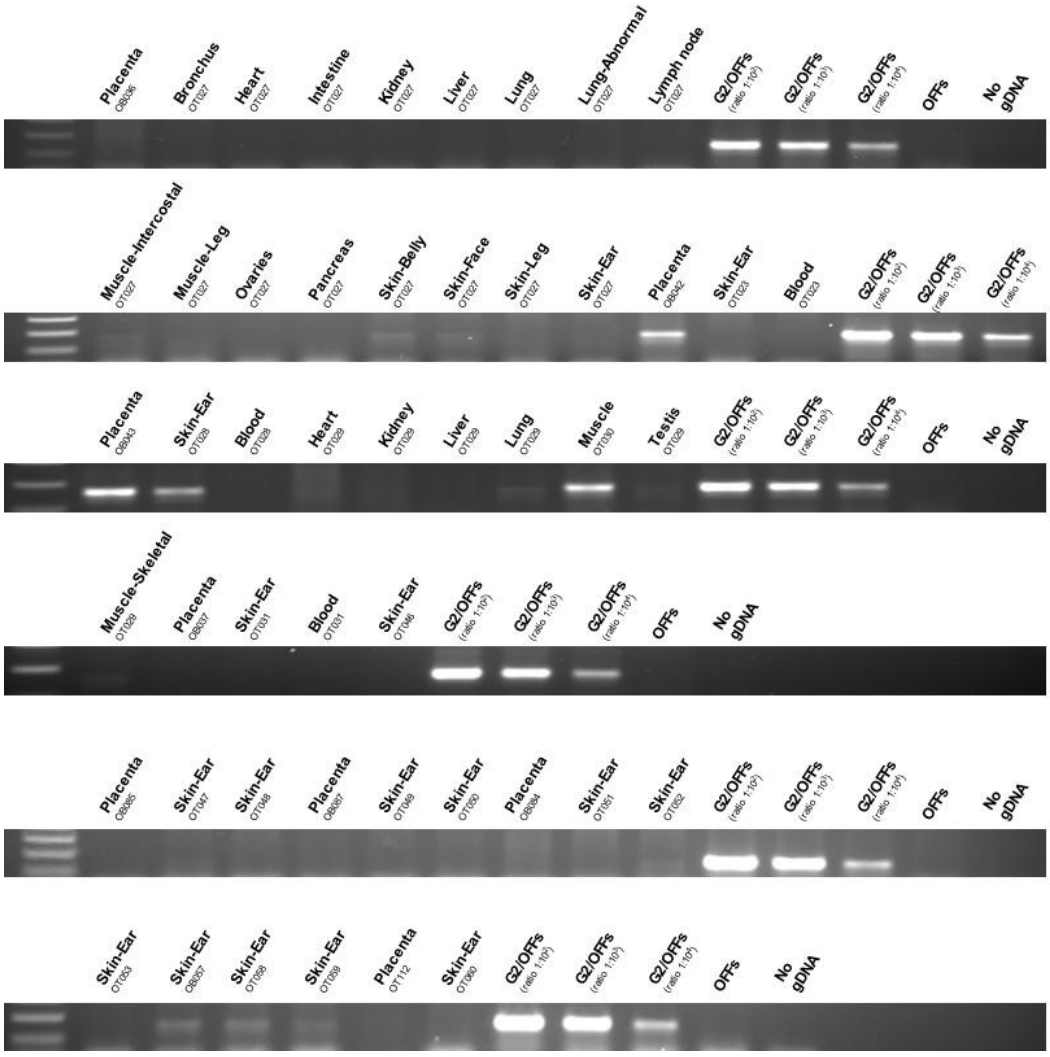


Figure 48. A representative Oct4 PCR assay on ovine tissues collected from the lambs. Negative control is OFFs gDNA, while positive control is represented by ovine iPSC gDNA spiked into the negative control at different concentrations.

The lambs were sacrificed at 5 to 16 weeks of age and multiple samples were collected from a wide range of tissues (Table 13), looked at under the stereoscopic fluorescent microscope and subjected to PCR specific to the Oct4 transgene.

No green tissues were observed at the microscope and the Oct4 PCR results showed a very low iPSC contribution in different tissues (Fig. 49a), which is summarised in table 13. The samples reproducibly positive to Oct4 belong to the three germ layers and also to the extraembryonic membranes. Again the contribution of the cells to the tissues was lower than 1 in 10^3 cells. Interestingly, while most of the G2-derived lambs had a wide range of tissues positive to Oct4 PCR, D1 cells achieved to weakly contribute to only one tissue of one lamb, suggesting that this second cell lines is less suitable for chimera formation, results which is consistent with the *in vitro* differentiation data.

In order to confirm the Oct4 results a viral cMyc PCR and a viral Klf4 PCR were performed on the samples positive for viral Oct4: only three tissues (skin from lamb OT028, muscle from lamb OT030 and placenta from ewe OB043) were positive for cMyc or Klf4 (Fig. 49b). Remarkably lamb OT028 and lamb OT030 are the offspring of ewe OB043. Lamb OT030, which displayed the higher contribution of iPSCs, was born mummified and, although different tissues (muscle, liver, heart and kidney) were collected from it, gDNA could be extracted only from muscle.

These results confirm that the ovine iPSCs are able to contribute to the generation of live chimeras, even though at low level.

	EWE	OB042	OB036	OB043			OB037	OB086	OB085		OB087		OB084	OB102		OB114		OB97	OB112
	LAMB	OT023	OT027	OT028	OT029	OT030	OT031	OT046	OT047	OT048	OT049	OT050	OT051	OT052	OT053	OT057	OT058	OT059	OT060
TISSUES																			
Placenta		+	-		+		-	\	-		-		-		\		\	\	\
Blood		-	\	-	\	\	-	\	\	\	\	\	\	\	\	\	\	\	\
Brain		-	\	-	\	\	-	-	-	-	-	+	+	+	-	+	\	\	+
Bronchus		\	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Gonads		-	-	-	+	\	-	-	-	-	-	+	-	-	\	+	\	\	-
Heart		-	-	-	-	\	+	-	-	-	-	-	-	-	-	-	\	\	-
Intestine		-	-	-		\	-	-	-	-	-	+	-	-	-	-	\	\	-
Kidney		\	-	-	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Liver		\	-	-	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Lung		-	-	-	-	\	-	-	-	-	\	-	-	-	-	-	\	\	-
Muscle - leg		-	+	-	+	+	+	-	-	-	-	+	-	+	-	+	\	\	+
Muscle - intercostal		\	-	-	\	\	\	\	\	\	\	\	-	\	\	\	\	\	\
Muscle - Neck		\	\	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Pancreas		\	-	-	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Skin - Belly		-	+	-	\	\	\	-	-	-	-	-	-	+	-	-	\	\	-
Skin - Ear		-	\	-	\	\	\	-	-	-	-	-	-	-	-	+	+	-	-
Skin - Face		\	-	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Skin - Leg		\	-	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Spleen		\	\	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Stomach		\	\	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\

Table 13. Table with a list of the tissues collected from the lambs born after ovine iPSC injection into blastocysts of sheep.

6.3 Discussion

In this chapter I have demonstrated that the ovine iPSCs I have generated are able to differentiate *in vivo* and to contribute to chimera formation.

At first sight the teratomas produced by the ovine iPSCs appeared to be extremely immature when compared to the mouse counterparts; nonetheless I was able to recognise many different cell types belonging to all three germ layers through immunohistochemistry. Among the immature tissues I could detect some organised structures, but initially I was not able to address whether these structures were derived from the ovine cells or were part of the mouse body, due to the lack of antibodies specific for ovine tissues. In order to trace ovine contribution we repeated the experiment marking the cells with EGFP and were able to demonstrate that the tumours, including some of the primitively organised tissues, were specifically formed by iPSCs.

The early differentiation state of our tumours might suggest a non-complete reprogramming of our ovine iPSCs; however other causes may be at the basis of the immature teratomas. Ovine pluripotent cells have been recently isolated and the literature about teratoma formation is very scarce. Only two reports are available which describe the injection of ovine iPSCs into SCID mice (Bao, 2011; Li, 2011), but in both cases there is a statement that the cells can give rise to different tissues belonging to the three germ layers, without commenting whether the teratomas were extremely differentiated or still naïve.

Culture conditions can be another explanation, even though EpiSCs and human ESCs, which are cultured in the same condition as our ovine iPSCs, do not exhibit this limitation.

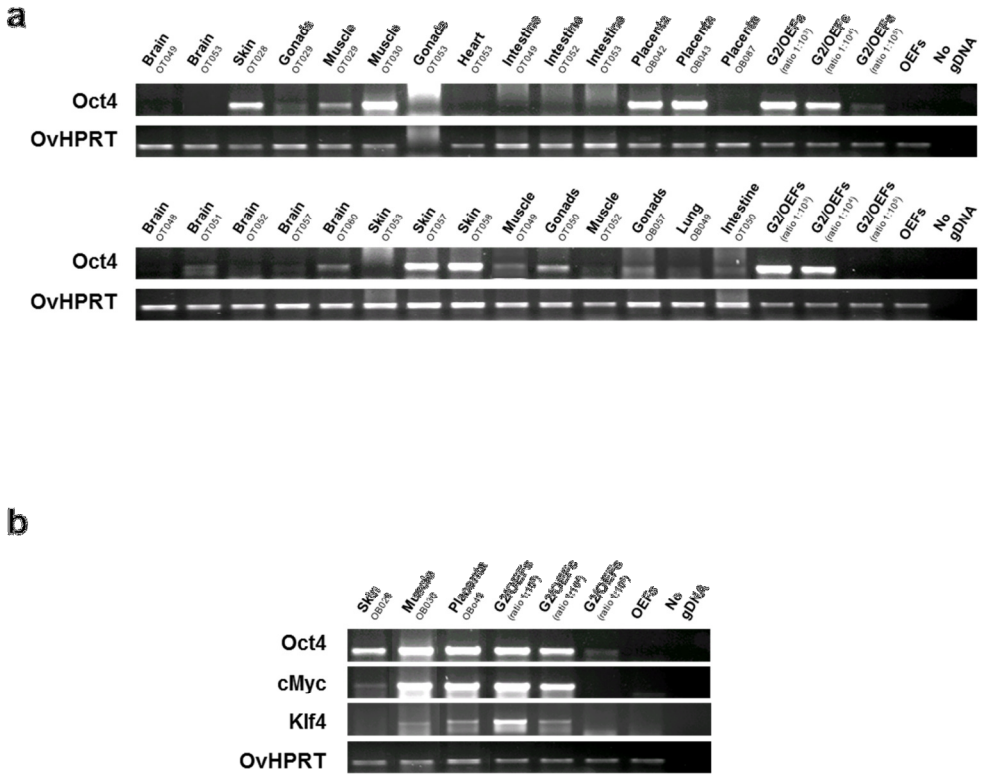


Figure 49. (a) PCR analysis targeting the viral Oct4 on a selected group of samples suggests that tissues coming from the different germ layers and from the germ cells have a low contribution of ovine iPSCs; (b) in order to confirm the assumption PCRs for the viral cMyc and viral Klf4 were performed: only few samples resulted positive to one or both genes.

Another possibility for the reduced differentiation that I observed can be linked to the site of injection: many reports (Cooke, 2006; Prokhorova, 2008) underline that teratoma formation from human ESCs is dependent on the graft site and the intramuscular injection is the least efficient method among those employed (Prokhorova, 2008); although the mouse ESCs employed as a control were inoculated in the same site producing widely differentiated tumours, the site-dependency might be a characteristic regarding only inter-species teratoma formation.

It is also possible that the ovine cells required more time to differentiate and the mice were sacrificed too early.

It also true that spontaneous *in vitro* differentiation of ICMs from ungulates does not give rise to the variety of different tissues seen in the mouse, but mostly neuroectoderm; this may suggest that we have not found yet the optimal culture conditions for the differentiation of pluripotent cells from ungulates.

EGFP however was not uniformly expressed among the teratomas, suggesting that either the PGK promoter have undergone silencing during the differentiation process towards some specific cell lines or simply that the EGFP-negative portions of the tumours had been generated by the few non transduced ovine iPSCs injected.

Both hypotheses are plausible: reports state that the PGK EGFP cassette introduced in ESCs is sometimes silenced upon differentiation (Hamaguchi, 2000); likewise it is important to bear in mind that the ovine iPSCs that we injected were 88% fluorescent, but there was still a small percentage of non-transduced cells among the

EGFP positive cells. The lack of fluorescence found in some areas of the teratomas may be the result of the combination of the two events.

A similar issue was encountered when performing the preliminary experiments for the ovine iPSCs injections in early stage embryos: while 2h after inoculation bright green cells were still detectable, at the blastocyst stage no fluorescence was visible. Again, this may be explained by inhibition of the PGK promoter that drives EGFP expression or by the presence of non-transduced cells among the green cells. Although at first sight it is very unlikely we inoculated mostly non fluorescent cells since they accounted only for the 22% of the total cells, it is possible that the EGFP cells had a slightly different morphology compared to the non-transduced cells and were unintentionally overlooked at the moment of the injection. This hypothesis might also explain why, although from 5 to 25 cells were inoculated into each embryos, only 1 or 2 green cells were noticeable only few hours post injection.

Analysing the embryos inoculated at zygote stage I noticed that already after 24h the fluorescence tended to fade greatly. This may be a consequence of cell death, which brought to loss of the cytoplasm content, including the EGFP protein; however the cell membrane looked apparently undamaged. Again the loss of fluorescence may be the result of inhibition of EGFP expression: EGFP half-life is roughly 26h (Corish and Tyler-Smith, 1999), so the fading could be explained as an intermediate state where the gene does not transcribed anymore, but the proteins are still in the cytoplasm and are slowly degraded or diluted by cellular division events.

Chimeric animals have been produced from mouse (Okita, 2007; Wernig, 2007) and rat iPSCs (Hamanaka, 2011). Among the ungulates a group has reported the generation of chimeric animals employing pig iPSCs (West, 2010), but the result has not been described yet in sheep.

Here I show that, albeit at low level, ovine iPSCs can contribute to live offspring. The sample showing the higher amount of iPSC-derived cells was collected from the mummified animal, raising the question whether the higher contribution was the responsible for the lack of viability of the lamb.

The low contribution to the chimeric animals may be the effect and the combination of different factors. The culture medium conditions in which our ovine iPSCs are grown could have had a major impact on the efficiency of the process: human ESC conditions are known to promote the EpiSC state both in mouse and in human. EpiSCs express the key pluripotency factors and can differentiate into numerous cell types *in vitro*, but they can hardly contribute to live animals after injection into blastocysts (Brons, 2007; Tesar, 2007). In the light of this consideration, the low contribution to the chimaeras is to be expected.

Another possibility may reside in differences in growth characteristics: even though the growth rate of our ovine iPSCs was comparable to established mouse ESCs, I observed that after trypsinization our ovine iPSCs took longer to recover than the mouse ESCs. This delay may reduce the proliferation capability of cells injected into the blastocyst.

Alternatively and reflecting the limited morphology observed in teratoma studies, our iPSCs may not be reprogrammed enough to allow for chimera formation. In a 2007 report Piliszek et al (Piliszek, 2007) demonstrated that embryonic fibroblasts can

_____*In vivo* characterization of ovine induced pluripotent stem cells contribute to full term mice; this might raise the question whether our ovine iPSCs are simply OFFs which succeeded to partially being incorporated into the offspring. However this would strongly be in contrast with the results presented in this and in the previous chapters, where the ovine iPSCs showed to express markers of pluripotency, to be able to partially silence the transgenes and to differentiate towards cells belonging to the three germ layers both *in vitro* and *in vivo*.

It is also important to underline that very little is known about the development of the early embryo in sheep; indeed there is no prior knowledge about how a pluripotent cell injected into the early ovine embryo will be incorporated.

CHAPTER 7

Final discussion and conclusions

7.1 Final discussion

The data described in this thesis illustrate the generation of iPSCs in sheep and their characterisation both *in vitro* and *in vivo*. The generated cells express some pluripotent markers, are able to differentiate into cells deriving from all three germ layers both in culture and after injection in an animal and can give rise to chimeras.

7.1.1 Viral vector preparation

The first step of the project was to repeat the original report of Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) in order to establish the novel iPSC generation protocol at The Roslin Institute. The expression plasmids we employed were those generated and used in the original iPSC work, while the packaging plasmids were purchased. In order to assess whether viable viruses could be produced and in order to verify that the reprogramming factors were indeed expressed in the target cells, the viruses were generated by transfecting HEK cells and were employed for the transduction of immortalised MFF lines. Those cells were fixed and the staining against Klf4, Oct4 and Sox2 confirmed that the viruses were functioning. We could not perform cMyc staining because of issues faced with the antibody we were employing: even after performing numerous optimizations varying the concentration of primary and secondary antibody, the negative control was always positive, indicating a poor specificity of the anti cMyc antibody. Being that the other three viruses were viable we assumed that also the cMyc virus might be able to express its reprogramming gene; since cMyc is not an essential factor for reprogramming (Nakagawa, 2008), we presumably could have isolated iPSCs albeit with a lower efficiency.

The immunocytochemistry assay was initially performed on cells transduced with a frozen virus. Since the protocol for the generation of iPSCs requires the use of fresh virus (Takahashi, 2007a), I repeated the transduction for the Sox2 virus and the staining confirmed that the use of a freshly prepared virus permits optimal cell transduction.

7.1.2 Generation of putative mouse induced pluripotent stem cells

Once confirmed the viruses were viable, they were employed for the reprogramming protocol in MFFs: the fresh cMyc, Klf4, Oct4 and Sox2 viral vector were transduced into MFFs which were grown on feeder layer. A week later small colonies appeared with a morphology similar to that of mouse ESCs; colonies were picked, expanded and tested for the basic features of pluripotency. The four clones evaluated were positive for Nanog, but, in contrast to mouse ESCs where the staining was strictly localised in the nucleus, Nanog appeared to be expressed in both nucleus and cytoplasm. This kind of Nanog expression pattern has been observed in ASCs (Zuk, 2009; Carlson, 2011), primitive germ cells (Goel, 2008) and tumours (Ezeh, 2005; Ye, 2008), suggesting that the mouse cells I have generated were in a partially reprogrammed state or were still in the middle of the reprogramming process and needed more time to fully reprogram.

The putative murine iPSCs were then allowed to differentiate *in vitro* through spontaneous differentiation via EB formation; the outgrowths derived from the plated EBs were stained against ectodermal, endodermal and mesodermal markers. The cells were positive for βIII-tubulin and Troponin T but no clear results could be

obtained for α-fetoprotein; the antibody optimisation required a long time and when I achieved it, I was already working on the ovine iPSCs.

Further characterisation of these cells might have been performed to have a more complete indication of whether the cells are authentic iPSCs (e.g. staining against a higher number of pluripotency markers, RT-PCR to verify whether the exogenous genes had been silenced, *in vitro* differentiation assay where alongside immunocytochemistry a RT-PCR was performed on a higher number of markers, blastocyst injection, germline transmission).

The efficiency of reprogramming was variable between different wells, ranging from 2.8% to 13.2%. This figure is much higher than those already published, but cannot be compared with them, because of the different techniques employed for the calculation of the efficiencies in the other studies. Indeed I counted all the colonies that had developed, while other studies calculated only those which had Oct4 or Nanog promoters activated or those positive to AP staining, which are substantially fewer than the total number of colonies generated.

The generation of mouse reprogrammed cells was performed in order to assess whether the protocol was functional and to find what may be improved. Indeed we found many points that could be changed in order to ensure a better outcome: the transduction protocol would have been more efficient without the presence of the feeder layer. Additionally, the presence of a retroviral vector carrying a marker in order to evaluate whether the transduction protocol had worked and the use of optimised number of feeder cells to expand the reprogrammed cells would have

probably improved the process. Again, the use of different culture conditions may have been interesting: the 3i or the 2i (Ying, 2008) media could have been employed, since they select only pluripotent cells, leaving the differentiating cells to die.

Although the characterisation of the mouse cells was not complete, we achieved the purpose of verifying that the Takahashi and Yamanaka's published protocol was able to reprogram embryonic fibroblast to a more naïve state; furthermore the feasibility of the method was proved by many other studies that were later published by other groups. We therefore decided to proceed to the main aim of my study: the derivation of iPSCs in sheep.

7.1.3 Generation and *in vitro* characterisation of ovine induced pluripotent stem cells

When I began the ovine iPSC generation experiment many reports had been recently published where new techniques were employed for the isolation of iPSCs. In particular, more efficient methods such as lentiviral transduction (Yu, 2007) or safer approaches, employing non integrating viruses (Stadtfeld, 2008c), plasmids or even cell permeable proteins (Zhou, 2009; Cho, 2010) have been developed. We evaluated the opportunity to employ these novel techniques, but decided to continue working with the MoML viral vectors for four main reasons. First, although not as efficient as the lentiviruses, retroviral vectors are more efficient than all other published protocols. Second retroviruses have a main advantage over lentiviruses for the production of iPSCs: they are silenced in pluripotent cells (Cherry, 2000), property which discerns between fully and partially reprogrammed iPSCs. Third, we knew the

retroviral protocol worked because of the mouse experiment, which moreover allowed us to be aware of the potential issues encountered during the process and of some hints to improve the protocol. Last, we had already all the reagents and plasmids we needed since the alignment of the murine and ovine reprogramming genes showed the mouse factors were highly homologous to those of the sheep.

The reprogramming protocol in sheep was based on that carried out on mouse cells, with minor modifications: OFFs were transduced on gelatin and only later passaged on feeder cells, a new packaging vector able to target all mammalian cells was employed and a GFP retroviral vector was employed in the negative control in order to verify whether the four genes, and not just the viral transduction, were the elements that brought to the colony formation; furthermore the GFP virus was useful to assess whether the viral vectors packaged with the new envelop were viable. After transduction the cells were passaged once at low density in order not to need to be passaged again, so that colonies derived from single cells were obtained. Since ovine ESCs had not been derived yet, I grew the cells in mouse ESC medium or in human ESC medium to test which was more suitable for the maintenance of pluripotent cells in sheep. Colonies were derived in both media, but the morphology was different: in mouse ESC medium the ovine colonies were granulated, while in the human ESC conditions the colonies looked more mouse ESC-like. In another study iPSCs were preferentially grown in FCS, because a higher number of AP-positive colonies were observed when ovine iPSCs were grown in these conditions (Li, 2011); however it is important to bear in mind that, although AP is a marker of pluripotent cells, it can also be found in other differentiated cell types (Weiss, 1986), so it cannot be

employed as the only marker to discriminate between fully and partially reprogrammed cells. Indeed this assumption is supported by the fact that the ovine iPSCs isolated by Li et al were not able to maintain their pluripotency without the sustained expression of the inducible transgene and could differentiate only upon withdrawal of drug employed to induce the reprogramming factors.

The cells growing in the human ESC medium were picked, expanded and tested for the main feature distinctive of ESCs and iPSCs. The characterisation was initially performed *in vitro*: ovine iPSCs displayed a morphology and a population doubling time similar to those of mouse ESCs; even after many passages they were able to retain a normal number of chromosomes, did not change their morphology and did not senesce, characteristics all typical of ESCs. The staining against markers of pluripotency revealed that some of the colonies were positive to both Nanog and AP, some others only to one of the two markers, but none of them was consistently positive to the surface markers SSEA1 or SSEA4. These last markers were chosen to understand whether the ovine iPSCs were closer to human or mouse ESCs, since SSEA1 is specific for the former, while SSEA4 for the latter. Although mostly negative for the two markers, some of the clones displayed a few cells positive for them, with a higher amount of cells positive to SSEA4, suggesting that, albeit ovine iPSCs have a morphology resembling that of mouse ESCs, their expression profile may be more similar to that of human ESCs.

After verifying that the transgenes were integrated into the different ovine iPSC lines, a RT-PCR was performed on ovine iPSCs to evaluate whether the reprogramming factors were still expressed: 4 out of 8 clones still expressed all 4

genes, while in 2 clones only one transgene was silenced, with the remaining 2 clones only expressing Oct4 (whose level was extremely low in the G2 cell line). Since the other clones had a strong expression, we supposed that the weak Oct4 expression found in G2 cells may be derived from only few cells that were not reprogrammed and strongly expressed the protein, even though we cannot exclude that all cells still expressed Oct4 at low level. Silencing of the transgenes is considered the final proof that full reprogramming had happened, at least in mouse ESCs. Silencing of the transgenes had not been accomplished in ovine or in porcine species so far.

The ovine cells were allowed to spontaneously differentiate *in vitro* in suspensions for about 5 days, until large EBs developed. The EBs were then plated and the outgrowths were allowed to spontaneously differentiate. Immunocytochemistry showed that the ovine iPSCs were able to give rise to cells belonging to the three germ layers, even though the differentiation potential was reduced when compared to mouse ESCs: for example beating cardiomyocyte which I had observed during mouse ESC and iPSC differentiation, were not seen for ovine iPSCs and I could not detect any troponin T staining.

7.1.4 *In vivo* characterisation of ovine induced pluripotent stem cells

The *in vivo* differentiation confirmed the *in vitro* data; teratomas generated by the injection of the ovine iPSCs formed primitive structures in some areas, yet the neoplastic tissue was mainly very naïve; on the other hand the control experiment performed employing mouse ESCs showed the formation of a wide variety of cells

belonging to different tissues, confirming the assay was effective. Although looking mostly embryonic, immunohistochemistry confirmed that our ovine iPSCs are able to differentiate along the three germ lineages and are therefore pluripotent.

Since no literature is available on ESCs from sheep and only two studies have been published on ovine iPSCs, we cannot determine if the limited potential of our iPSCs compared to that of mouse cells depends upon a not complete reprogramming of the cells, wrong culture conditions applied to the differentiating cells or it is intrinsic of pluripotent cells in sheep.

Finally, ovine iPSCs were injected in early stage embryos where they demonstrated the ability to contribute to animals, although the contribution of the ovine iPSCs in live born animals was very low. While our ovine iPSCs have a normal karyotype and show features similar to that of ESCs (such as morphology, population doubling time and ability to differentiate *in vitro* and *in vivo*) very little is known about the development of the early embryo in sheep. Growth conditions are likely to play a role in this; for example mouse EpiSCs, grown in a medium similar to that employed for human ESCs, have been shown to seldom contribute to chimeras (Brons, 2007; Tesar, 2007). Optimization of culture conditions for the maintenance of ovine iPSCs may be pivotal for the achievement of a higher contribution of the cells to chimeric animals.

7.1.5 Comparison with induced pluripotent stem cells from other species

The establishment of pluripotent cells from species different from mouse has remained an elusive goal for many years. Human ESCs were derived in 1998, about

20 years after the generation of the mouse counterparts, while the isolation of rat ESCs was reported only 3 years ago. The establishment of authentic ESCs from ungulates has never been achieved; the derivation of pluripotent cells from those animals has been hampered by the costs and the technical challenges of the embryo production in these species: while many mouse embryos can be obtained in a short amount of time and with relative low expenses, the *in vitro* fertilization necessary to obtain blastocysts from these commercially-important livestock species is a costly process, the number of embryos is limited and the timing is longer.

The generation of iPSCs has circumvented the need for a large number of animals and is seen as a useful alternative to the blastocyst-derived pluripotent cells. The first report describing the derivation of iPSCs from ungulates was published in 2009 (Esteban, 2009), 3 years after Takahashi and Yamanaka's original study. Esteban and his group transduced porcine foetal fibroblasts (PFFs) isolated from Tibetan miniature pig with MoML viral vectors carrying the four original reprogramming factors; they employed three different media (mouse ESC medium, human ESC medium and a mixture of the two conditions) and at day 8-10 observed that colonies were generated in all conditions, but at day 16 these colonies were maintained only in human ESC conditions. These pig iPSCs showed human ESC-like morphology (colonies with clear-cut borders and with flat cells). They were positive to AP, SSEA4, Nanog, Rex1, endogenous Sox2 and Lin28 and had an increased TERT expression compared with the parental cells. Esteban's iPSCs were also able to give rise to teratomas, but did not achieve the silencing of the transgenes.

Esteban's study was shortly followed by other three reports employing a similar approach (Ezashi, 2009; Wu, 2009; West, 2010): cells were transduced with lentivirus carrying different combinations of the reprogramming factors: the four original factors in Ezashi's study, the four factors plus Nanog and Lin28 in West's report and both combinations in Wu's work. The iPSCs derived were maintained in human ESC cell culture medium.

The cells generated by the different groups did not have identical phenotypes, at least in terms of morphology and markers. The porcine iPSCs generated by Wu et al were positive to SSEA3, SSEA4 and Tra-1-81, but negative to SSEA1, while the cells generated by West et al were mainly negative to both SSEA4 and Tra-1-81. Esteban's cells also were negative to SSEA4, while Ezashi et al only stained for Oct4, Nanog and Sox2.

Notwithstanding these differences, all cell lines were able to differentiate along the three germ layers and one group also reported the production of chimeric offspring (West, 2010); however, no groups were able to maintain the cells in an undifferentiated state without continued expression of the reprogramming factors.

Early this year the first description of ovine iPSCs was reported (Bao, 2011; Li, 2011) by two groups employing different combinations of the pluripotent factors: Bao et al utilised Oct4, Sox2, cMyc, Klf4, Nanog, Lin28, SV40 large T and hTERT and stated in their hands it was not possible to generate the ovine iPSCs using only the four original genes; however shortly afterward, Li et al reported the establishment of ovine iPSCs employing only cMyc, Klf4, Oct4 and Sox2.

Bao's cells were positive to many markers of pluripotency (AP, Oct4, Nanog, Sox2, Rex1, SSEA1, TRA-1-60, TRA-1-81 and E-cadherin); Li's iPSCs were positive to AP, Oct4, Sox2, Nanog and SSEA4. The morphology of the cells reflected the expression pattern of the surface markers: Bao's cells, which were positive to SSEA1 and mouse-like, while Li's cells, positive to SSEA4, had a morphology more similar to that of human ESCs. Both groups demonstrated that the iPSCs were capable of cellular differentiation *in vitro* and *in vivo*. However like porcine iPSCs, maintenance of the cells in an undifferentiated state required continual transgene expression.

The iPSCs I have generated have a morphology similar to that of mouse ESCs, stain for AP and Nanog, but only few cells are positive to SSEA1 and SSEA4. These cells are also able to give rise to cells belonging to the three germ layers both *in vitro* and *in vivo* and can contribute to live animals when injected into early stage embryos. Moreover, in contrast to the previous reports, my ovine iPSCs were able to substantially silence the transgenes.

Likewise porcine iPSCs, ovine iPSCs generated by the different groups express dissimilar markers of pluripotency; yet they are able to differentiate towards all lineages *in vitro* and *in vivo*. The reason may stem from the different breed of the animals employed, from the stage of the parental cell lines (adult versus foetal) or from the differences of the medium utilised for the derivation and the maintenance. Also horse iPSCs have been reported (Nagy, 2011). Equine iPSCs are positive to AP, Oct4, Nanog, SSEA1, SSEA4, TRA-1-60 and TRA-1-81, but only one report is

available, it is not therefore possible to estimate whether cells derived by different groups in this species have distinct expression profiles as happens in pig and sheep.

As said above, iPSCs from ungulates able to give rise to chimeras have already been established (West, 2010); however, they were derived from porcine mesenchymal stem cells and employing six reprogramming factors (the original cMyc, Klf4, Oct4 and Sox2 with the addition of Nanog and Lin28). We now report the first demonstration that iPSCs derived from a differentiated non rodent cell type (fibroblast) by employing the four Takahashi and Yamanaka's genes can contribute to live born following blastocyst injection.

Since ESCs from sheep have not been established yet, these ovine iPSCs hold great promise for the understanding of how pluripotency functions in species other than mouse, rat and human. They may also allow the production of transgenic modified animals which could be employed to study disease which are not reproducible in mouse or simply for agricultural purposes.

7.2 Conclusions

In conclusion we demonstrated that the Takahashi and Yamanaka's protocol for the establishment of iPSCs in mouse (Takahashi and Yamanaka, 2006) can be successfully applied also to the sheep. Indeed we have generated ovine iPSCs which have a morphology and a population doubling time similar to that of mouse ESCs, stain for markers of pluripotency and can differentiate along the three lineages both *in vitro* and *in vivo*. Those cells have maintained their pluripotency and a normal

karyotype over more than 24 passages and were able to contribute to live animals after injection in ovine blastocysts, albeit at low level.

Although many assays were performed to characterise the ovine iPSCs generated, numerous other tests should have been carried out in order to achieve a better depiction of the features of the cells. An important trait in iPSC establishment is the ability of the differentiated cells to reactivate the expression of the endogenous counterparts of the exogenous factors upon reprogramming. While the immunocytochemistry would not be able to discern between viral and cellular genes, a RT-PCR specific for the ovine sequences could be easily performed.

Microarrays comparing the whole gene expression profile of the ovine iPSCs to the parental OFFs, to the ovine ICM and to mouse ESCs would give an interesting data, useful to evaluate whether the generated ovine iPSCs are similar to the ovine pluripotent cells in the embryo and have characteristics comparable to ESCs in mouse.

The generation of iPSCs from sheep hold great promise for the understanding of the mechanisms at the bases of pluripotency in species other than mouse, rat and human; beside, ovine iPSCs can be exploited for the generation of animal models for human disease which do not have a correspondence in the species normally employed for this purpose.

Although the establishment of ovine iPSCs able to contribute to chimeras is a significant step forward, the generation of iPSCs with higher ability to contribute to

the offspring would be desirable in order to have a more efficient platform for the derivation of transgenic animals for research and medical purposes.

Increasing the efficiency of iPSC generation through the optimization of the current protocol or the use of a lentiviral-mediated protocol could be useful; however the generation of ovine iPSCs by employing integrating viral vectors to drive the expression of the reprogramming factors does not allow full exploitation of the potential of the cells either in research or in the clinical field. Ovine iPSCs derived without the use of integrating vectors or even with proteins may provide a valuable resource for transgenesis.

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Appendix

Ovine induced pluripotent stem cell response to mouse LIF

Introduction

Ovine iPSCs were established and maintained on SNL feeder layer. SNL cells are STO cells (immortalised MFFs) that were genetically modified in order to express mouse LIF and neomycin phosphotransferase (McMahon and Bradley, 1990). As reported in the general introduction, LIF is the key cytokine for the maintenance of mouse ESCs in their pluripotent state, I therefore aimed to understand whether LIF produced by the feeder layer played a role in the pluripotency of the ovine iPSCs I have generated.

Results

Through RT-PCR I first confirmed that the SNL cells expressed LIF (Fig. 50a). I then grew the ovine iPSCs on SNL feeder layer or gelatin, in order to address whether the iPSCs undergo differentiation without supplementation of LIF (Fig. 50b). Ovine iPSCs maintained on SNLs retained their pluripotency, while the same cells growing on gelatin showed a strong weakening of AP staining, which is the first signal of cell differentiation.

The addition of exogenous LIF, which rescued mouse ESCs growing on gelatin, did not produced the same outcome on the sheep cells. This suggests that the mouse LIF does not have an effect on the ovine iPSCs.

In order to confirm that LIF is not able to trigger a response in sheep I performed an induction assay: as already mentioned in the general introduction, LIF fulfils its role by binding to the heterodimer composed by LIFR and gp130, which in turn induces STAT3 phosphorylation; therefore measuring the proportion of phospho-STAT3 levels provides an indirect assay for whether LIF is able to interact with the cells.

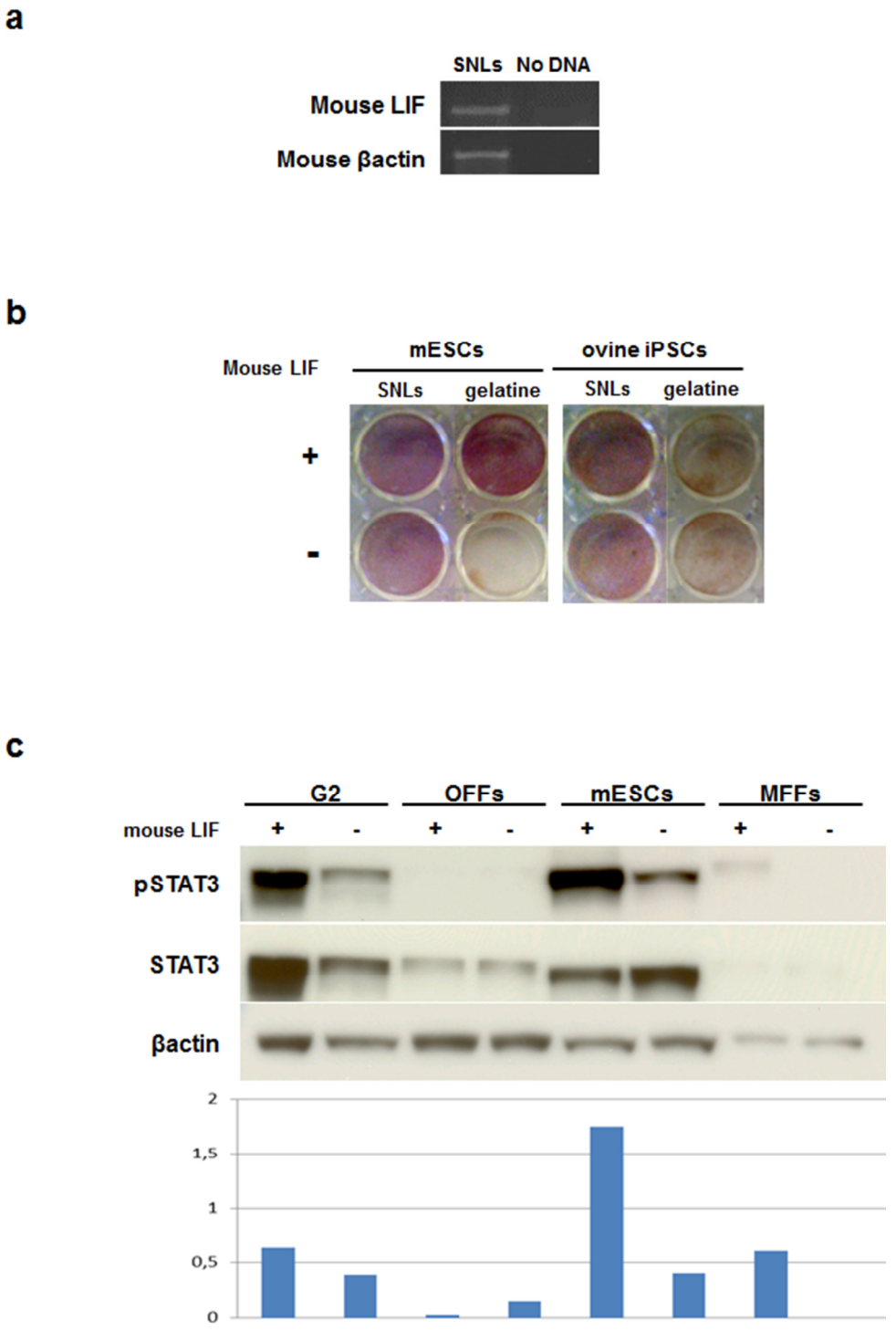

Figure 50. (a) RT-PCR showed that SNL feeder cells express LIF; no band was detected in the no template control (NTC). (b) LIF withdrawal assay: ovine iPSCs were grown in human ESC medium on SNL feeders or gelatin; the cells were cultured both in presence or absence of exogenous LIF and mouse ESCs were employed as control. (c) LIF induction assay: ovine iPSCs and OFFs were starved for 4h and induced with LIF for 10min, the cells were then lysed in the wells and collected; the lysate was employed in a western blotting where phospho-STAT3 levels were evaluated, while STAT3 and βactin were utilised as loading controls. LIF-induced mouse ESCs and MFFs represented the positive control, while the same cells non-induced were the negative control.

A STAT3 induction assay was performed on ovine iPSCs and OFFs: the cells were initially starved for 4h with medium depleted of bFGF and SR; then the medium was replaced with induction medium, which was composed of starving medium with the addition of 1000U/ml of mouse LIF. For each cell line a non-induced negative control was performed. The Western blotting (Fig. 50c) against STAT3 and phospho-STAT3 (pSTAT3) revealed that the assay had worked: indeed both positive controls (mouse ESCs and MFFs) strongly increased the proportion of pSTAT3 upon treatment with LIF. The OFFs showed a very low amount of STAT3, which was consistent with the result obtained in MFFs; in contrast to the mouse, however, the LIF-induced OFFs did not display any rise in the phospho-STAT3 proportion compared to the untreated controls. This might suggest that ovine cells do not respond to mouse LIF. Yet the result obtained with the ovine iPSCs was unexpected: while no difference was detectable in the phospho-STAT3/ STAT3 ratio between induced and non-induced cells, the induction increased the amount of both phosphorylated and unphosphorylated proteins.

Conclusions

While SNL cells truly expressed LIF and were able to maintain mouse ESCs proliferating without loss of phenotype, the LIF withdrawal assay demonstrated that that ovine iPSCs do not respond to it. A STAT3 induction assay was performed in order to confirm this data, but, instead, generated an unpredicted result: while mouse cells (ESCs and MFFs) and OFFs reacted as expected, the ovine iPSCs did not: while the proportion of phospho-STAT3 over STAT3 was not different in induced or non-induced ovine iPSCs, the use of an additional loading control (βactin) indicated that both protein populations underwent a considerable increase. Since only one replicate

of the assay was performed, this may be the result of errors happened during the induction assay, even though the other cell lines responded as expected; furthermore, also the βactin control of the induced iPSCs did not show any anomaly, nor did the non-induced ovine iPSCs.

The antibodies did not present any issues either, since they successfully worked for the other cell lines, nor could it be a species matter, since the antibodies stained the OFFs and the non-induced iPSCs as expected.

In some cell lines STAT3 autoregulates its own expression, acting through a composite response element in its promoter that contains a STAT3-binding element (Narimatsu, 2001). It is therefore possible that in ovine iPSCs LIF does not induce the phosphorylation of STAT3, but its synthesis.

Since it is demonstrated that also the unphosphorylated form of STAT3 can drive a wave of gene expression (Yang, 2005), our data do not allow us to conclude whether the mouse LIF produced by the SNL feeder layer influences the pluripotency of the ovine iPSCs. The assay should be repeated in order to exclude a possible error during LIF induction and to confirm that the STAT3 synthesis is truly upregulated after cytokine treatment.

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