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"Manipulation of signalling to promote reprogramming of pig cells into a naïve pluripotent state"

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Contents

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ABSTRACT

For years and years, attempts have been made to isolate porcine embryonic stem cells (ESCs). Although porcine ESCs would be invaluable, – since the pig represents an attractive large animal model that could be used in human medicine , derivation of authentic porcine ESCs has proven to be elusive. Induced pluripotent stem cells (iPSCs) provide a unique resource of pluripotent cells and are a useful alternative to embryo-derived cells. The Burdon lab has generated pig iPSCs by transducing foetal fibroblasts with retroviruses expressing c-Myc, Klf4, Sox2 and Oct4. The resulting lines expressed core pluripotency factors and embryoid bodies could be formed. Interestingly, all of the pig cell lines the Burdon lab has screened retained expression of the four retroviral transgenes, which consequently indicates that these cells are not fully reprogrammed. Lack of transgene silencing is thought to be indicative of incomplete reprogramming. It has previously been reported that culturing mouse and human stem cells at a low oxygen concentration instead of the atmospheric 21% oxygen concentration, may also aid reprogramming. The aim of this project is to investigate whether manipulation of signalling by modifying the culture conditions will enhance reprogramming of porcine iPSCs as well. The major finding from this project is a consistent difference in the growth rate between the two O_2 concentrations. Additional differences between 5% and 21% O_2 – which were likely caused by the O_2 concentrations – were exclusively found in cell line A1 and included a difference in colony morphology, AP-staining and the appearance of the plated EBs.

1. INTRODUCTION

Pluripotency is defined as the ability to make all cell types found in the body[1, 2]. Embryonic stem cells (ESCs) represent the gold standard for pluripotency[3]. ESCs are unique because of their continuous, long term self-renewal capacity without loss of their stable karyotype and their potential to differentiate into cell types of all three germ layers, in vitro as well as in vivo (provided that the appropriate culture conditions are applied)[2, 4-7].

1.1 Applications of embryonic stem cells

The discovery of ESCs resulted in a tremendous scientific and public interest in these cell types and from then, the research field concerning ESCs rapidly expanded[7, 8]. But why are we so interested in ESCs?

Firstly, an ESC has the potential to proliferate for an unlimited period of time and lends itself for precise genomic modifications, like the introduction of gene knock-ins or knock-outs in cells (often established through homologous recombination)[2, 4, 5]. Secondly, the modified cells can be differentiated in vitro into any cell type. By generating knock-in or knock-out cells and letting them differentiate, the function of certain genes and their encoded proteins could be accurately studied and a population of cells with specific desired properties could be created[2, 4, 9]. These genetically manipulated cells could then be used in multiple applications including the basic study of developmental biology and (early) embryogenesis, processes involved in nuclear reprogramming, regulation of the differentiation processes, pharmaceutical drug screening and a variety of diseases[2-4, 6, 10-12].

Furthermore, genetic engineering of pluripotent stem cells derived from *animals*, could lead to the production of genetic animal models for several human diseases, including genetic disorders, cancerous diseases and infections[3, 4, 6]. These genetically modified (GM)animals are made through germline transmission (NB. The term 'germline transmission' is explained later in this report).

Additionally, certain transgenic animals are able to produce and secrete proteins in their organs or body fluids, which are of pharmacological interest in human medicine[4].

Currently genetically engineered animals – especially the pig – are not only used to produce relevant proteins, but even cells, tissues or whole organs, which are then transplanted back into humans (xeno-transplantation)[4].

Using pluripotent stem cells from animals could also be relevant in commercial agricultural branches, since animal production traits – like growth rate, reproductive performance and feed utilization – , animal products - like milk and carcass composition – and disease resistance could be improved using ESCs[2, 4, 6, 13, 14]. In short, animals with desirable traits could be generated.

But probably the most important reason that explains the interest in ESCs, is that pluripotent stem cells could potentially be used in human medicine[2, 6]. Human ESCs could be a particular

important tool in transplantation medicine; they would for instance be useful in innovative gene therapies or cell replacement therapies like cell therapy, regenerative medicine or tissue repair[2, 4, 6, 12].
Quicker generation of GM animals or animal models and more precise modifications are some of the advantages of using ESCs in this research field.

1.2 Derivation and maintenance of rodent and primate ESCs

To date, authentic embryonic stem cells have only been successfully established from rodent species and primates[2, 12, 13].
Mice were the first species from which derivation of embryonic stem cells was described, in 1981[15]. From then it took less than two decades, till the first ESCs from (rhesus) monkeys were isolated, followed shortly thereafter by the isolation of human ESCs, in 1998[16, 17]. Most recently, ESCs have been generated from the rat[18, 19].
It applies to both rodents and primates that ESCs are predominantly isolated from either the inner cell mass (ICM) or – in the slightly older embryo – from the epiblast of pre-implantation stage embryos[4-7]. The epiblast is the structure that will ultimately give rise to the entire foetus itself[6, 20]. Nevertheless, isolation of ESCs has also been established from earlier embryonic stages[12].
Substantial differences exist between ESCs from those two best studied species[7].
To start with, morphological differences are evident. Mouse ESCs (mESCs) strongly resemble the cells from the pre-implantation embryo, while human ESCs (hESCs) are more closely related to the late epiblast stage of the post-implantation embryo[21].
Colonies formed from mESCs are tightly packed, show well-defined borders and have a three-dimensional dome-shaped appearance[11]. Cells within these colonies rapidly proliferate[7].
hESC colonies, on the other hand, grow in a two-dimensional monolayer-way, which causes their colonies to be more flattened than mESC colonies, and the proliferation rate is slower[7].

Apart from their morphological characteristics, marked differences also exist in their culture medium requirements[2, 7, 22]. The existence of differences in the culture condition needs is explained by the differences in dominant signalling pathways[7]. For instance, in rodent species, the LIF-JAK-STAT signalling pathway is most important and mESCs therefore require the addition of leukemia inhibitory factor (LIF)[2, 7, 8, 22, 23]. It has been shown that mouse embryonic fibroblasts (MEFs) – the cells commonly used as a feeder layer for the culture of rodent ESCs – are responsible for the production of LIF, which is a cytokine of the IL-6 family[24]. Because, mESCs are LIF-dependent, co-culture of mESCs with MEFs originally lead to the establishment of authentic ESCs[20]. For mESC culture and maintenance to be successful, mESCs should be co-cultured with MEFs[4]. LIF acts through the activation of the transcription factor STAT3, and is thus responsible for the stimulation of self-renewal and the inhibition of differentiation or maintenance of pluripotency in pluripotent stem cells of mice/rodents[4, 10, 20, 23]. In addition, LIF is also known to activate the RAS/MEK/MAPK pathway which paradoxically promotes differentiation in mESCs[7]. The possibility of 'feeder free culturing' of mESC (in the absence of LIF-producing MEFs) has also been described, and is successful as long as LIF is added to the culture medium[4, 5, 11, 24].
In contrast, human pluripotent stem cells do not need the addition of LIF (LIF-independent), but rely on Activin and fibroblast growth factor (FGF2) instead[23, 25]. In hESCs the MEK-ERK pathway plays a dominant role and therefore these cells require Activin and FGF2 (also known as bFGF)[2, 7, 8, 22].
A final remarkable difference between mESCs and hESCs is the profile of expressed markers of pluripotency. This will be discussed in *paragraph 1.4*.
NB. In addition, intra-species variation is also likely to exist. For instance: the ease of isolation of ESCs varies significantly from one mouse strain to another, suggesting the involvement of a strong genetic component[4].

Intermezzo 'The distinction between mouse ESCs and EpiSCs'

Recently, a new class of murine stem cells has been defined, the epiblast stem cells (EpiSCs)[21, 26]. It is thought that the features of hESCs are comparable to those of the EpiSCs rather than to those of mESCs[21].
EpiSCs are heterogeneous cell lines obtained from the post-implantation embryo, under conditions parallel to hESCs isolation conditions [7, 20, 23, 27]. EpiSCs express some pluripotency markers and are capable of embryoid body and teratoma formation, but unlike true ESCs, they fail to produce chimeras[23, 28]. Furthermore EpiSCs express certain markers indicative of differentiation (like Brachyury) and lost expression of a few early embryonic (pluripotency) markers, which both indicates that EpiSCs are in a more differentiated state than ESCs[23]. Like hESCs, EpiSCs form flattened colonies, are not tolerant to passage as a single cell, depend on Activin/bFGF signalling rather than LIF signalling, proliferate slower than mESCs, and have one inactivated X-chromosome[7, 28-30].
ESCs turn into EpiSCS upon withdrawal of LIF in combination with supplementation of Activin/FGF to the feeder free culture medium[23, 28]. The opposite has also been reported: when EpiSCs are switched to a culture system including feeders and LIF-supplemented medium, then these cells convert into ESCs[23, 28].
Because of all the features mentioned above, it has been suggested that EpiSCs are in a 'primed' state of pluripotency[28-30]. Authentic ESCs on the other hand, are believed to be in a naïve state of pluripotency[28-30].
Because of the similarities between hESCs and EpiSCs, it could be possible that cells currently termed 'hESCs' are in fact also in a primed state of pluripotency, and are yet incompletely pluripotent[29].

1.3 The search for the appropriate animal model

As stated above, one of the most promising tools of ESCs is their potential use in human medicine[2].
Many countries have laws impeding the use of human cells and embryos to a greater or lesser extent[11, 24]. Understandably, the study and use of authentic hESCs for various implications, is limited for ethical and moral reasons, but this seems to contradict the goal of using human pluripotent stem cells in medicine branches[10, 14]. Apart from the fact that using human embryonic tissue for research is considered unethical, isolation of ESCs from embryos is technically difficult and scarcity of human oocytes and embryos would also be a matter of concern[8, 10, 24]. In addition, before these cells could be used for human medical treatment in a clinical setting, it needs to be thoroughly tested that treatment with pluripotent stem cells would be safe and efficient[2, 3, 6, 10, 12].
As a solution for the difficulties just mentioned, it is possible to use animals and animal ESCs as a model for humans, provided that these animals are so similar to humans, that the information obtained from animal experiments could be extrapolated. In pluripotent stem cell technology, rodents are very often used as experimental models; especially mice are well-studied [3]. Indeed, mice and rats are perhaps the most common and simplistic models to study the basic principles of reprogramming[3]. But the rodent species seems to be an inappropriate model for humans since profound differences between rodents and humans exist[6, 12]. Important differences include the life span and whole animal physiology[6, 31].
Naturally, non-human primates would be a very reliable model for human medical research because of great (phylogenetic) similarities between the two species[3, 6, 8]. Nevertheless, monkeys are expensive and the use of monkeys also raises some ethical concerns[3, 8]. Besides, monkeys normally need to be imported and are rather hard to maintain and breed, which also make this species less suitable[3, 8, 31].
The porcine species represents an attractive alternative large animal model that could be used for preclinical trails[3, 5, 6, 8].
For a long time, the pig has been used as an animal model in many branches of human medicine[6, 13, 22, 31]. For instance, porcine insulin has been used worldwide as a treatment for diabetes[3, 22, 31]. The pig is also a very important species in respect to xeno-transplantation, a technique whereby an organ of one species is transplanted into an individual of another species. Accordingly, porcine organs – like heart valves and skin – have been successfully transplanted into humans for many decades now[3, 22, 31]. Regarding its similarities with humans in size, organ function and morphology, whole animal physiology and immunology, the pig would be a rather reliable

experimental model in pre-clinical applications[2, 5, 6, 12-14]. The pig is useful as well because of its longevity (18-25 years), which permits more targeted experiments and makes it possible to evaluate the course of a transplant over a longer period of time and investigate the long term effects of therapies[2, 3, 5, 6, 8, 13, 31].
Compared to monkeys, pigs are better affordable and ethical issues are less relevant in porcine species since pigs are an agricultural commodity, and commonly used for human consumption on a large scale[8, 22].
In addition, in contrast to primate embryos, porcine embryos are abundant[22].
Finally, by using pig cells, improvement of pig farming might be established as well, as just discussed in *paragraph 1.1*. This could naturally be beneficial from the agricultural point of view[2, 6, 8].

1.4 Pig ESCs: invaluable but unavailable

For years and years, attempts have been made to isolate ESCs of species other than rodents and primates[2, 3, 8]. After the generation of authentic ESCs from non-human primates (i.e. rhesus monkey), research groups from all over the world attempted to derive ESCs from a wide range of other species, such as the cat[32], and dog[33], horse[34], rabbit[35] and hamster[36], mink[37] and livestock species like the goat, sheep and cattle [38, 39] . Yet, derivation of ESCs from additional species – also including ungulates like the pig – has proven to be elusive . For reasons mentioned above, derivation of pig ESCs, would be of great value.
Although many reports have described the generation of ESC-like or putative ESC cultures from species considered as 'difficult' or 'non-permissive', so far no cell type has been produced from these species which has the ability to proliferate continuously nor the capacity of full pluripotency[10]. These ESC-like cultures did not meet the full criteria or were characterized as ESCs using vague criteria, and could therefore not be defined as true ESCs[2, 5].
Nine years after the derivation of mESCs it was claimed by Evans et al. (1990) that the first porcine ESCs (pESCs) were isolated from day 7-9 in vivo produced hatched blastocysts[40]. The colonies produced showed some similarities with mESCs. But unfortunately, ill-defined and unreliable (mainly morphological) criteria were used in order to characterize these cell cultures in vitro and in vivo[5, 6] From then, other research groups also tried to generate pESCs, with a similar lack of success [2, 3, 22].
From the initial studies performed on porcine ESC-like cells, it was concluded that despite the fact that colonies of porcine ESC-like cells could be established, these colonies could not exactly be characterized – thus true pluripotency could not be proven – and they could not be sustained in vitro for a long term[2, 22].
The main problem encountered in deriving, culturing and sustaining porcine putative ESCs (ppESCs) seems to be the observed loss of pluripotency after only a few passages[9].
However, the reasons for the difficulties regarding the derivation of true ESCs from early embryos of livestock species, like the pig, are largely undefined[5, 13].
The three main factors that might explain the capricious, poor research outcomes are as follows:

1) Early embryonic development;

As previously discussed, stem cells from the rodent and primate embryo, are principally derived during the pre-implantation stage from an embryological structure known as the epiblast[2]. However, the events that occur during early developmental biology differ considerably between different species[2].
In mice, for instance, the actual implantation is a rapid process[12]. But when the pig is scrutinized, one will find that the pre-implantation stage of porcine embryos is relatively long compared to the pre-implantation stage of mouse and human embryos[2, 6, 8, 22]. During this prolonged pre-implantation stage, the conceptus stretches, a characteristic relatively extensive expansion of the trophoblast takes place and the pig epiblast develops significantly later (not until hatching) than the epiblast in mice and human embryos (*Figure 1*)[6, 8, 22]. In addition, it takes four days for the porcine epiblast to fully differentiate thus formation of the epiblast in the pig is

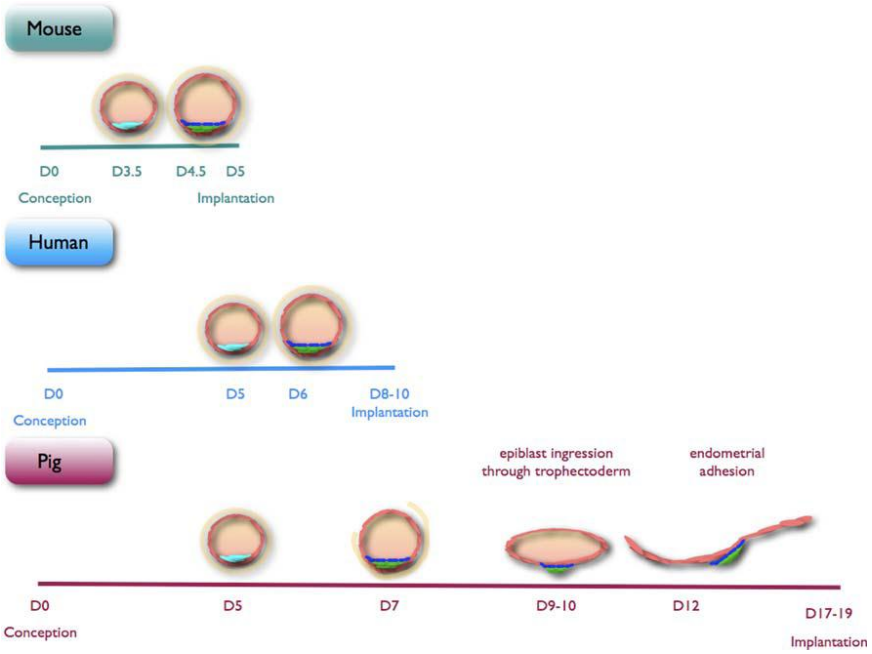

Figure 1. Pre-implantation stage in mouse, human and pig embryo [22]

also a slower process and the epiblast structure persists much longer[2, 22]. As a result, contaminating cell types – like cells from the trophoblast or primitive endoderm – could be isolated together with the actual ESCs and these cells could eventually overrule outgrowth of ESCs [2, 5, 11].

In conclusion, the porcine epiblast is available for a protracted period of time, which makes it rather difficult to decide what exact moment is best to obtain ESCs from the porcine embryo[2, 6, 8]. The choice of the wrong stage of pig embryo development could thus be an important factor[5, 31, 41].

2) Cell culture conditions;

For ESC culture and maintenance to be successful, it is absolutely essential that the culture conditions support the growth and maintenance[11]. The culture of ppESCs has largely depend upon adopting culture strategies used in rodents and primates, since little information is available on media for pig cell culture[2, 6, 8].

It has been concluded that pESCs need a feeder layer for their survival and growth, even if LIF is added to the culture medium [5, 6]. In previous studies it has been investigated whether culturing ppESCs on a feeder layer of porcine embryonic fibroblasts (PEFs) or porcine uterine cells (PUCs) – instead of the generally used mouse embryonic fibroblasts (MEFs) or STO-cells (a murine embryonic fibroblast cell line, frequently used for culturing mESCs) – would have a beneficial effect[2, 6, 22]. It was suggested that common heterologous feeders could be responsible for the failure of sustaining ppESCs because of the evolutionary gap between murine and porcine species[2]. Indeed, the type of growth factors secreted by MEFs and STO-cells or their binding capacity to porcine receptors could have limited the successful support of porcine cells. Nonetheless, the studies performed showed that there appeared to be no clear advantage of the autologous PEFs or PUCs over heterologous MEFs or STO-cells [42-46]. Nevertheless, it might be possible that alternate feeder types or an adapted density of feeders could improve the support of ppESCs, but further research is needed[2].

Moreover, it is not clear yet, what signalling pathways are involved in the regulation of pluripotency in pig cells[8].

It has been shown that signalling pathways differ significantly between mouse and human ESCs and thus part of the debate surrounding the culturing of pig ESCs focuses on the

question as to whether porcine cells need addition of specific growth factors to their culture medium, and if yes, what type of factors[6, 22]. Although on-going research is being carried out, it is not entirely clear which supplements are essential and which are dispensable in culturing pluripotent pig cells, and it has to be stated that most of the protocols used in pig cell culturing to date are extrapolated from murine experiments[6]. As just discussed, at the moment, mouse feeder cells are the most common cell type used as a feeder layer in culturing pig cells[6]. These feeders are used in combination with medium containing a variety of supplements, like for instance LIF, FGF2 (bFGF), or EGF[6]. Different studies report various kinds of supplements. From earlier reports it appears that the addition of LIF or FGF2 (bFGF) alone to the culture medium is not sufficient to prevent differentiation of pluripotent pig cells[5, 6]. It should be noticed that the role of LIF varies among different species[24].

It is very likely that lack of knowledge about appropriate culture conditions is involved in the failing of sustaining growth and pluripotency of the ppESCs[5, 31].

3) *The lack of specific markers exclusive for pESCs*;

One of the first molecular markers demonstrated to identify ESCs in sheep and pigs, was alkaline phosphatase (AP)[6]. AP is a common marker indicative for pluripotency; expression is observed in early embryonic stages and is lost as soon as the cells differentiate[12].

Additionally, in order to identify authentic ESCs in mice and primates, three important proteins are used, namely Oct4, Sox 2 and Nanog[2, 6, 8]. These proteins seem to be exclusively expressed in ESCs of these species, and are therefore trustworthy markers of ESCs[2, 6]. In porcine species, on the other hand, in has not been confirmed that Oct4, Sox2 and Nanog could be used as reliable markers to recognise true ESCs[5, 22]. For instance, in mice it has been proven that the expression of Oct4 is restricted to the ICM of the embryo, in contrast to the expression in the porcine embryo[6]. In the pig, Oct4 (mRNA as well as the protein itself) can be detected in the ICM but also in the oocyte and in trophectoderm, during all stages of embryonic development prior to implantation[2, 6, 12]. During embryonic development, Oct4 expression is down-regulated and expression of β-tubulin III – which is one of the markers of neural differentiation – replaces Oct4[6]. Only at the point of hatching, the expression of Oct4 seems to be exclusive to the pre-implantation epiblast[6]. And although expression of Sox2 has been detected in the porcine embryo, it has not been proven that the expression of this transcription factor is exclusive to the inner cell mass or epiblast[2, 22]. Like Oct4, the expression of Nanog – an ESC-specific marker in mice and primates – is not restricted to pluripotent cells in the pig. It has been reported that the protein is also expressed in the porcine trophoblast and in some differentiated cell types like cardiac cells, muscle cells and in ovary[2, 6]. Prior to hatching, Nanog is not even detectable in the porcine embryo[22].

In addition to the transcription factors Oct4, Sox2 and Nanog, certain proteins expressed on the cell surface of pluripotent cells are used to identify pluripotency in rodent and primate cells[2]. These cell surface antigens mainly include Stage Specific Embryonic Antigens (SSEA)-1, -3 and -4, TRA-1-60, TRA-1-80 and TRA-1-81, whereby the expression of SSEA-1 is a characteristic of mESCs, while in human derived ESCs the expression of SSEA1 is absent, and instead the surface markers SSEA-3 and -4, TRA-1-60, TRA-1-80 and -1-81 are expressed[2, 5, 8]. Nevertheless, expression of these cell surface markers seems to be of less use in pig cells, since their expression is not thought to be exclusive to pluripotent cells in this species[2].

The topographic and temporal expression of Oct4, Sox2 and Nanog and the non-specific expression of the cell surface markers just mentioned, indicate that there might be other, more accurate and exclusive factors, which can be used to identify ESCs in ungulates[6, 8, 22]. Among the genetic factors that might be involved is the gene Rex1, but further research is needed to confirm this[6]. However, the

identification of pluripotency (surface) markers and the definition of morphological properties in itself could be problematic since validated, true pECSs – which should be used for comparison – have not been generated yet[8, 13, 41].

1.5 Reprogramming by defined factors: induced pluripotent stem cells

In order to find alternative methods to generate pluripotent stem cells, a variety of methods which could induce nuclear reprogramming and hence pluripotency, has been explored. These include somatic cell nuclear transfer (SCNT) whereby a somatic cell nucleus is transplanted into an enucleated egg, fusion with ES cells and – since a few years – reprogramming by defined factors (*Figure 2*)[1, 8, 10, 11, 24]. Cells produced by one of these three methods are also called ESC-like cells[8].

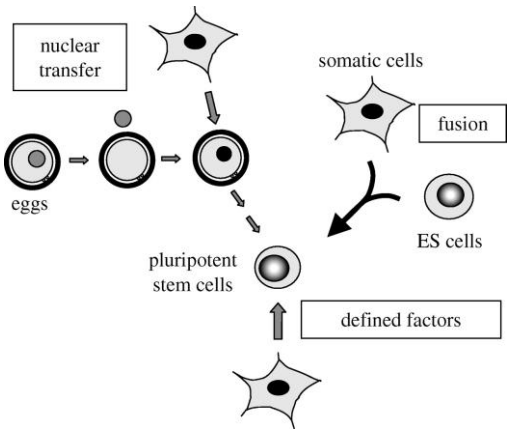

Figure 2. Methods to establish nuclear reprogramming of somatic cells[24]

From the SCNT-experiments and from studies concerning fusion with ES cells, it became apparent that embryonic stem cells must contain factors which are of great importance for the induction of pluripotency (although it has not been determined if these factors reside in the nucleus or in the cytoplasm[1, 24, 47]. In general, the efficacies and cloning efficiencies of the SCNT and ESC fusion techniques are not very high[3, 5, 10, 13]. Therefore it is not likely that these techniques will become commonly applied in the medical industry[10].

In 2006, Takahashi and Yamanaka reported a major breakthrough in the derivation of pluripotent cells: the generation of so-called induced pluripotent stem cells (iPSCs)[48]. iPSCs provide a unique resource of pluripotent cells and are a useful alternative to embryo-derived cells.

The principle of induced pluripotency is based on the ectopic overexpression of exogenous factors, which are abundant in and exclusive to ESCs[5]. iPSCs are derived by means of transduction of only a few defined reprogramming factors into differentiated mature cells, which causes these cells to return to their embryonic stem-cell-like pluripotent state within a limited period of time[1, 49].

In previous years, 24 gene-candidates were identified in Yamanaka's laboratory; a group which was later reduced to only 4 transcription factors[11, 25, 49]. In the original experiments these 4 factors Oct4, Sox2, Klf4, and c-Myc– to date referred to as the Yamanaka factors or the 'original reprogramming cocktail' – were brought into mouse fibroblasts using retroviruses[2, 3, 8, 10, 49].

Oct4 and Sox2 are transcription factors which are expressed in ESCs, germ cells and early embryos[24]. In addition, the level of Sox2 expression is also high in neural stem cells[24] (fresh). Both of the factors are thought to act through the inhibition of genes associated with differentiation, as well as the stimulation of genes involved in the induction of pluripotency[24]. Klf4 might act as a co-factor for both Oct4 and Sox2[24]. Klf4 is a transcription factor which is paradoxically associated with tumour suppression on one side, and oncogenesis on the other side[24]. Finally, c-Myc is a transcription factor involved in important signalling pathways in pluripotent cells, but is – like Klf4 – also associated with oncogenesis and is known to be a potent proto-oncogene[24, 50]. The rapid proliferation characteristic of ESCs, might be explained by the involvement of c-Myc[24].

The main factors commonly used to reprogram the cells include the genes Oct4, Sox2, Klf4, c-

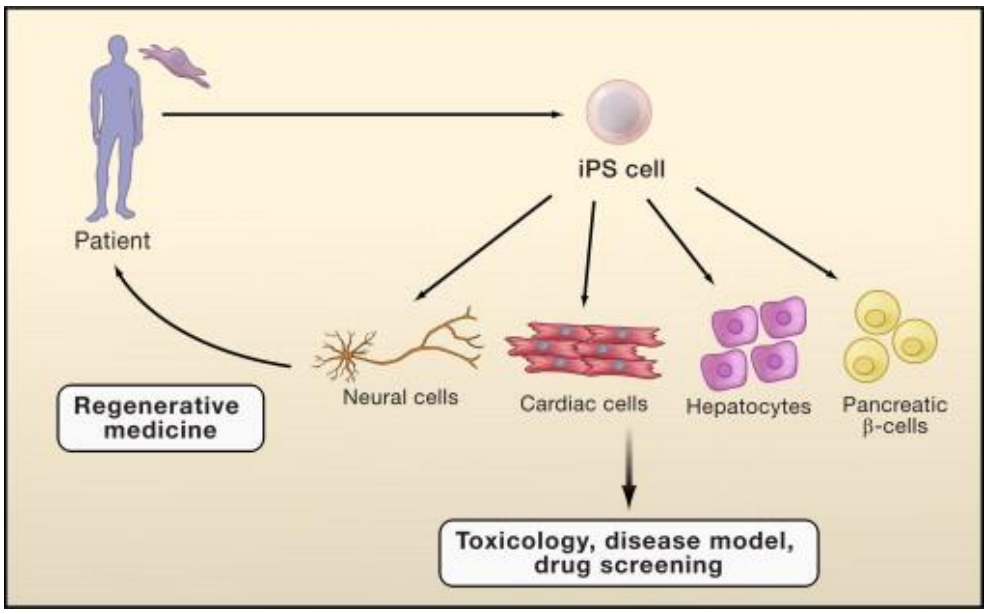

Figure 3. The generation of patient- specific cells from iPSCs, which could be used for many applications, including transplantation treatment[51]

Myc, Lin28 and Nanog[1, 3, 8, 11]. Different reports describe the use of different combinations of reprogramming factors and different cell types, indicating that the method used in Yamanaka's original experiments, is flexible and not unique[1, 3, 8, 25].

The vast amount of potential applications of iPSCs is invaluable[11]. In fact, most – if not all – of the advantageous hopeful potential applications of ESCs, which are listed in *paragraph 1.1* of the introduction, apply to iPSCs as well.

Nevertheless, induced pluripotent stem cells have some important advantages over embryonic stem cells.

First of all, using iPSCs rather than ESCs would circumvent the ethical concerns regarding the use of ESCs – especially those derived from human embryos – and SCNT techniques with human cells[3, 10, 25]. Secondly, the somatic cells used in iPSC-technology are far more abundant than embryo-derived cells[8, 10, 24]. Besides that, the technique will eventually be much less invasive, especially when easily accessible somatic cells, like keratinocytes or skin fibroblasts, would be used[2, 3, 10].

Thirdly, iPSCs could be used to build genetic models for human diseases[5]. Up till now, differentiated cells have been reprogrammed from patients suffering from a variety of diseases, including Parkinson disease, Huntington, Down Syndrome and diabetes mellitus[10].

And fourthly, in theory, when clinically applied in human medicine, highly personalized patient-specific cells could be made from iPSCs since the DNA in iPSCs would be the patient's own[2, 3, 9, 25]. All without the use of embryonic material[11]. This is in contrast to ESCs, in which the genetic background of the donor egg will differ from the genetics of the recipient, and thus immune rejection remains an issue[10, 24]. Naturally, when transplanted cells match perfectly with the patient's cells, the chance of immunological rejection or graft versus host disease – a problem currently encountered after transplantation treatment and commonly solved with strong immunosuppressive medicines – is minimized[6, 9, 24 11, 31]. Therefore, one of the main future implications of iPSCs is the generation of highly specific cells which could be used as a therapeutic tool in transplantation treatment (*Figure 3*)[31]. Future donor cells have to be easily accessible, less likely to contain genetic lesions, and easy to reprogram[2, 11, 31]. Also with regards to xeno-transplantation – which is mainly important in the pig species – , iPSC technology could be meaningful, since genetically manipulated porcine iPSCs could result in the production of prominent tissues which can be transplanted

into humans[3].
And lastly, another potential advantage that needs to be highlighted is the promising use of iPSCs in agriculturally important species; the iPSC-technology might improve the cloning efficiency of SCNT or ES cell fusion experiments[5, 13].

1.6 The key properties of iPSCs

The key properties of iPSCs are practically indistinguishable from those of ESCs[5]. In fact, all of those key properties can be traced back to the two main characteristics of true pluripotent cells, namely their immortal nature in vitro and their capability to differentiate into every conceivable cell type[11]. This means that iPSCs can be maintained over several cell passages without losing their broad differentiation capacity[1]. The rapid proliferation rate of iPSCs is roughly similar to the proliferation rate of ESCs[10, 12, 14].
In respect to morphologic characteristics one can conclude that these are (virtually) identical to ESCs: cell colonies are compact, with each individual cell showing a high nuclear:cytoplasm ratio[1, 4, 11, 12]
The gene expression profile of iPSCs is highly similar to the profile of ESCs[11]. DNA and histone methylation patterns are highly similar between ESCs and iPSCs[1].
Expression of key factors involved in pluripotency, including for instance, the transcription factors Nanog and Oct4 and the glycoprotein surface markers SSEA-1 and SSEA-3 and -4 (in human) and TRA-1-60 and TRA 1-81 (human) are known to be expressed in ESCs and iPSCs[1, 11]. Up-regulation of these markers involves the loss of markers specific to differentiated cells[25]. And naturally, upon loss of pluripotency, the expression of pluripotency markers like Oct4, SSEA-4 and AP is gradually replaced by markers indicating differentiation[6].
The expression of Nanog – which is a homeobox protein – is up-regulated by the transcription factors Oct4 and Sox2[24]. Expression of Nanog is specific to pluripotent cells, including the inner cell mass of early embryos[24].
Furthermore, certain enzymes exclusively expressed in ESCs are also expressed in iPSCs, including alkaline phosphatase (AP) and telomerase[4, 11, 12, 25]. AP – already briefly mentioned – is an early embryonic stage marker, whereas telomerase – a ribonucleoprotein enzyme normally present at high levels in cells of the germ-line as well as in embryonic tissues – plays an essential role in the maintenance of the telomere length of cells[4, 12, 13]. This enzyme elongates DNA strings by adding telomere repeats to chromosomes, thereby improving the life-span of the cells[13]. The activity of both of these enzymes is measured in iPSC-experiments, since enhanced activity of these enzymes is one of the hallmarks indicating pluripotency.
An additional property in murine pluripotent cells is the presence of two active X-chromosomes in female derived cells[7, 11]. Differentiated female cells possess one active and one inactive X-chromosome, and as a consequence of reprogramming the inactive X-chromosome must become reactivated again[1, 25]. In hESCs on the other hand, one inactive X-chromosome often remains detectable, indicating that these cells are in a more differentiated state than their murine counterparts[27].
Another very important feature indicating full pluripotency, is the absence of gene expression of those genes that were transduced into the cell in order to establish reprogramming[11]. This is called transgene silencing[11]. In pluripotent stem cells integrated viral genes are silenced, whereas in somatic cells this isn't seen. Thus if a reprogrammed cell becomes a true iPSC cell it should result in silencing of the retroviral genes. Transgene silencing is of extreme importance for a few main reasons.
In the first place, some of the genes used for direct reprogramming – for example c-Myc – belong to a class of genes called oncogenes[2, 9]. When these genes are continuously (over)expressed, they are capable of increasing the risk of malign cell mutations, which could lead to cancer[2, 50]. Silencing therefore reduces the risk of spontaneous cell mutations and unlimited cell proliferation, which could otherwise result in the formation of tumours[2, 9, 14].
And secondly, silencing might help to allow

the cells to differentiate once they have been reprogrammed, since continued expression of the transgenes or later reactivation might keep the cells in a more or less pluripotent state[3, 9, 10, 25]. The persistent expression of transgenes may therefore influence the readiness of reprogrammed cells to differentiate in an optimal way[2, 3, 5, 25]. Lack of transgene silencing may thus imbalance the reprogrammed cells and silencing is therefore thought to be indicative for full pluripotency[5, 9]. Subsequently it would be ideal to generate transgene-free iPSCs[10].

Functional capacities of the cells must be proven by in vitro and in vivo differentiation experiments[11]. A variety of assays can be performed, and when arranged at increasing levels of stringency, these include the following[11]:

In vitro: *Embryoid bodies*

In vivo: *Teratoma; chimerae; germline transmission; tetraploid complementation*[25]

Differentiation capacity can be examined in vitro by the formation of embryoid bodies (EBs)[1]. EBs are embryo-like aggregates which grow in suspension[4]. Once these EBs have been cultured, staining with specific markers for all three germ layers, as well as RT-PCR for the analysis of the RNA-profile, can be applied to confirm their pluripotency[1].

To prove the ability of cells to differentiate into any cell type in vivo, a variety of methods exist[1].

Teratoma or teratocarcinoma formation is considered to be the less stringent method used to measure differentiation of iPSCs in live animals[4]. The formation of teratomas is mediated through the injection of iPSCs into immunodeprived severe combined immune deficiency (SCID) or nude mice[6]. Teratomas are benign tumours containing cell types of all three germ layers[4, 6]. These cell types include for example neural epithelium, neural fibers (ectoderm), striated muscle, connective tissue, adipose tissue (mesoderm) and epithelium with brush border (endoderm)[3, 5]. Differentiation into all three germ layers is usually confirmed through the application of histological and immunohistochemical tests[11].

Chimera formation is regarded to be a more reliable test of in vivo pluripotency than the formation of teratomas. Indeed, the initial mouse iPSC-generation could give rise to embryoid bodies and teratomas and were thus defined as pluripotent, but these iPSCs were unable to contribute to live chimeric offspring, although contribution of iPSCs to foetal development was seen[11, 14]. From this it was concluded that the first iPSCs derived from mice were only partially reprogrammed, since true pluripotency can only be confirmed after chimera formation[5, 14]. Chimera can be produced by blastocyst injection or morula aggregation, in either way an embryo is combined with the pluripotent cells[4]. As a consequence, the resulting offspring is formed from host embryonic cells and injected iPSCs. The iPSCs in true chimeras contribute to cell types of all three germ layers, including functional gametes[1, 4]. When iPSCs also contribute to the formation of gonads, so-called germ line chimeras develop: iPSC-DNA will be passed on to the next generation after reproduction of the germline chimeric animal, a phenomenon which is called germ-line transmission[1, 4, 12].

Lastly, the tetraploid complementation assay is the most stringent in vivo pluripotency test[11]. In this assay, the ability of iPSCs to create a whole, fertile individual which entirely consists of iPSCs, is investigated[1]. Mice are the only species so far in which tetraploid complementation experiments haven been carried out[1, 11].

Obviously, these robust pluripotency tests cannot be performed with human iPSCs for ethical reasons[25]. The use of human iPSCs in chimera-studies is considered non-acceptable, though this would confirm their definite pluripotent state[14, 20]. Actually, not only chimera-studies in humans are taboo, in fact in vivo experiments are not at all performed in humans at this moment. In humans, the only method to demonstrate multi-lineage pluripotency in vivo is by teratoma formation[1, 3, 14, 20]. This is done by transplanting human derived cells into nude animals (commonly mice), so that these cells can be tested in vivo[1, 6]. Human iPSCs are therefore pluripotent in the sense that they can proliferate indefinitely

in vitro, and that they can lead to teratomas once they are injected into nude mice[1, 5, 6]. But in view of the issue with the first generation of mice iPSCs previously discussed, true pluripotency of hiPSCs remains questionable[20]. Finally, it should be noticed that different iPSC lines sometimes show different characteristics[1]. Therefore a general, minimal set of criteria often consists of the following:

- the morphological characteristics,
- the ability of continuous self-renewal,
- up-regulation of pluripotency genes,
- down-regulation of genes indicating a differentiated state
- evident transgene silencing,
- X-chromosome reactivation in female lines
- differentiation capacity in vitro,
- differentiation capacity in vivo, proven by the most stringent assay possible for a given species

1.7 Initial pig work published on iPSCs

Currently, iPSCs have only been successfully produced from rodents (i.e. mouse and rat), monkeys, humans and most recently the pig[1-3, 31]. Research teams have attempted to overcome the lack of pESCs by generating porcine induced pluripotent stem cells (piPSCs). Recently, three independent laboratories succeeded in the derivation of piPSCs. The three papers were published within a few months of each other. In each study, the differentiated cells used for reprogramming were transfected by retroviruses[3], lentiviruses[5], or a drug-inducible lentivirus[13], all expressing the original, most standard combination of four reprogramming genes Oct4, Sox2, Klf4 and c-Myc[8].

Estaban et al. (2009) as well as Ezashi et al. (2009) describe the generation of piPSCs from porcine embryonic fibroblasts, the latter group used cells from a Tibetan miniature breed of pig[3, 5] . Wu et al. (2009) used either pig primary ear fibroblasts or primary bone marrow cells, derived from a breed of pig called Danish Landrace[13]. The resulting cell lines met most of the standard criteria used to confirm pluripotency, which is of great importance since previously derived ESC-like cells from swine did not convincingly fulfil these criteria[9]. In all three reports, cells were cultured in the presence of FGF2 and needed a feeder layer of MEFs [2]. Moreover, all three research teams described the LIF-independent growth of the piPSCs[8].

Table 1 summarizes the cell characteristics obtained from those three studies. Given the results from these studies, one will find that the position of the porcine species is somewhat in between the rodent and human species with regards to their iPSC-characteristics.

According to all three reports the resulting piPSC colony morphology resembled the morphology of hiPSCs/hESCs colonies (i.e. well defined bordered and flat colonies) rather than it resembled the miPSCs/mESCs colony morphology [3, 5, 8, 13]. Colonies retained their initial morphologic characteristics and flat, big-nucleated cells (i.e. a high nuclear: cytoplasm ratio) were observed[3]. A feature common to primate ESCs, namely the presence of big nucleoli in the cells, was also seen in the piPSCs[3].

Wu et al. reported that the level of expression of endogenous Oct4, Nanog and Sox 2 in pig cells, was comparable to the level of expression of these markers in human cells[13].

Results regarding the expression of surface markers in piPSCs are controversial. According to Ezashi et al. (2009) the pig cells were positive for SSEA-1, but expression of SSEA-3 and -4 was (almost) not detectable[5]. These piPSCs also lacked the expression of TRA1-60 and TRA1-80, two markers expressed in human pluripotent cells. Thus the expression profile described resembles the expression profile of murine pluripotent cells rather than human pluripotent cells[5]. In contrast, Wu et al. reported the opposite: expression of SSEA3- 4, TRA1-60 and TRA1-80 was evident in piPSCs and hence these cells resembled human cells rather than murine cells[13]. The observed expression of markers of the piPSCs obtained by Estaban et al. (2009) also mimic human pluripotent cells[3].

Because of the conflicting data, the pluripotency markers SSEA1, -3, and -4, and

Article	Morphology	Karyotype	Alkaline phophatase	Telomerase activity	Important genetic ESC-like markers	Cell surface markers	Differentiation in vitro	Differentiation in vivo	Transgene silencing
Estaban et al. (2009)	hESC-like colonies	Normal	Positive	High	Nanog, Rex1,Lin28, SOX2	- SSEA4	Not reported	Teratoma	no
Ezashi et al. (2009)	hESC-like colonies	Normal in 1 cell line	Positive	High	Oct4, Nanog, Sox2, TDGF1 TERT,KLF4, c-Myc	- SSEA1 -Weak to negative SSEA3 and 4	Embryoid bodies	Teratoma	no
Wu et al. (2009)	hESC-like colonies	Normal	Positive	High	Oct3/4, Nanog, Sox2, Rex1,Lin28	-SSEA3 -SSEA4 -TRA1-60 -TRA1-80	Embryoid bodies		

Table 1. Overview of the properties of the first piPSCs [3, 5, 13]

TRA1-60, 1-80, and 1-81 are sometimes considered to be non-specific in the pig[8].
None of the three papers on piPSCs reported, screening of the lines for their ability to contribute to chimaeras.
In 2010, West et al. reported the generation of chimeric offspring from porcine iPSCs[14]. It was the first report of live chimeric offspring production of a species other than the rodent species[14]. Offspring could be produced with a rather high efficiency (85,3%)[14]. The cell type used to reprogram, was the porcine mesenchymal cell, and the cells were transfected by lentivirus expressing all six reprogramming factors (Oct4, Sox2, Klf4, c-Myc, Lin28 and Nanog[14]. Again, the resulting cells and colonies were comparable to hiPSCs/hESCs cells and colonies[14]. Foetal and live born chimeras were investigated for specific marker-expression[14]. iPSCs were later shown to have also contributed to the trophoectoderm (placenta)[14].
West et al. (2010) also reported lack of transgene silencing, probably due to lentiviral integration into the genome of the reprogrammed cell[14].
Up till now, all research groups working on piPSCs, reported the persistent expression of the transcription genes used to reprogram the cells[3, 5, 9].

1.8 Increasing the efficiency of induced reprogramming

With an efficiency in the range of 0.01% to 0.50%, reported in the majority of studies performed, it is evident that reprogramming of somatic cells into iPSCs can be improved[25]. The main factors thought to influence the efficiency of reprogramming, include the combination of factors used, the delivery method of reprogramming factors and the culture conditions. These factors all seem to be associated with the chosen cell type, so the efficiency of reprogramming depends – at least in part – on the chosen cell type:

Combination of reprogramming factors

It might be possible that the number of factors and the ideal combination of factors required for reprogramming, depends on the chosen cell type[11, 25, 52]. But not only the properties of the chosen cell type and the combination of factors influences the efficiency of reprogramming, but also the level and ratios of different factors as well as the timing are important aspects of reprogram ability[2, 5, 11, 52] .
The first cell type to be used in reprogramming experiments in mice as well as in humans, was the fibroblast[11]. Thus far, a wide range of cell types has been used in reprogramming experiments, including embryonic, foetal and adult fibroblasts, hepatocytes, adipocytes, gastric epithelial cells, hematopoietic (stem)cells, pancreatic β-cells and neural (progenitor or stem) cells[1, 10, 11, 25].
In addition to the cell type, the age or passage number and differentiation status of the cells used also plays an essential role[11, 52]. The efficiency of cloning is inversely correlated to

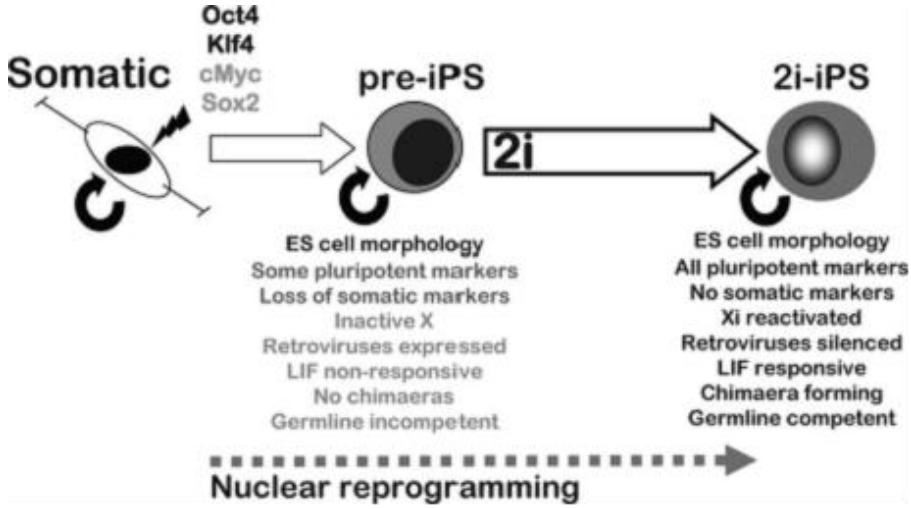

Figure 4. Switching partially reprogrammed pre-iPSCs towards full pluripotency by culturing in 2i medium[52]

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the differentiation state of the cell used: in previous experiments it has been shown that the more differentiated the cell type used, the more difficult it is to reprogram the cell[1, 8]. This explains why tissue stem cells might be easier to reprogram[52].

For instance, Silva et al. (2008) used retroviral transfection and reported that MEFs could be reprogrammed by transducing a cocktail consisting of three factors (Oct4 Sox2 and Klf4) into the somatic cell, whereas neural stem cells could be reprogrammed using only Oct4 and Klf4[52]. This could be explained by the fact that neural stem cells already have high endogenous levels of Sox2 and c-Myc, and thus do not need the addition of exogenous Sox2 and c-Myc for reprogramming[1, 10, 11]. Moreover, reprogramming of these neural stem cells occurred faster, at higher frequency and with better transgene silencing compared to the MEFs[52]. Furthermore, it has even been reported that neural stem cells could be reprogrammed by Oct4 alone, although the efficiency was very low[10].

On the other hand Wu et al. (2009) reported that the use of all six reprogramming factors increases the efficiency of reprogramming in pig-derived ear fibroblasts or primary bone marrow cells, in a sense that the colonies obtained grew better and appeared to maintain undifferentiated for a longer period of time[13]. Several studies have shown that the addition of c-Myc to the combination of reprogramming factors is not necessary for reprogramming fibroblasts of mice and primates, although c-Myc tends to increase the efficiency of reprogramming[10, 11, 25]. c-Myc could also be replaced by small molecules – like valproic acid (VPA), which could also replace the reprogramming factor Klf4 – as has been described for reprogramming of human fibroblasts[1, 10, 50].

At the moment, the only indispensible reprogramming factor seems to be Oct4, which cannot be replaced and is thus crucial in the establishing and maintenance of pluripotency[1, 10, 20, 51].

Delivery method

Regardless of the combination of reprogramming factors used, all factors must be brought into the cell to carry out their function. There are various ways to deliver these factors to the cell that has to be reprogrammed.

The most common method is the use of viruses (mainly retroviruses and lentiviruses, the latter which are a subclass of retroviruses) that will cause integration into the genome of the cell, resulting in expression of the transgene(s)[9, 11]. Regardless, viral systems are criticized because of their permanent genome integration [11].

Recent work has shown that in order to reprogram a cell successfully, integrating into the genome is not essential[1, 11, 25, 52]. Permanent insertion of transgenes and hence modification of the genome could best be avoided, in view of the increasing risk of insertional mutagenesis[11, 25]. By randomly integrating into the genome of the cell, inserted transgenes could cause insertional mutagenesis, thereby disrupting the expression and thus the function of other genes[10, 11, 25] Therefore, alternate – and preferentially non-integrating – methods are now established, including non-integrating

adenoviruses, small molecules which could enhance the efficiency of reprogramming or even replace certain transcription factors (like VPA or 5'-azacytidine), plasmids, episomal vectors and the administration of proteins which function directly as a reprogramming factor itself[1, 2, 5, 10, 11, 31, 50].

In future, reprogramming will preferentially rely on altering the host cell machinery, rather than using genetic materials; ideally, the majority of reprogramming techniques will become non-nucleic acid-based[25]. Again, the ease and efficiency of delivery methods also depends on the cell type[11].

Culture conditions

In addition to the variability in ways of delivery and reprogramming factors used, variety in culture conditions can also play an crucial role in the efficiency of reprogramming[5, 52]. Evidence for the involvement of the culture medium as well as the oxygen-concentration with regards to the efficiency of reprogramming, has been reported and will be briefly discussed below.

In various reports, it has been shown that the addition of specific chemicals, small molecules or growth factors to the culture medium, influences the outcome of experiments significantly[2, 5, 11, 52].

Hanne et al. (2010) succeeded in the derivation of naïve hESCs, and reported that these cells are more closely related to mESCs than primed hECS/iPSCs[29]. They described that human iPSC lines derived in medium containing LIF, required addition of inhibitors to several signalling pathways before the cells could be maintained in the absence of transgene expression [29]. Similarly, Buecker et al. (2010) reported that naïve human iPSCs could be generated using applying LIF and chemical inhibitors into the culture medium[7].

In addition, it has been reported that switching partially reprogrammed mouse cells into medium containing specific chemicals or inhibitors to specific signalling pathways can promote complete reprogramming[52]. Silva et al (2008) reported the rapid progression towards pluripotency of neural stem cells, which initially seemed to be only partially reprogrammed[52]. At first, these cells showed properties of an undiffentiated state like the loss of somatic marker expression, colony morphology and up-regulation of pluripotent markers[52]. But characteristics of these partially reprogrammed pre-iPS cells also included the persistent inactive X-chromosome in female cells, the lower expression of pluripotency markers like Nanog and Rex1 compared to true ESCs, the lack of transgene silencing, and the failure of chimera formation, indicating that full reprogramming had not occurred[52]. Silva et al (2008) transferred the pre-iPS cells then into a modified culture medium, containing two small molecule inhibitors plus LIF, and by doing so, revealed that the pre-iPS cells could be further reprogrammed[52]. In this 2i-medium, the first inhibitor is known to block the MEK/Erk signalling pathway in cells, while the second component inhibits GSK3-beta[23, 52].

From additional experiments in this study, it was concluded that further reprogramming obtained after applying the 2i medium and LIF-stimulation, was mediated via the induction of a final transition to full pluripotency rather than it was a strict selection for those cells that were already fully reprogrammed[52]. In a way, pre-iPS cells were 'primed' and had to be pushed over a threshold to gain full pluripotency ('naïve' state)(*Figure 4*)[52].

It has also been reported that the small molecule inhibitors of the MEK/Erk pathway and GSK3-beta can replace some of the reprogramming factors itself, in the process of iPSC derivation[7].

Likewise, Li et al. (2009) applied the 2i-medium to successfully generate rat-iPSCs and naïve human iPSCs with characteristics similarly as mESCs instead of Epi-SCs[53].

Recently, Telugu et al. (2011) reported the establishment of naïve porcine iPSCs upon the use of LIF and 2i-medium in the culture medium. All piPSCs generated so far resembled hiPSCs/hESCs and EpiSCs, and – as previously discussed – those cell types are thought to be in a primed state of pluripotency rather than a naïve condition[54].

Culturing the cells at a concentration of 1- 5% oxygen (O_2) instead of the atmospheric 21% O_2 -concentration may also aid reprogramming[27, 50]. It has previously been

demonstrated that the O_2 tension in the mammalian female reproductive tract, in which embryos in vivo develop, varies from 1.5% up to 5.3% O_2 concentration[55]. In fact, an ambient 21% O_2 concentration is considered hyperoxic as opposed to physiological O_2 levels and it is thought that a low O_2 microenvironment would be beneficial for the culture of cells, including whole embryo's, ESCs and iPSCs[27].
Various reports exist in which the beneficial effects of a low O_2 -concentration in hESCs are described, such as reduction of chromosomal abnormalities and increasing cloning efficiency[27, 56].
Furthermore, Yoshida et al. (2009) showed that hypoxic (1%) and physiological (5%) O_2 conditions promote the efficiency of reprogramming of MEFs and human dermal fibroblasts as opposed to standard 21% O_2 levels[50].
In addition, Lenger et al. (2009) reported that the derivation of hESCs under 5% O_2 promotes isolation of cells that display a more developmentally naïve state of pluripotency, like displayed by mESCs[27, 50]. One of the hallmarks of a more developmentally naïve state is the X-chromosome-reactivation; the hESCs derived under a 5% O_2 concentration displayed two active X-chromosomes, whereas hESCs cultured and maintained in 21% O_2 did not[27]. Therefore they concluded that physiological O_2 tension helps to establish a more developmentally naïve state in hESCs[27].
Kurosawa et al. (2005) reported the inhibiting effects of 40% O_2 concentration in mESCs on differentiation and found that those cells expressed AP at a relatively high level compared to 20% and 5% O_2 conditions[57].
Although more evidence exists which supports the hypothesis that low O_2 helps to maintain fully pluripotent state, it might also be useful to investigate the impact of hyperoxic conditions on ESCs/iPSCs.

1.9 Promotion of reprogramming of piPSCs into a naïve pluripotent state

The Burdon lab has generated pig iPSCs by transducing foetal fibroblasts with retroviruses expressing the four traditional Yamanaka transcription factors c-Myc, Klf4, Sox2 and Oct4 (unpublished data). Colonies were obtained, picked and expanded and the resulting lines were AP-positive, expressed endogenous Nanog and were SSEA4-positive (unpublished data). Embryoid bodies could be formed and differentiation into ectoderm and endoderm was observed (unpublished data). Teratoma formation was also established, but cell lineages were poorly differentiated (unpublished data). Some of these cell lines have an active LIF signalling pathway and their growth is stimulated by this cytokine. Interestingly, all of the pig cell lines the Burdon lab has screened retained expression of the four retroviral transgenes, which consequently indicates that these cells are not fully reprogrammed (unpublished data).
The aim of this project is to investigate whether manipulation of signalling by modifying the culture conditions will enhance their reprogramming. The hypothesis is that culturing piPSCs at 5% O_2 will help the piPSCs to reach a fully reprogrammed (naïve) state rather than a partially reprogrammed (primed) state of pluripotency. This research project involved culturing pig iPSCs – generated at the Burdon Lab – at 5% versus environmental 21% O_2 concentrations respectively. The cells were screened at various time points for expression of markers that indicate (further) reprogramming. Expression of these markers, including SSEA-1 and Nanog, as well as transgene silencing were determined using mainly reverse transcriptase (RT)-PCR (RNA expression) and immunohistochemistry (protein expression). The differentiation potential of the cells was investigated in vitro by culturing cells as embryoid bodies (EB) The EBs were analysed for the up-regulated expression of certain differentiation markers.
The two most important research questions in this project, are as follows:
1. Is there any difference detectable between cells that were cultured in the two conditions (i.e. 5% versus 21% O_2)?
2. Does anything change over time, after repeated passages?
Contemporaneously, the differences between different cell lines were investigated.

2. MATERIALS AND METHODS

2.1 Cells and tissue culture

Mouse feeder cells

piPSCs were cultured on a feeder layer of irradiated mouse embryonic fibroblasts Feeder cells were plated out at least half a day prior to the passage of piPSCs.

Feeder medium used for the cell culture of Snl 76/7 mouse feeder cells contained 500 mls Glasgow Minimal Essential Media (GMEM, G5154 SIGMA®, Lot RNBB4047), 50 mls fetal calf serum (FCS), 5.6 mls non-essential amino acids (NEAA), 5.6 mls L-glutamine (L-glut) and 5.6 mls sodium pyruvate (NaPy). FCS, NEAA, L-glut and NaPy were added to the GMEM solution using 2.0 µl filters and 50 ml syringes.

Feeder cells were cultured in gelatin-coated T25, T75 and T175 flasks, at room oxygen (~20%) and at 37ºC . Confluence was regularly determined using light microscopy.

To passage the feeder cells:

- feeder medium was aspirated
- cells were washed with PBS
- cells were detached using trypsin/TVP (which was incubated for ±3 min. at 37ºC) and picked up from the flasks using feeder medium
- cell suspension was transferred to a 15ml tube and centrifuged for 3 min. at 1000rcf
- medium + TVP was aspirated, pellet was re-suspended and cell suspension was divided over new gelatin coated flasks.

When feeder layers for piPSCs were set up:

- cells were washed, trypsinized and centrifuged according to the protocol just described
- re-suspended cells were collected in a 50 ml (volume: up to 20 mls cell suspension) tube
- cell suspension was irradiated (20 mls at a time) for 8 min. and 10 seconds (at least 97 Gray)
- cells were counted using a hemocytometer and either frozen down in cryovials or directly plated out*

Cryopreservation of feeder cells:

- 2.0 x10^6 irradiated cells were transferred to every 1 cryovial
- suspension was diluted in an equal volume of 20% freezing mix DMSO (FM = 2,5 mls DMSO added to 10 mls FCS) for feeders, which was added (slowly!) to the suspension, so that the final concentration FM-DMSO in each cryovial was 10%.
- cryovials were brought to the -80ºC freezer
- after a few days the cryovials were transferred to the -150ºC freezer

* Feeders were plated out at densities of either 0.5x10^5 (in wells of 12-well plates) or 1.5x10^5 (in wells of 6-well plates) cells per well. Plating of feeders was done at least half a day prior to the passage of piPSCs

piPSCs

All 3 piPSC lines were cultured in wells of 12-well plates and 6- well plates, in SR medium+LIF and every cell line was kept in two conditions: either 5% or 21% oxygen.

All piPSCs were cultured using serum replacement (SR)-medium, which was prepared with filter units. SR-medium was made using 400 mls KNOCKOUT™ Dulbecco's Modified Eagle Medium (DMEM) which included NaPy (10829, Invitrogen™, GIBCO®, REF 10829-018, LOT 803588), 100 mls KNOCKOUT ™ Serum Replacement (KO-SR), 5.0 mls NEAA, 5.0 mls L-glut and 1.1 mls β-mercaptoethanol. For the piPSC culture, 50 µl recombinant human LIF (concentration: 10^6 units/ml, Millipore™) was added to 50 mls aliquots of SR-medium (final concentration of LIF: 10^3 units/ml). Cells were cultured in 6-well and 12 well tissue culture plates, at 37ºC. Confluence was daily determined, bright field images were taken regularly using light microscopy (1 day after plating, prior before passaging and additionally at various time point in between). Furthermore, images were taken of unfixed living cells using UV/fluorescence microscopy (green channel) to evaluate the level of Green Fluorescent Protein (GFP) expression in the piPSCs. Cells were passaged every ~4 days.

To passage the piPSCs:

- SR-medium was aspirated
- cells were washed with PBS
- cells were detached using accutase and transferred to 15 ml tubes containing SR-medium
- tubes were centrifuged for 3 min. at 1000rcf
- medium + accutase was aspirated, pellet was re-suspended and cells could be passaged to gelatin coated wells containing feeder cells

Cells were plated out in a certain dilution or later (in the experiments) after cell counts. Based on cell counts, cells were passaged at a cell density of 2.0x10^5 per well in the wells of the 6-well plates, and 1.0x10^5 cells per well in the wells of the 12-well plates.

NB. Feeder cells were stored in feeder medium;

before passage of piPSCs, this medium was aspirated and wells were washed with SR-medium first.

Preparation of 2x FM for cryopreservation of piPSCs:

When piPSCs had to be frozen down, 2x freezing mix was made by slowly adding 2.5 mls DMSO to 10 mls serum replacement (SR). DMSO + SR was then transferred to a 10 mls syringe and pushed through a 2.0 µl filter into a 15 ml tube. 3.0 mls aliquots were made, final DMSO concentration was 10%.

For the cryopreservation of piPSCs:

- 2.0 x10^6 piPSCs were transferred to every 1 cryovial
- suspension was diluted in an equal volume of 20% freezing mix DMS for piPSCs, which was added (slowly!) to the suspension, so that the final concentration FM-DMSO in each cryovial was 10%.
- cryovials were brought to the -80ºC freezer
- after a few days the cryovials were transferred to the -150ºC freezer

2.2 Cell count and statistics piPSCs

Bright field images were taken prior to counting, using light microscopy. Cells were accutased and spun down according to the protocol in *paragraph 2.1.* Pellets were re-suspended in SR-medium and Trypan Blue (50 µl : 50 µl). The number of cells alive and dead cells was counted using a haemocytometer. Then a two-sample-t-test was performed, to see if there were any significant differences in cell number between the two oxygen conditions.

2.3 Alkaline phosphatase (AP) staining

AP staining was done with an ALKALINE PHOSPHATASE Kit (SIGMA-ALDRICH™, 86R-1KT, Lot. 020M4337) An AP-solution was made using 10 mls citrate, 26 mls acetone and 3.2 mls formaldehyde.

For staining of 12 wells of a 12-well plate, the following protocol was used:

- preparation of staining solution: 300 µl sodium citrate and 300 µl FRV alkaline solution were mixed in 15 ml tube wrapped in foil.
- mix was incubated for 2 min.
- 12.0 mls double destiled water (ddH_2O) was added to mix
- media was aspirated from wells and 1.0 ml AP-solution was added to each well
- wells were incubated for 30 seconds
- AP-solution was aspirated and 1.0 mls ddH_2O was added to each well
- wells were incubated for 45 seconds
- 300 µl naphtol (light sensitive) was added to staining mix
- 1.0 ml staining solution was added to each well
- plated were wrapped in foil and incubated in the dark for 10 min.
- stain was aspirated and 1.0 ml ddH_2O was added to wells
- images were taken using light microscopy
- ddH_2O was aspirated and plates were dried overnight to be scanned

2.4 Double immunofluorescent antibody staining protocol

In order to fix the cells:

- wells were washed with PBS
- 4% PFA was added to each well
- wells were incubated for 10 minutes at room temperature
- PFA was aspirated and well were washed briefly with PBS three times
- wells were stored in PBS at 4 ºC.

In order to stain the cells:

- wells were washed with PBST (PBST = 1xPBS plus 0.3% Triton, to permeabilize the cell membrane) 4x5minutes

NB. Wells that had to be co-stained for SSEA-1 and -4 were washed with 1xPBS alone, since these markers are cell surface markers and thus do not need permeabilization of the membrane.

- blocking solution was prepared: 27 mls PBST, 10% (3 mls) goat serum from Sigma® and 1% (0.3 gram) Bovine Albumin Serum 96-99% (BSA, SIGMA®) were mixed
- blocking solution was added to each well, wells were incubated for 1 hour
- primary antibodies were diluted in blocking solution (1:200 dilution)*
- primary ab solution was added to each well, wells were incubated overnight at 4 ºC (fridge)
- wells were washed with either PBST or PBS alone, 4x5minutes
- secondary antibodies (light sensitive) were diluted in blocking solution (1:1000 dilution)**
- secondary ab solution was added to each well, wells were incubated for 2 hours in the dark
- wells were washed with either PBST or PBS alone, 4x5minutes (kept dark)
- DAPI (10mg/ml, light sensitive) was diluted in 1xPBS (1:5000 dilution)
- DAPI solution was added to each well, wells were incubated for 5 min. in the dark

- DAPI was aspirated, wells were washed with PBS 1x5minutes in the dark
- PBS was aspirated, new PBS was added and images were taken using the bright field and fluorescence microscope

Details concerning the antibodies used, are described below.

* *Primary antibodies*

*O*ct4: C-10 Santa Cruz-5279, 1:200
Nanog: Ab21603, Abcam, 1:200
SSEA-1: MC-480, DSHB, 1:200
SSEA-4: MC-813-70, DSHB, 1:200

** *Conjugated secondary antibodies (Alexa Fluor, Invitrogen™)*

Oct4: Goat Anti-mouse IgG2b 488 (green)
Nanog: Goat Anti-rabbit IgG 568 (red)
SSEA-1: Goat Anti-mouse IgM 488 (green)
SSEA-4: Goat Anti-mouse IgG3 594 (red)

NB. The SSEA-1 staining was repeated; in that assay, two different secondary antibodies were compared to confirm the previous results. The primary SSEA-1 antibody used was the same antibody as in the protocol described. Details regarding the two secondary antibodies:
Green secondary: Anti-mouse IgM 488 (green)
Red secondary: Anti-mouse IgM 568 (red)

2.5 RNA and cDNA isolation/quantification from piPSCs for expression of pluripotency markers and level of transgene silencing

Cells were frozen down and stored in the -80ºC freezer until RNA isolation was performed. Subsequently, cDNA was prepared from those RNA samples.

In order to freeze piPSCs down:

- cells were accutased and spun down according to the protocol in appendix 2.
- pellets were re-suspended with PBS
- tubes were centrifuged again for 3 min. at 1000rcf
- PBS was aspirated
- pellets were put onto dry ice and were taken to the -80 ºC as soon as possible

RNA purification from cell pellets was done with the RNeasy® Mini Kit, QIAGEN (Cat No. 74104, Lot No. 136243798) :

- A RTL buffer + β-mercaptoethanol (β-ME) solution was made: 10 µl β-ME was added per 1.0 ml RTL buffer
- 350 µl RTL+ β-ME solution was added to each 15 ml tube containing thawed piPSC pellet and tubes were vortexed to mix
- lysate was homogenized by pipetting it into a QIA-shredder spin column placed in a 2.0 ml collection tube
- columns were centrifuged for 2 min. at full speed (13.000rpm)
- 350 µl of 70% ethanol was added to homogenized lysate and it was mixed by pipetting (do not centrifuge)
- 700 µl of the sample was transferred to an RNeasy spin column placed in a 2.0 ml collection tube
- columns were centrifuged for 15 seconds at ≥10.000 rpm, flow through was discarded

Optional: DNase digestion with RNase free DNase set

- 350 µl buffer RW1 was added to RNeasy spin column
- columns were centrifuged for 15 seconds at ≥ 10.000rpm to wash the spin column, flow through was discarded
- DNase I stock solution was prepared by injecting 550 µl RNase free water into a vial containing lyophilized DNase I (with an RNase free needle and syringe)
-vial was gently mixed by inversion
- per one sample: 10 µl DNase I stock solution and 70 µl buffer RDD were gently mixed by inversion
- per one sample: 80 µl DNase incubation mix was added directly to the RNeasy spin column membrane
- samples were placed on benchtop (20-30ºC) for 15 min.
- 350 buffer RW1 was added to each RNeasy spin column
- columns were centrifuged for 15 seconds at ≥10.000 rpm, flow through was discarded
- 500 µl buffer RPE was added to RNeasy spin column, columns were centrifuged for 15 seconds at 10.000 rpm to wash the column, flow through was discarded
- again, 500 µl buffer RPE was added to RNeasy spin column
- columns were centrifuged for 2 min. (long centrifugation time ensures that no ethanol – which is part of the buffer RPE – is carried over during RNA elution) at 10.000 rpm
- RNeasy spin column was placed in a new 2.0 ml collection tube and centrifuged for 1 min. at full speed (optional)
- RNeasy spin column was placed in a new 1.5 collection tube
- 35 µl RNase free water was directly added to the spin column membrane
- columns were centrifuged for 1 min. at 10.000

rpm to elute the RNA
- ½ of the eluted RNA was transferred to another tube, so that 2 RNA samples per original cell pellet-sample were made
- all RNA samples were stored in the -80ºC freezer

Preparation of cDNA and minus RT samples from piPSC RNA samples for the RT-PCRs (starting material 1-5 µg total RNA), was done with the cDNA Kit Superscript® First-Strand (Invitrogen™) Synthsis System for RT-PCR, Part No. 11904-018, Lot No. 797343).

A protocol for first-strand synthesis using oligo (dT) (Invitrogen™) was used:
- all components were mixed and briefly centrifuged before use
- for each reaction sample, 6.0 µl RNA, 1 µl 10 mM dNTP mix and 1 µl primer (o.5 µg/ µl oligo (dT)12-18) and 2.0 µl DEPC-treated water, were combined into a 0.5 ml tube
- the RNA/primer mixture was incubated for 5 minutes at 65ºC, and thereafter placed on ice for 1 minute
- in a separate tube, a 2x reaction mix was prepared (each component was added in the indicated order). Per sample 2.0 µl 10x RT buffer, 4.0 µl 25 mM $MgCl_2$, 2.0 µl 0.1 M DTT and 1.0 µl RNaseOUT (40U/ µl) were combined
- 9.0 µl of the 2x reaction mix was added to each RNA-primer mixture and this was gently mixed and briefly centrifuged
- tubes were incubated for 2 minutes at 42ºC
- 1.0 µl of Superscript ™ II RT was added to the cDNA tubes, and for the preparation of the minus RT controls, 1.0 µl DEPC-treated water was added instead of the RT
- tubes were incubated for 50 minutes at 42ºC
- the reaction was terminated by placing the tubes for 15 minutes at 70ºC, thereafter, the tubes were chilled on ice
- to collect the reaction, the tubes were briefly centrifuged
- 1.0 µl of RNase H was added to each tube
- tubes were incubated for 20 minutes at 37ºC
- 2.0 µl of each cDNA sample was transferred to a separate tube, which could be used for the quantification of cDNA
-all tubes were stored at -20ºC

In order to quantify the amount of cDNA from piPSCs:
- the amount of cDNA in each piPSC cDNA sample was quantified using a spectrophotometer (Nanodrop®)

2.6 Reverse Transciptase (RT)- PCR protocol

Details on the primers used, can be found in *Appendix 1*. Protocol applying to the RT-PCRs performed:

Master mix	**1x** (µl)
10x TBE buffer	10
DNTP's (25 mM)	0.8
$MgCl_2$ (50 mM)	3.0
Actin primer forward	5.0
Actin primer reverse	5.0
ddH_2O	74.7

- 99 µl master mix was put into each PCR tube
- 1.0 µl of each sample was added (NB. For the Rex1 and Nanog RT-PCR, 2.0 µl of each sample was used and therefore 73,7 µl ddH_2O per 1x master mix was added)
- 0.5 µl Taq DNA polymerase enzyme (Invitrogen™, 5U/ µl , Cat No. 18038-026, Lot No. 831465) was added to each tube and volumes were mixed.
- PCR machine Actin:

95ºC 2'
94ºC 30''
63ºC 1' } 30 cycles
72ºC 30''
72 ºC 10'
4ºC hold

Pluripotency factors
- PCR machine Rex1:

95ºC 2'
94ºC 30''
60ºC 1' } 30 cycles
72ºC 30''
72 ºC 10'
4ºC hold

- PCR machine Nanog:

95ºC 2'
94ºC 30''
64ºC 1' } 35 cycles
72ºC 30''

72 ºC 10’
4ºC hold

- PCR machine pig endogenous Oct4 (Oct4UTR):

95ºC 2’
94ºC 30”
67ºC 1’ } 30 cycles
72ºC 30”
72 ºC 10’
4ºC hold

- PCR machine pig endogenous Klf4:

95ºC 2’
94ºC 30”
64ºC 1’ } 35 cycles
72ºC 30”
72 ºC 10’
4ºC hold

Transgenes

- PCR machine transgenic Oct4:

95ºC 2’
94ºC 30”
56ºC 1’ } 30 cycles
72ºC 30”
72 ºC 10’
4ºC hold

- PCR Machine transgenic Klf4/transgenic Sox2:

95 ºC 2’
94 ºC 30”
58 ºC 1’ } 30 cycles
72 ºC 30”
72 ºC 10’
4 ºC hold

- PCR machine transgenic c-Myc:

95 ºC 2’
94 ºC 30”
65 ºC 1’ } 30 cycles
72 ºC 30”
72 ºC 10’
4 ºC hold

- samples were spun for few seconds to get rid of condensation
- 22.5 µl 5xTBE loading buffer (= 4 gram sucrose (40%), 5.0 ml 10xTBE, ddH_2O up to volume of 10 mls, bromophenol blue) was added to each PCR tube sample and mixed
- Samples were run on a 2% agarose gel (made of 1 gram agarose powder and 50 ml TBE buffer), at 50 Volt, therefore: 5.0 µl Tracklt™ (0.1 µl/ml, 1 kb Plus DNA ladder, Invitrogen™, Cat No. 10488-085, Lot No. 800963).

It applies to all RT-PCRs carried out to screen for transgene silencing, that 7.0 µl sample was loaded on the gel.

It applies to all RT-PCRs carried out to screen for the expression of pluripotency factors, that 15 µl sample was loaded on the gel.

-Gel was stained for 15 min. in ethidium bromide bath
-Image was taken

2.7 gDNA isolation/quantification from piPSCs for sex-determination

For the derivation of genomic DNA, piPSCs were cultured on feeder free, gelatin-coated 6-well plates till maximum confluence.

- cells were lysed using ESC-lysis buffer containing 10mg/ml stock Proteinase K (lysis buffer contains 100mM Tris, 5mM EDTA, 200M NaCl, 2% SDS and pH=8,5)
- wells were incubated for 24 hours at 37ºC (incubator)
- lysate was transferred to eppendorph tubes
- 1.0 ml isopropanol was added to each tube (ratio lysis buffer: isopropanol is 1:1)
- tubes were mixed by inversion for 5-10 min.
- tubes were centrifuged for 10 min. at 10.000 rpm in bench top centrifuge
- pellets were washed twice with 70% ethanol, with a two-minute spin after each wash
- ethanol solution was aspirated, pellets were air-dried for ± 10 min.
- pellets were re-suspended in 340 µl ddH_2O
- tubes were incubated for ± 15 min.
- samples were run on a 0.8% agarose gel (50 Volt) in order the quantify the amount of gDNA and thereafter stored at -20ºC

2.8 gDNA isolation/quantification from tissue sample: pig testis

Genomic DNA had to be isolated from a pig testis tissue sample, in order to use it as a positive control in the PCR for sex determination of the piPSC lines.

- a pig testis tissue sample (stored in -80ºC freezer) was thawed, transferred to two eppendorph tubes and lysed with 1.0 ml lysis buffer /tube, containing 10mg/ml stock proteinase K (lysis buffer contains 100mM Tris, 5mM EDTA, 200M NaCl, 2% SDS and pH=8,5).
- eppendorph tubes were incubated overnight at 55º

- tubes were centrifuged for 5 min. at 10.000 rpm to clear the lysates of debris
- supernatant was transferred to fresh tube

To extract the supernatant:

- an equal volume (1.0 ml)of phenol was added to each tube and mixed, and tubes were centrifuged for ± 1 min.
- again, 1.0 ml of phenol was added to each tube and mixed, and tubes were centrifuged for ±1 min.
- 1.0 ml of Chloroform was added to each tube and mixed, and tubes were centrifuged for ± 1 min.
- contents of the two tubes were put together in one tube
- final aqueous phase was precipitated in 2x volumes (600 µl) of ice-cold 100% ethanol and 1/1oth volume (30 µl) 3M Sodium Acetate by centrifuging for 10 min. at 10.000 rpm
- pellet was washed twice with ethanol
- pellet was air-dried and re-suspended in 200 µl ddH_2O
- samples were run on a 0.8% agarose gel (50 Volt) in order the quantitate the amount of gDNA and thereafter stored at 4ºC

2.9 Formation of embryoid bodies (EBs)

Embryoid bodies were formed from mESCs and the piPSC lines in both oxygen conditions. EB-medium was prepared, by adding 100 µl β-mercaptoethanol to 50 ml aliquots of feeder medium *(appendix 1*).Cells were fed ± 2 hours prior to starting with the appropriate medium (mESC medium or SR+LIF). Cells were either trypsinized (mESCs) or accutased, and then centrifuged following the protocol described in *paragraph 2.1.* Pellets were re-suspended in EB-medium, and counted using a haemocytometer. For mESCs and each piPSC line in both conditions, approximately 2.0x10^6 cells diluted in 10 mls EB-medium were added to each 10cm petri dish (non-treated so low attachment bacteriological plates), and 0.33x10^6 cells diluted in 3.0 mls EB-medium were added to each well of a 6-well ultra-low attachment plate. Cells were incubated at 5% and 21% O_2 at 37ºC. Medium was changed every couple of days by transferring the medium + EBs with a 10 ml pipet to a 15 ml tube, letting the EBs settle, sucking off the old medium, adding new EB-medium and transferring the medium + EBs back into fresh dished/plates. Bright field images were taken regularly using light microscopy. EBs were kept in suspension for ± 7 days.

The EB-formation of all three cell lines in both conditions was repeated three times.

NB. The third time, the EBs were kept in SR-Media + LIF for the first two days, thereafter the EBs were switched to EB-medium.

2.10 Plating of EBs

EBs were plated out on gelatin-coated 6-well plates. The amount of EBs used per well, varied from approximately 20 up to 150. Plated EBs were maintained in EB-medium (which was changed every couple of days) and cultured at 37ºC in either 5% or 21% O_2 to grow out and differentiate for at least 14 days, before fixing them. The plating of EBs of all three cell lines in both conditions was repeated three times.

2.11 Immunohistochemistry on plated piPSCs and mECS EBs

Staining of plated piPSC and mESC EBs was done following the protocol described in *paragraph 2.4*, with three exceptions:

- blocking solution contained 3% goat serum instead of 10%
- blocking solution was incubated for 2 hours instead of 1 hour.
- primary antibody was added to the wells in a 1:500 dilution, instead of a 1:200 dilution. Class III b-tubulin, Tuj1, Covance, was used as a primary β-tubulin antibody. Goat Anti-mouse IgG2a 594 (red) was used as a secondary antibody.

2.12 Actin and SRY PCR for sex-determination of piPSC lines

Actin

Master mix	**1x** (µl)
10x TBE buffer	10
DNTP's (25 mM)	0.8
$MgCl_2$ (50 mM)	3.0

Actin primer forward	5.0
Actin primer reverse	5.0
ddH_2O	74,7

- 99 µl master mix was put into each PCR tube
- 1.0 µl of each sample was added
- 0.5 µl Taq DNA polymerase enzyme (Invitrogen™, 5U/ µl , Cat No. 18038-026, Lot No. 831465) was added to each tube and volumes were mixed
- PCR machine:

95ºC 4'
94ºC 2' ┐
63ºC 1' ├ 30 cycles
72ºC 30'' ┘
72 ºC 10'
4ºC hold

- samples were spun for few seconds to get rid of condensation
- 22.5 µl 5xTBE loading buffer (= 4 gram sucrose (40%), 5.0 ml 10xTBE, ddH_2O up to volume of 10 mls, bromophenol blue) was added to each PCR tube sample and mixed
- Samples were run on a 2% agarose gel (made of 1 gram agarose powder and 50 ml TBE buffer), at 50 Volt, therefore: 5.0 µl Tracklt™ (0.1 µl/ml, 1 kb Plus DNA ladder, Invitrogen™, Cat No. 10488-085, Lot No. 800963) and 15 µl of each sample were loaded on the gel
- Gel was stained for 15 min. in ethidium bromide bath
-Image was taken

SRY

Master mix	**1x (**µl)
10x TBE buffer	5
DNTP's (25 mM)	0.8
$MgCl_2$ (50 mM)	1.5
SRY primer forward	1.0
SRY primer reverse	1.0
ddH_2O	36.2

- 47.5 µl master mix was put into each PCR tube
- 2.0 µl of each sample was added
- 0.5 µl Taq DNA polymerase enzyme (Invitrogen™, 5U/ µl , Cat No. 18038-026, Lot No. 831465) was added to each tube and volumes were mixed
- PCR machine:

95ºC 2'
94ºC 30'' ┐
65ºC 1' ├ 30 cycles
72ºC 30'' ┘
72 ºC 10'
4ºC hold

- samples were spun for few seconds to get rid of condensation
- 12.0 µl 5xTBE loading buffer (= 4 gram sucrose (40%), 5.0 ml 10xTBE, ddH_2O up to volume of 10 mls, bromophenol blue) was added to each PCR tube sample and mixed
- Samples were run on a 2% agarose gel (made of 1 gram agarose powder and 50 ml TBE buffer), at 50 Volt, therefore: 5.0 µl Tracklt™ (0.1 µl/ml, 1 kb Plus DNA ladder, Invitrogen™, Cat No. 10488-085, Lot No. 800963) and 15 µl of each sample were loaded on the gel
-Gel was stained for 15 min. in ethidium bromide bath
-Image was taken

NB. Primer sequences used:
SRY-BF
5'-TGAACGCTTTCATTGTGTGGTC-3'
SRY-3R
5'-GCCAGTAGTCTCTGTGCCTCCT-3'

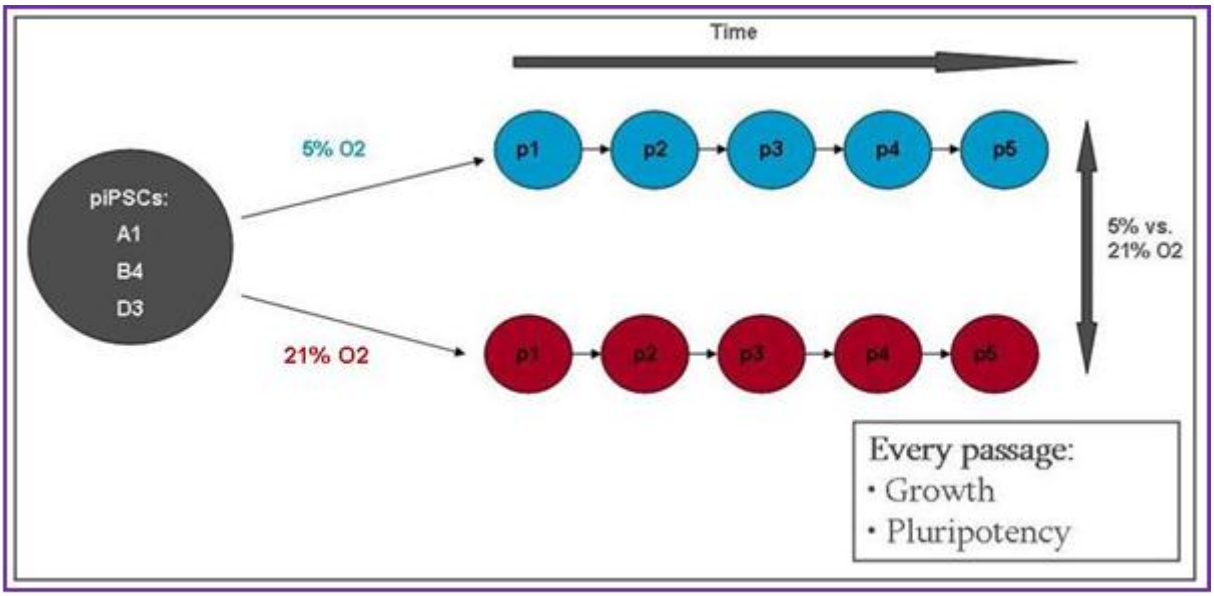


Figure 5. First experimental designs to investigate growth and pluripotency differences of cells kept in 5% versus 21% O2

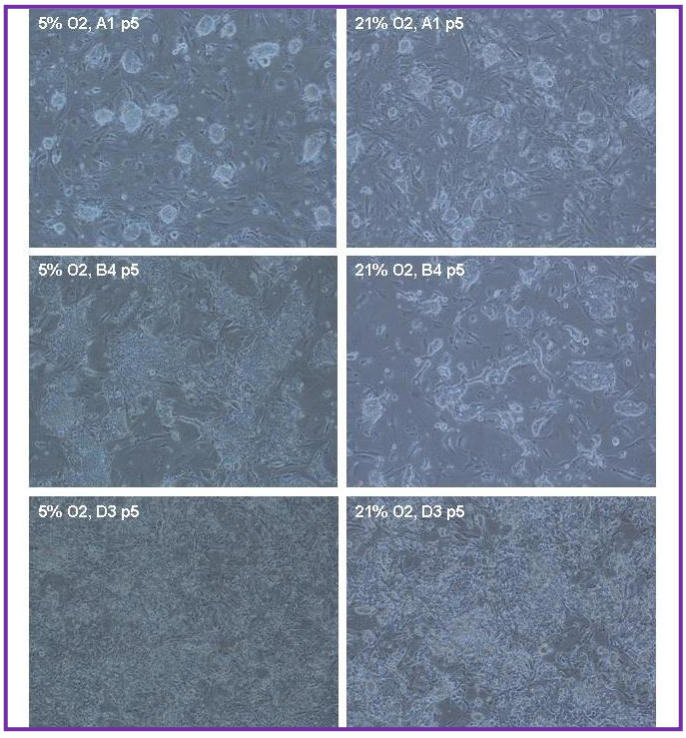


Figure 6. Colony-morphology of piPSC lines A1, B4 and D3, cultured in 5% and 21% O_2 respectively

3. RESULTS

3.1. Experimental designs

The piPSCs used have been derived in the Burdon lab. Three early passage piPSC lines were used, named A1, B4 and D3. Both, the feeder cells and (later) the early passage piPSCs were thawed and maintained for a few passages in 5% (piPSCs) and 21% O_2 (feeders and piPSCs), prior to the actual experiments.

The first part of the project focused mainly on tissue culture aspects, whereby in the first experiment growth and pluripotency properties of the cells were investigated, while in the second experiment the differentiation capacity of the cells was analysed.

The first experiment contained the following tests for all three cell lines in each condition *(Figure 5)*:

Growth

Cells were counted at regular time points during tissue culture; cells were counted prior to passaging and 1 day after plating.

Pluripotency

- alkaline phosphatase staining
- immunohistochemistry: staining with antibodies against Oct4, Nanog, SSEA-1 and SSEA-4
- freezing down cells for RNA elution (which would later be used to perform reverse transcriptase (RT)-PCR).

This experiment was repeated over five passages, after every passage, all cell lines in both condition were plated out at the same densities. When the first experiment (*Figure 5*) started, the passage-numbers were as follows: A1: p3+13, B4: p3+12 and D3: p3+13. In the

remainder of this report, the passage numbers in the first experiment will be referred to as passage 1 to 5.
The second experiment comprised the formation, maintenance, plating and antibody staining of EBs, to analyse the in vitro differentiation capacity of the 3 cell lines. Attention was paid not only to the differentiation capacity of individual cell lines, but also to any possible differences between the two O_2-conditions.
The third experiment focused principally on microbiological aspects and comprised the following assays:
- the elution of RNA from frozen piPSC pellets from the first and fifth passage
- preparation/quantification of complementary DNA (cDNA) from eluted RNA (passage 1 and 5)
- RT-PCRs for screening of pluripotency factors (endogenous Oct4, Nanog, Rex1, Klf4) and level of transgene silencing (transgenic Oct4, Sox2, Klf4 and c-Myc)
In addition, a fourth experiment was included in which gDNA from piPSCs and a pig testis tissue sample was isolated and quantified, in order to determine the sex of all cell lines with an SRY PCR.

3.2 Results obtained from cell count, just before passage of the cells

Cells were plated out at the same densities throughout the project, which made it possible to compare the growth of each cell line in each oxygen condition. At the end of each passage, cells were counted using a haemocytometer. For four passages, the number of cells counted is shown in the graphs of *Figure 7*. From these graphs it can be concluded that in general there are significantly more cells at the end of each passage in the wells kept in 21% O_2. There are two reasons which could explain this observation. First of all, cells kept in 21% O_2 could simply proliferate faster than cells kept in 5% O_2. Secondly, a concentration of 21% O_2 might cause a higher plating efficiency. If the latter is the case, one might expect less cell death and more living cells that plated down in the 21% O_2 wells, versus the 5% O_2 wells within a limited interval after plating.

3.3 Results obtained from Trypan Blue cell count, 1 day after plating

In order to investigate the role of the plating efficiency in the piPSCs, a cell count 24 hours after plating was included in the first experiment. The results are shown in the graphs of *Figure 8*. Although the number of cells tends to vary slightly, there seems to be no advantage of one oxygen concentration over the other. This makes it likely to conclude that the involvement of plating efficiency is limited.

3.4 Results from Alkaline Phosphatase (AP) staining

From two 12-well plates (one kept in 5% and one in 21% O_2), two wells per cell line per plate were stained for AP over five passages (1-5, *Figure 9*). Cells from all stained wells were found to be AP-positive. Macroscopically, in the cell lines B4 and D3, no very clear difference in staining results between cells cultured at 5% versus 21% O_2 was observed, although the staining results tended to vary slightly per passage number (i.e. sometimes the staining seemed more intense in the wells cultured at 5% O_2, while at other times, the 21% O_2 wells stained stronger, *Figure 9.A*) In cell line A1, over all five passages and in both conditions, the intensity of the staining was much weaker compared to the staining intensity in B4 and D3. Nevertheless, a more pronounced difference between the two conditions was found in this cell line. Macroscopically, A1-wells cultured in 21% O_2 seemed to stain more intense than the 5% O_2 wells, mainly in the first three passages. The microscopic analysis of A1 also showed more intense staining per well in the 21% O_2 wells compared to 5% O_2 wells (*Figure 9.B*) Furthermore, a morphologic difference was found to exist between A1 colonies cultured in different oxygen concentrations. Colonies grown in 21% O_2 appeared to be bigger and rather flattened with rough colony-borders, whereas cells cultured in 5% O_2 formed well-bordered, smaller but more compact dome-shaped colonies. Therefore staining in the flattened 21% O_2 colonies was more widespread, whereas the staining in the compact 5% O_2 colonies seemed more intense, probably due to the higher cell density in the latter colonies.

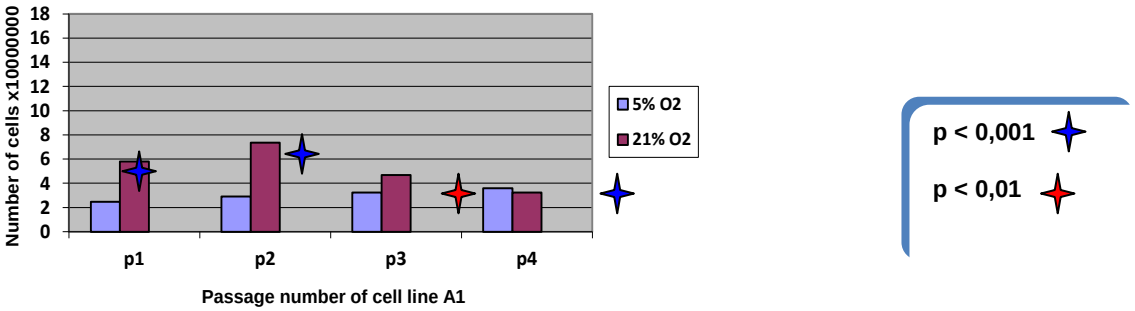


Figure 7.A. Cell line A1: number of cells (x10^6), counted just before passaging

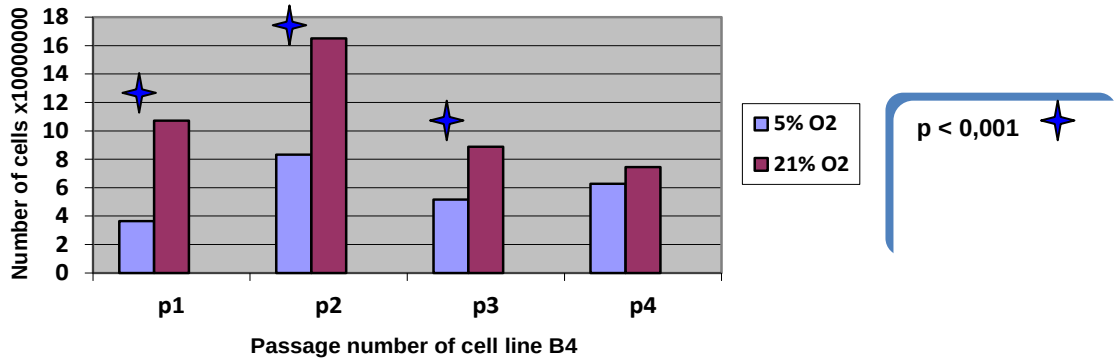


Figure 7.B. Cell line B4: number of cells (x10^6), counted just before passaging

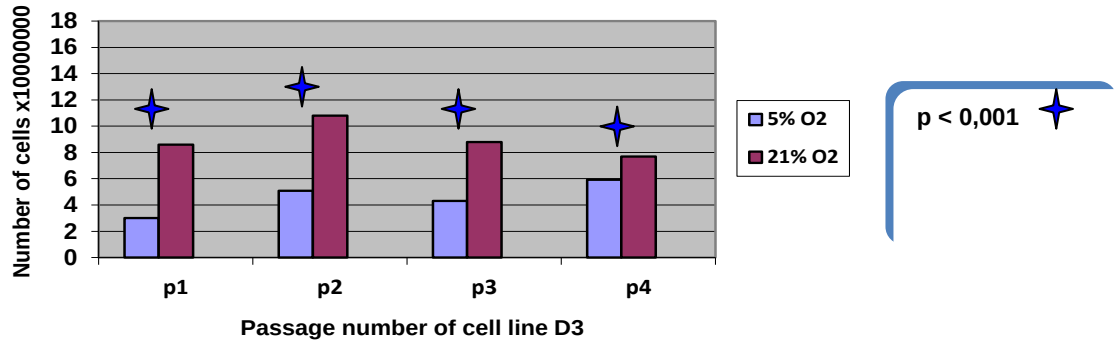


Figure 7.C. Cell line D3: number of cells (x10^6), counted just before passaging

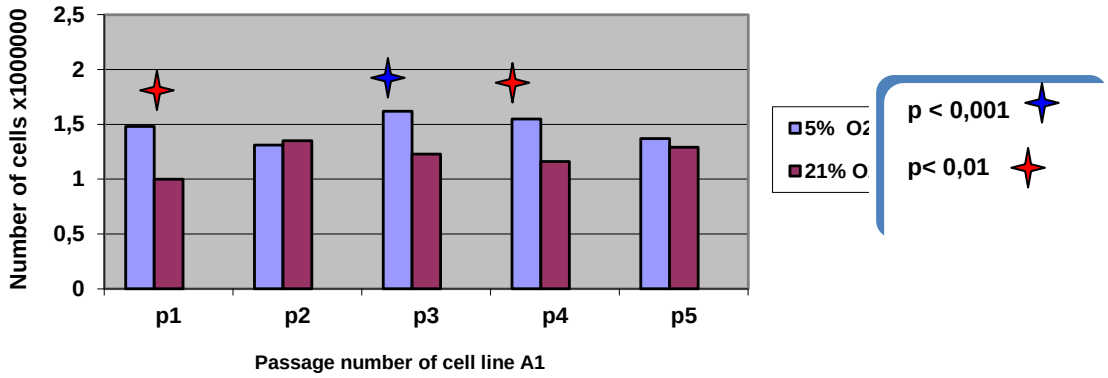


Figure 8.A. Cell line A1: number of cells alive (x10^5), counted 1 day after plating

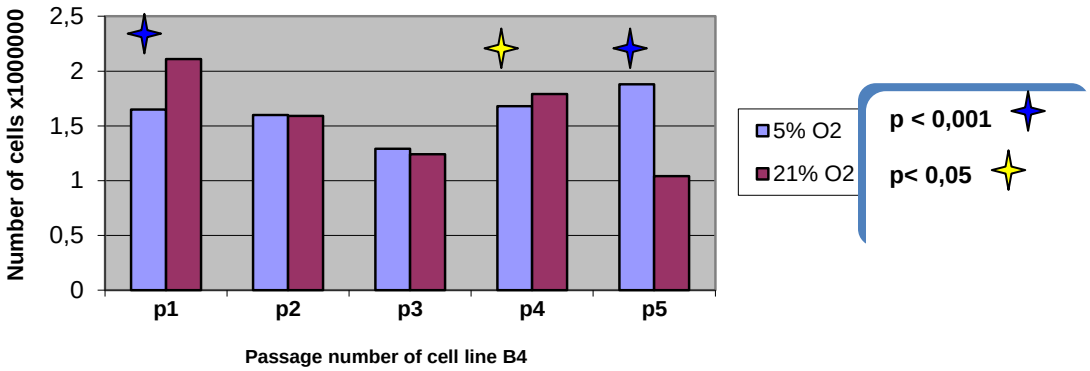


Figure 8.B. Cell line B4: number of cells alive (x10^5), counted 1 day after plating

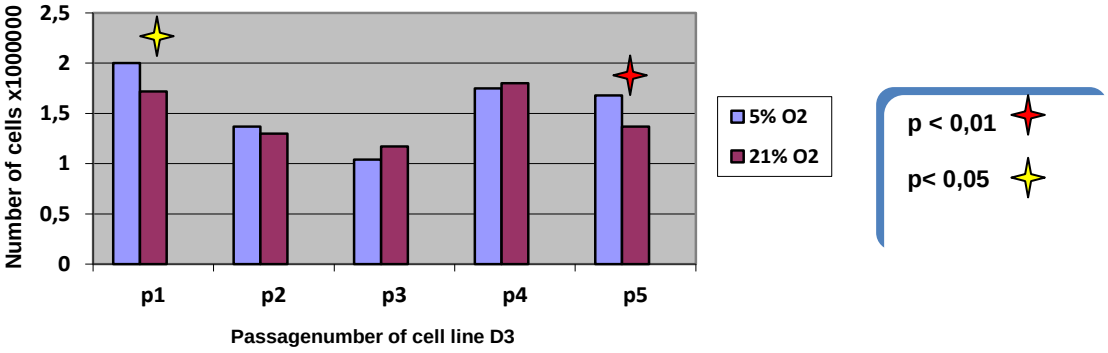


Figure 8.C. Cell line D3: number of cells alive (x10^5), counted 1 day after plating

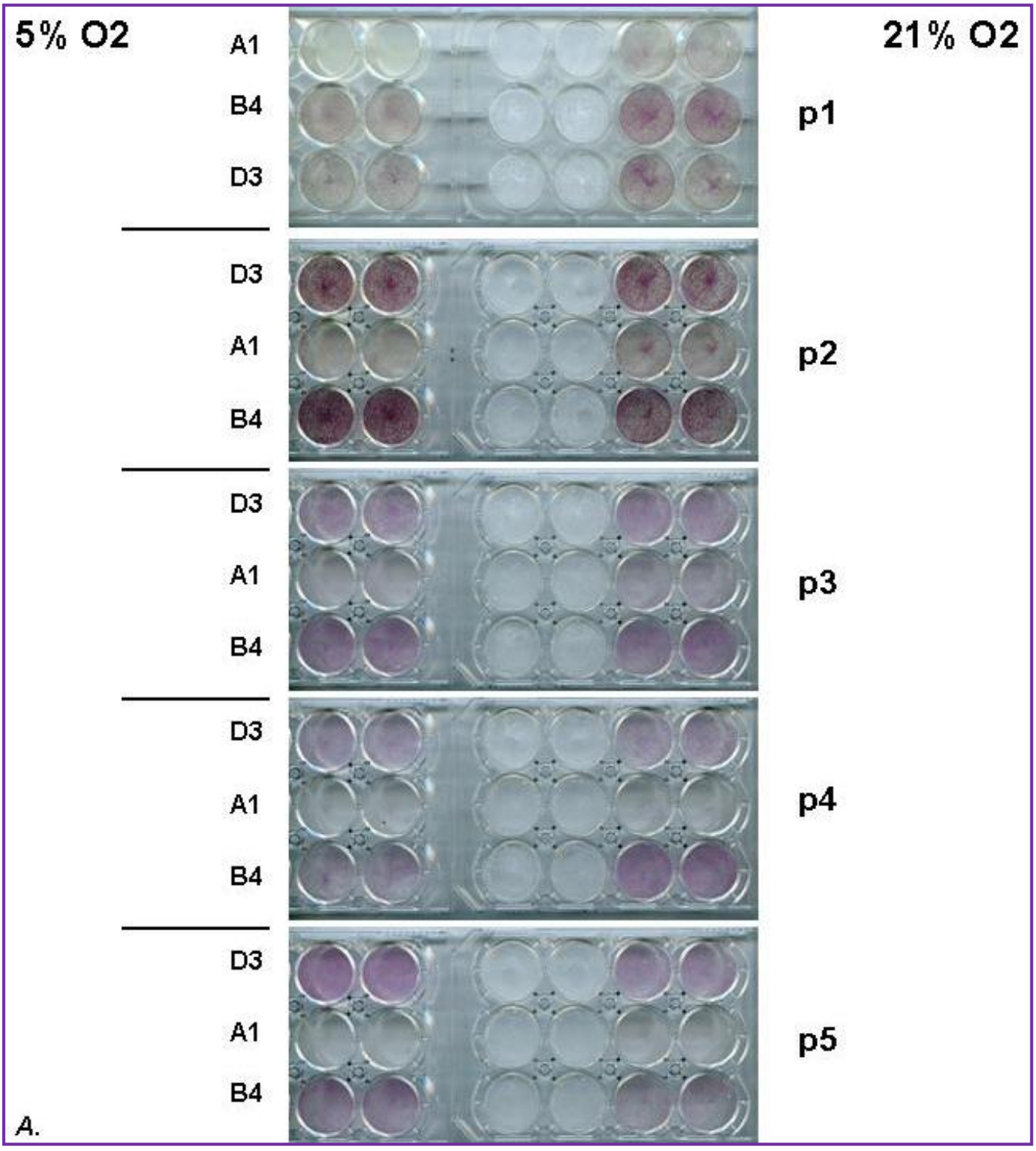
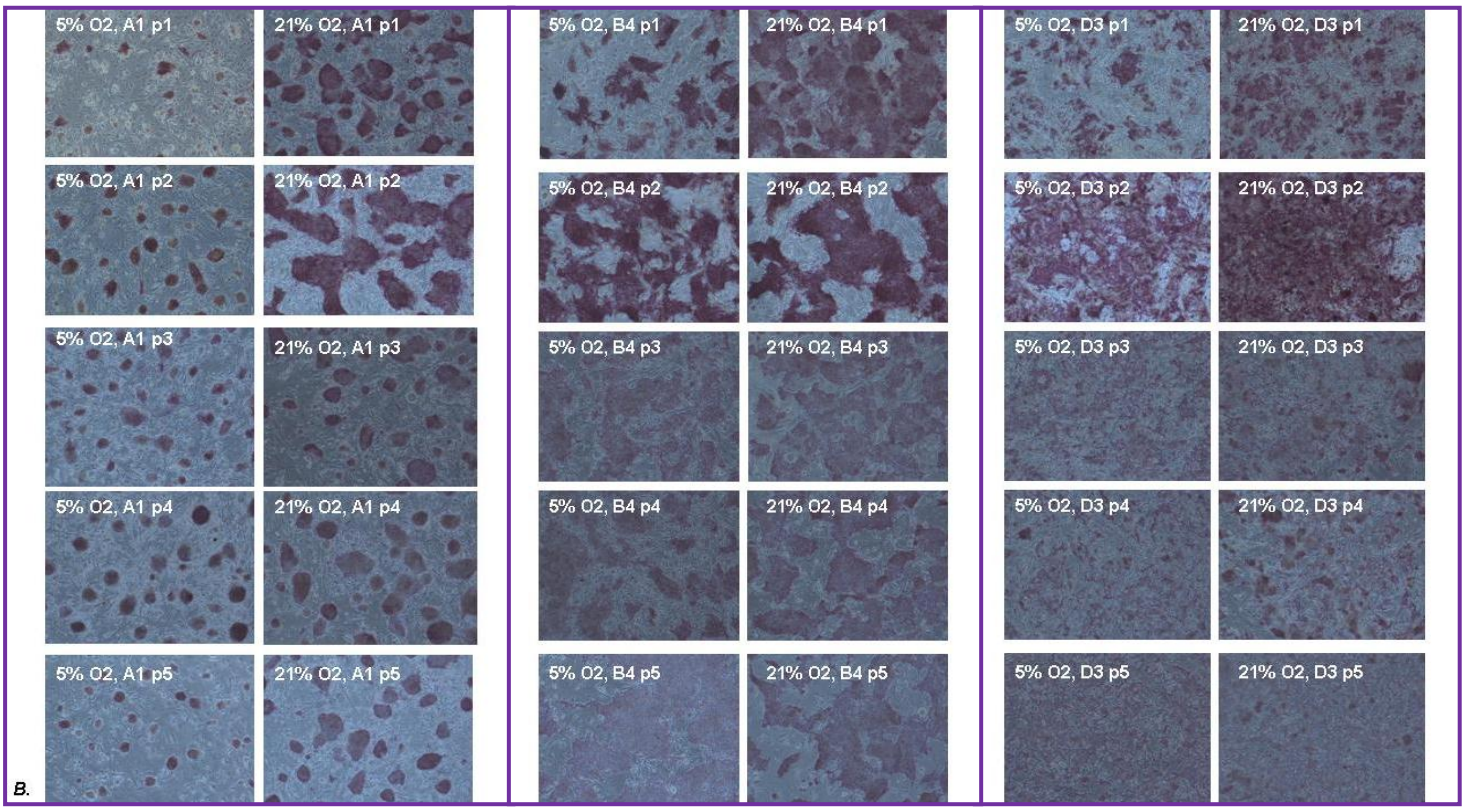
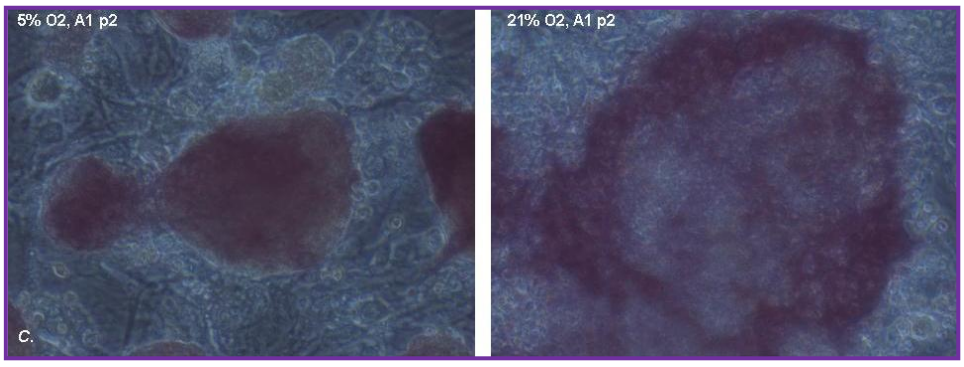
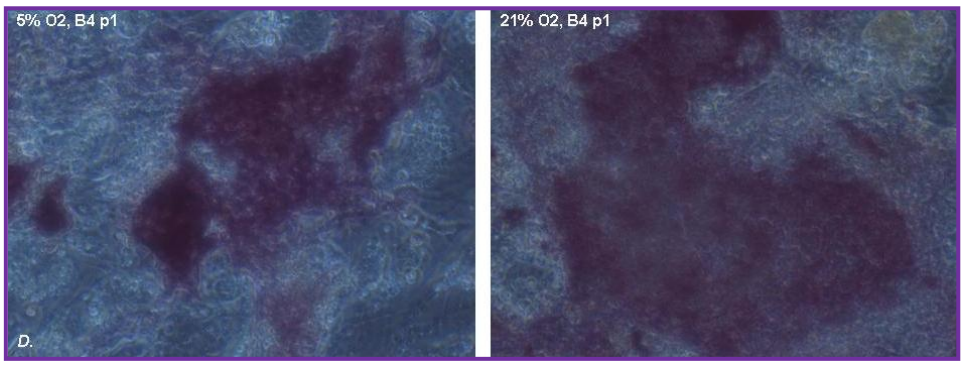
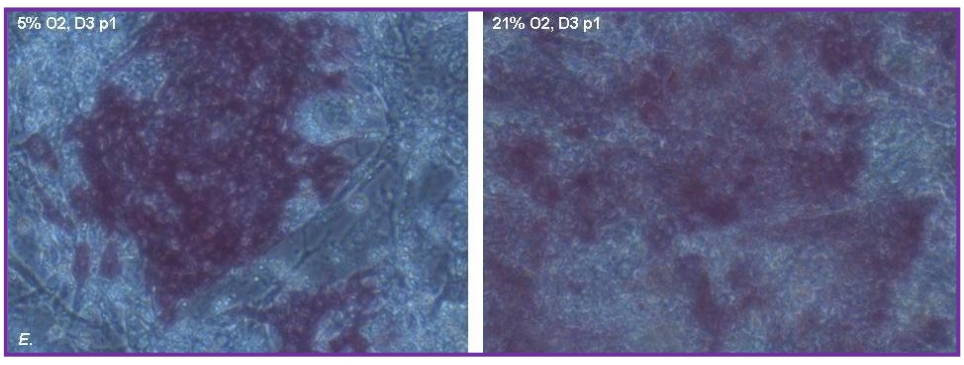


Figure 9.
A. **Macroscopic overview of the results obtained from the alkaline phosphatase staining of piPSCs, scanned plates. From two 12-well plates, two wells per cell line per condition were stained for AP over five passages;**
B. **Overview of the results from the alkaline phosphatase staining in all 3 piPS cell lines, kept in both conditions (5% and 21% O_2), microscopic images, 10x objective;**
C. **Comparison of AP-positive colonies grown in either 5% or 21% O_2, piPS cell line A1, 40x objective;**
D. **Comparison of AP-positive colonies grown in either 5% or 21% O_2, piPS cell line B4, 40x objective;**
E. **Comparison of AP-positive colonies grown in either 5% or 21% O_2, piPS cell line D3, 40x objective.**


5% O2, A1 p1
21% O2, A1 p1
5% O2, B4 p1
21% O2, B4 p1
5% O2, D3 p1
21% O2, D3 p1
5% O2, A1 p2
21% O2, A1 p2
5% O2, B4 p2
21% O2, B4 p2
5% O2, D3 p2
21% O2, D3 p2
5% O2, A1 p3
21% O2, A1 p3
5% O2, B4 p3
21% O2, B4 p3
5% O2, D3 p3
21% O2, D3 p3
5% O2, A1 p4
21% O2, A1 p4
5% O2, B4 p4
21% O2, B4 p4
5% O2, D3 p4
21% O2, D3 p4
5% O2, A1 p5
21% O2, A1 p5
5% O2, B4 p5
21% O2, B4 p5
5% O2, D3 p5
21% O2, D3 p5
B.


5% O2, A1 p2
21% O2, A1 p2
C.


5% O2, B4 p1
21% O2, B4 p1
D.


5% O2, D3 p1
21% O2, D3 p1
E.

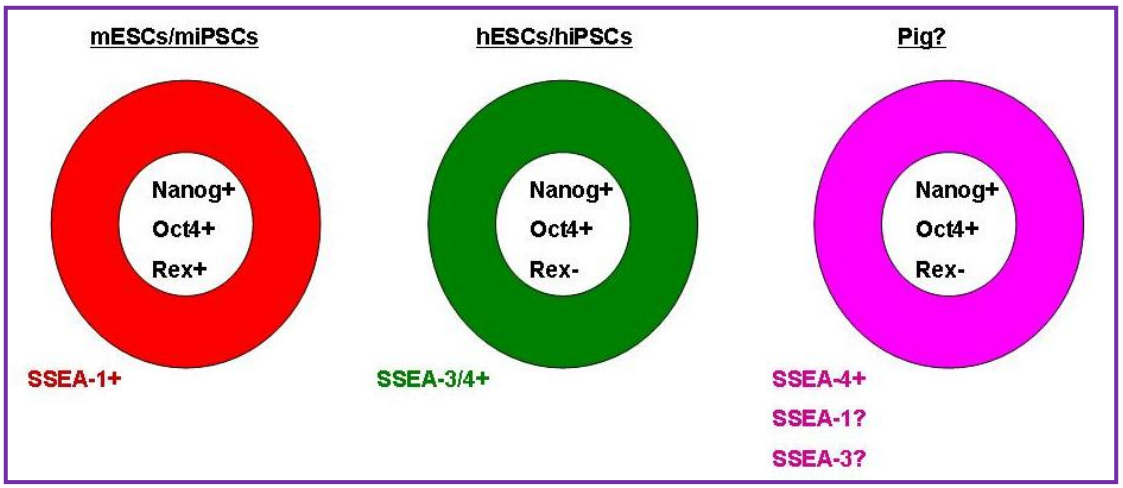


Figure 10. Expression of endogenous pluripotency factors in mESCs/miPSCs and hESCs/hiPSCs and piPSCs from the Burdon lab. At the start of the project the piPSCs generated in the Burdon lab were known to express endogenous Oct4, Nanog and SSEA-4. In previous assays, these piPSCs were not found to be positive for the pluripotency factors REX1 and SSEA-1 and -3.

3.5 Results from immunohistochemistry staining of piPSCs, mESCs and mouse feeder cells

With regards to the immunohistochemistry, every well was stained for pluripotency factors as well as for DAPI. piPSCs were stained with antibodies to detect and analyse the expression of the nuclear factors Oct4 and Nanog, and the cell surface markers SSEA-1 and SSEA-4 (*Figure 10*). For the antibody staining of piPSCS, mESCs (cell lines HM1 and E14) were used as a positive control and mouse feeder cells (cell lines DIAM and Snl 76/7) were used as a negative control. Details concerning the antibodies are mentioned in 'materials and methods'.

Results Oct4/Nanog co-staining

The mESCs (cell lines HM1 and E14) were used as a positive control and stained positive for both markers (*data not shown*). The mouse feeder cells (DIAM and Snl 76/7) used as a negative control, stained negative, but the cytoplasm of the Snl 76/7 feeders showed bright Nanog-positive staining *(visible in Figures 12 B,C ; 13 B,C ; 14 B,C*). However, since all feeder nuclei were clear and since Nanog is known to be a nuclear marker of pluripotency, the feeders could be used as a valid negative control. All 3 piPSC lines in both conditions (i.e. cultured in 5% and 21% O_2) stained positive for Oct4 (green secondary antibody) and Nanog (red secondary antibody). From the images taken, it could be concluded that the staining is restricted to the nuclei of the cells (*Figure 11-14*). With regards to the cell lines B4 and D3, there seemed to be no significant difference in staining between the two conditions, neither a change over time after five passages within each cell line. In cell line A1, a remarkable feature in the Oct4 staining was found: cell nuclei from cells located in the periphery of colonies stained brighter compared to cells in the centre (*Figure 11*). This difference in the intensity of staining was found in both conditions, and was especially evident over the first three passages of the experiment. Later, the difference could still be found, but it was less evident than before, again in both conditions. The bright staining cells stained positive for Nanog as well, but not necessarily as bright as the Oct4 staining. Furthermore, the morphologic difference between A1 colonies cultured in 5% versus 21% O_2 just mentioned in *paragraph 3.3*, was accentuated again (*Figure 11.A*).

Results SSEA-1/SSEA-4 -staining

Again, mESCs (cell lines HM1 and E14) were used as a positive control. The mESCs stained positive for SSEA-1, and negative for SSEA-4, conform the pluripotency marker-expression of mESCs – mESCs are known to be negative for SSEA-4 – (*date not shown*). Mouse feeder cells (DIAM and Snl 76/7) used as a negative control, stained negative like expected (*data*

not shown). All 3 piPSC lines in both conditions (i.e. cultured in 5% and 21% O_2) stained positive for SSEA-4, however, staining was weak in A1 (over the first two passages, in both conditions) and B4 (in the first passage, in both conditions)(*Figure 14*). Overall, the expression of SSEA-4 does not seem to be influenced by the oxygen concentration in which the cells are cultured (*Figure 14*).
Based on the results generated from these tests, the SSEA-1 staining was repeated. Initially, co-staining with SSEA-4 – for which a red secondary antibody was used – made it necessary to use a green secondary antibody for the SSEA-1 staining. All cell lines in both conditions, were found to be green fluorescent after staining, however, the staining results for this marker were dubious for three main reasons. Firstly, the staining pattern did not seem to be exclusive to the cell surface, which should be expected since SSEA-1 is a cell surface marker. Secondly, until now SSEA-1 staining has not been observed in these 3 piPSC lines in previous experiments. Cells were derived from foetuses of a mating with a boar that was transgenic for CMV-GFP and so, fluorescent microscopic analysis of living (i.e. unfixed) cells confirmed that cells of all 3 cell lines were still expressing GFP (*Figure 16.C.*). In conclusion, the presence of true SSEA-1 positive piPSC colonies was not confirmed, because it could not be excluded that the green fluorescent dye observed was caused by the GFP. An additional experiment was done to test the expression of SSEA-1 again, thereby using two different secondary antibodies (green and red) in order to compare the outcomes obtained from staining with the red versus the green secondary antibody. Like observed in the first SSEA-1 assay, cells from all cell lines in both conditions, were found to be green fluorescent (*Figure 16.A.*). However, in cell line A1, the expression pattern of the green fluorescent dye appeared to be similar to the pattern observed in the positive control (in both conditions). When looking at the wells that were stained with the red secondary antibody, this observation was confirmed; again, the expression pattern in cell line A1 matched the pattern of staining found in the mESCs . Thus A1 was found to be positive for SSEA-1, in both conditions. There seemed to be no difference between the expression in colonies grown in 5% versus 21% O_2. In contrast to A1 colonies, cells from the B4 and D3 colonies were found to be negative for the SSEA-1 marker when using the red secondary antibody. This indicates that the previous results obtained from the first experiment with the green secondary antibody, were in fact false-positive since the green fluorescent dye was probably background, caused by the GFP-expression. A1 was the only cell line in which colonies were tested positive for the SSEA-1 staining (*Figure 17*). No obvious difference between the two oxygen conditions was found.

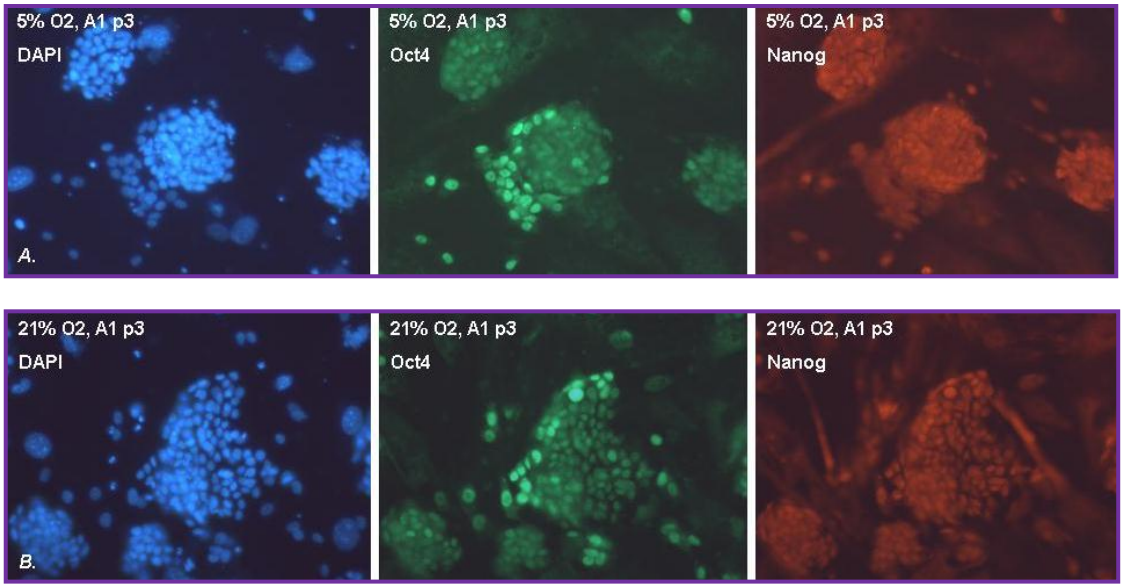


Figure 11. Bright Oct4 staining in peripheral cells of A1 colonies. Bright Oct4 staining does not necessarily corresponds to bright Nanog staining.
A. **DAPI, Oct4 and Nanog-positive staining in A1 colony, cultured at 21% O_2, microscopic images, 40x objective;**
B. **DAPI, Oct4- and Nanog-positive staining in A1 colony, cultured at 5% O_2, microscopic images, 40x objective.**

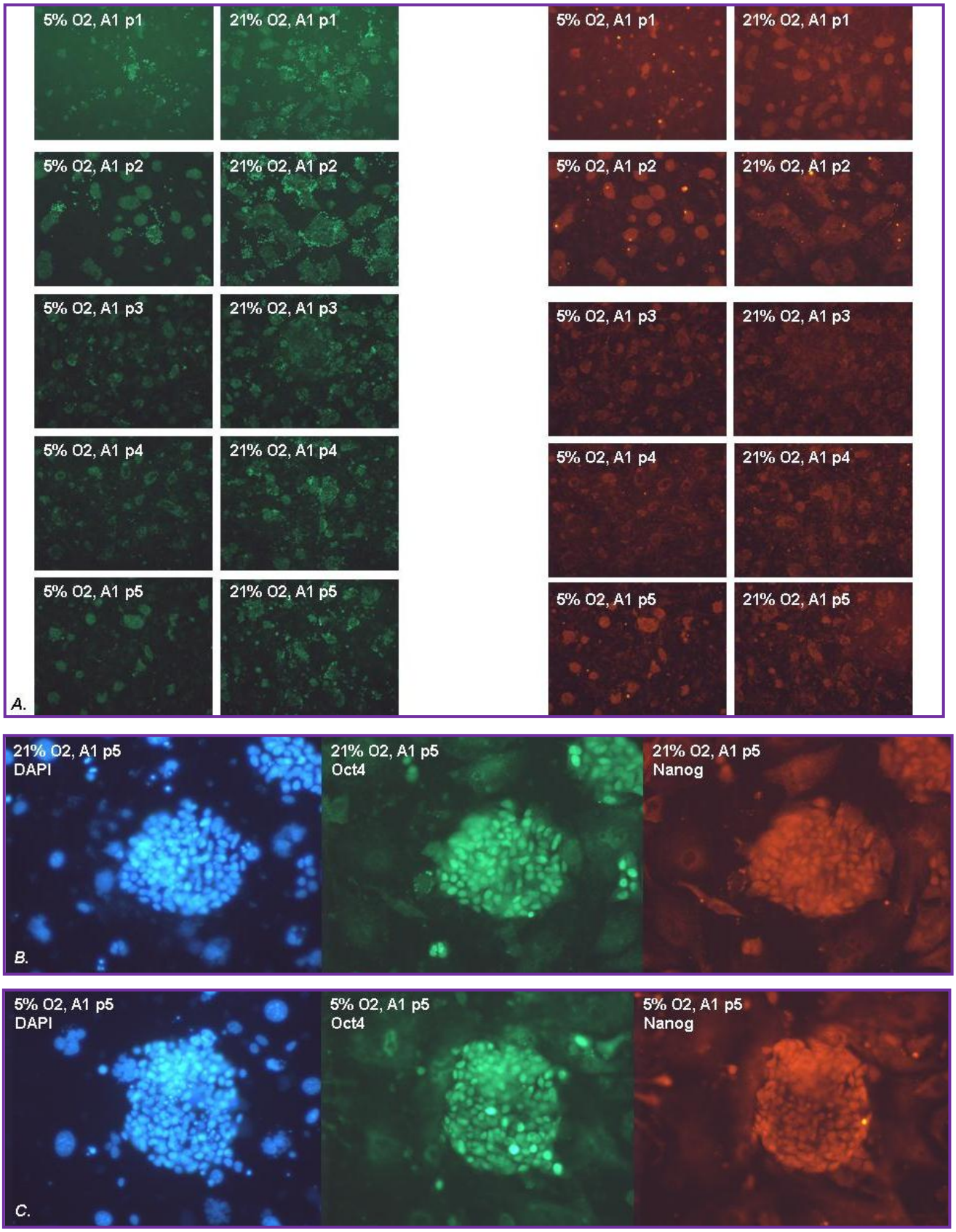
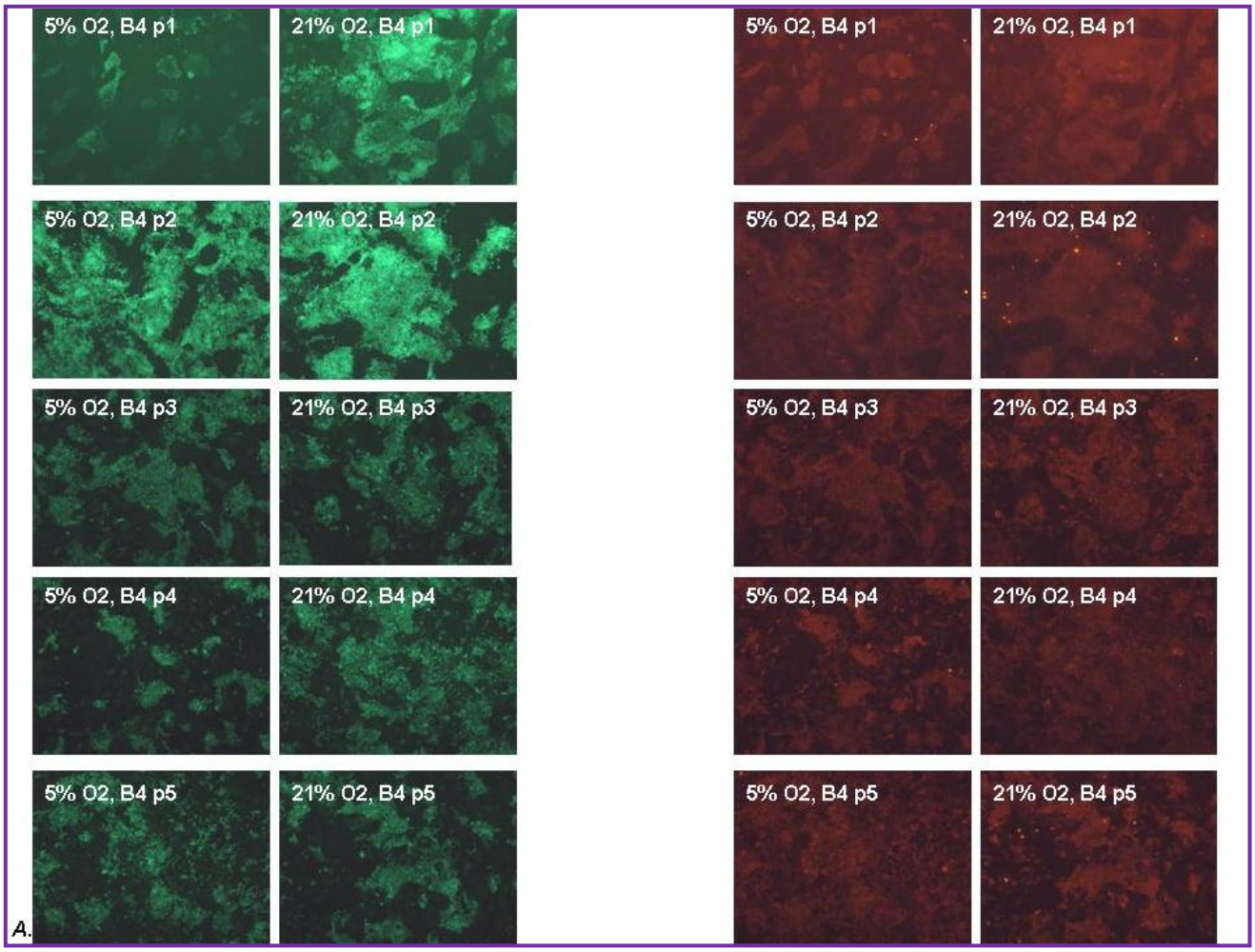
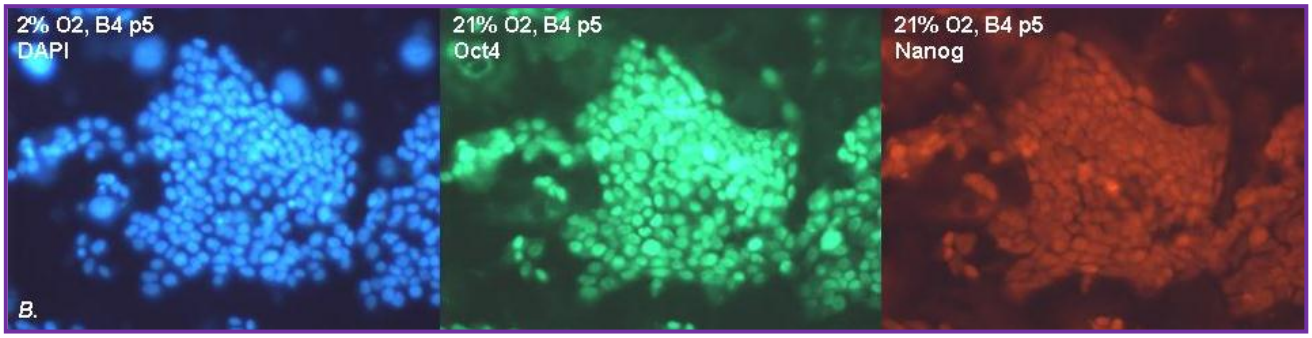


Figure 12.
A. **Overview of the results obtained from the Oct4 (green secondary antibody) and Nanog (red secondary antibody) staining in piPS cell line A1, kept in both conditions (5% and 21% O_2), over five passages, microscopic images, 10x objective;**
B. **Oct4- and Nanog-positive A1 colony, cultured at 21% O_2, microscopic images, 40x objective;**
C. **Oct4- and Nanog-positive A1 colony, cultured at 5% O_2, microscopic images, 40x objective.**





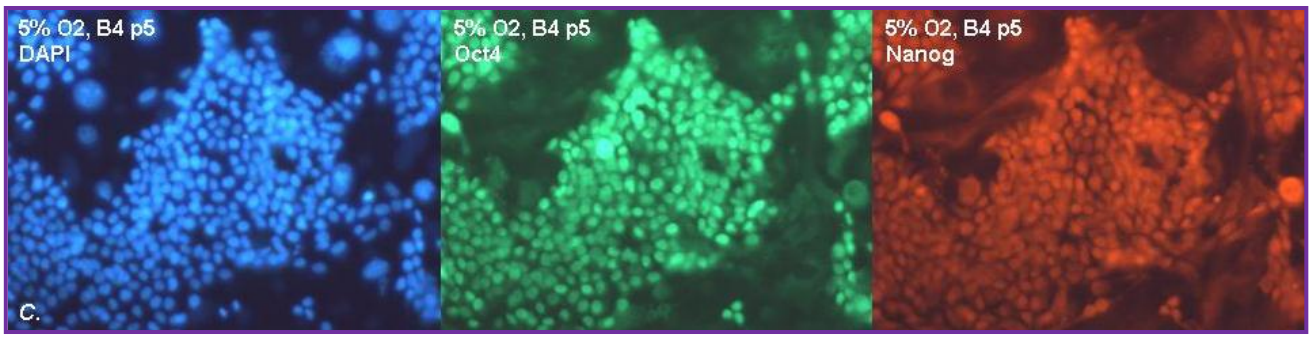


Figure 13.
A. **Overview of the results obtained from the Oct4 (green secondary antibody) and Nanog (red secondary antibody) staining in piPS cell line B4, kept in both conditions (5% and 21% O_2), over five passages, microscopic images, 10x objective;**
B. **Oct4- and Nanog-positive B4 colony, cultured at 21% O_2, microscopic images, 40x objective;**
C. **Oct4- and Nanog-positive B4 colony, cultured at 5% O_2, microscopic images, 40x objective.**

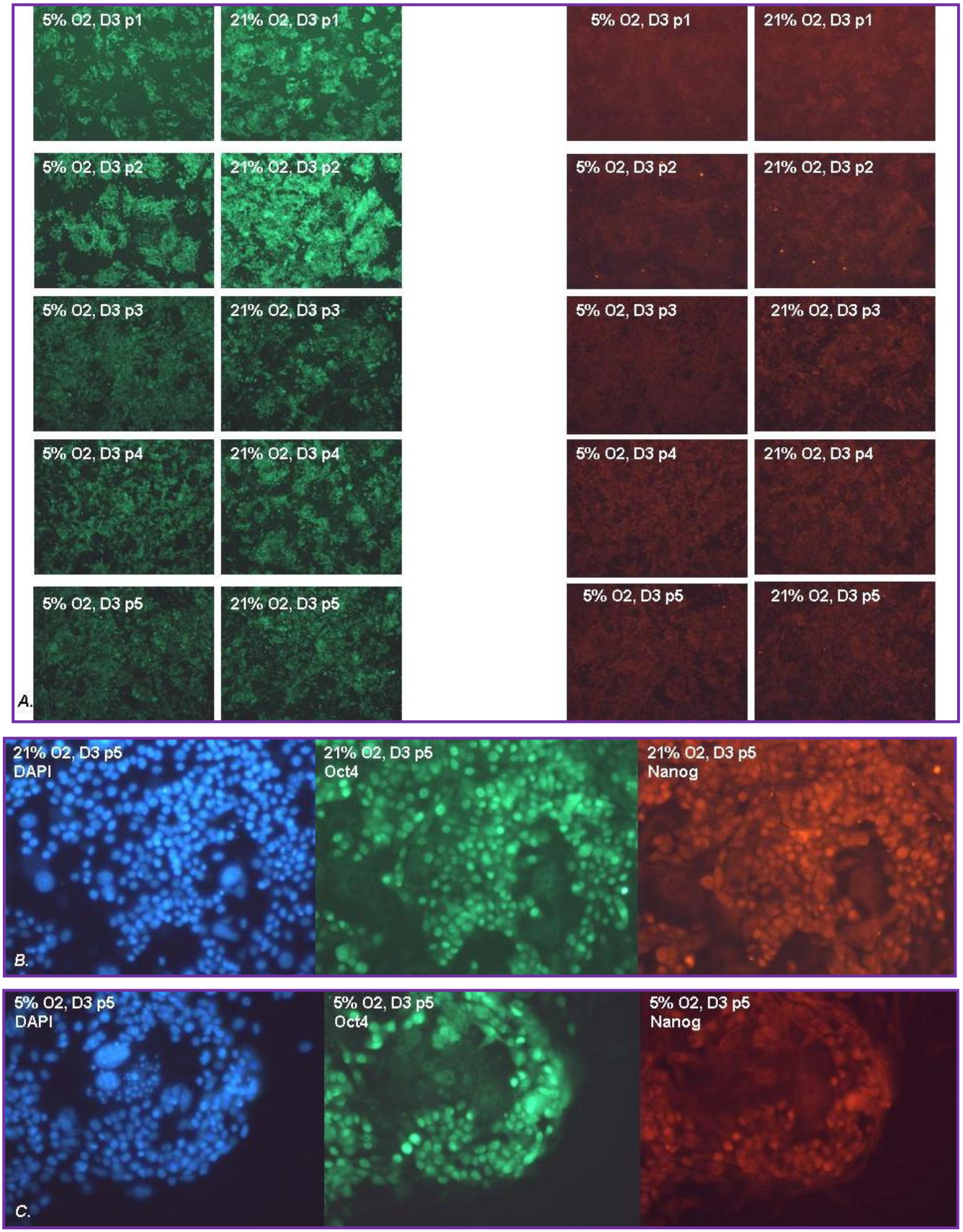
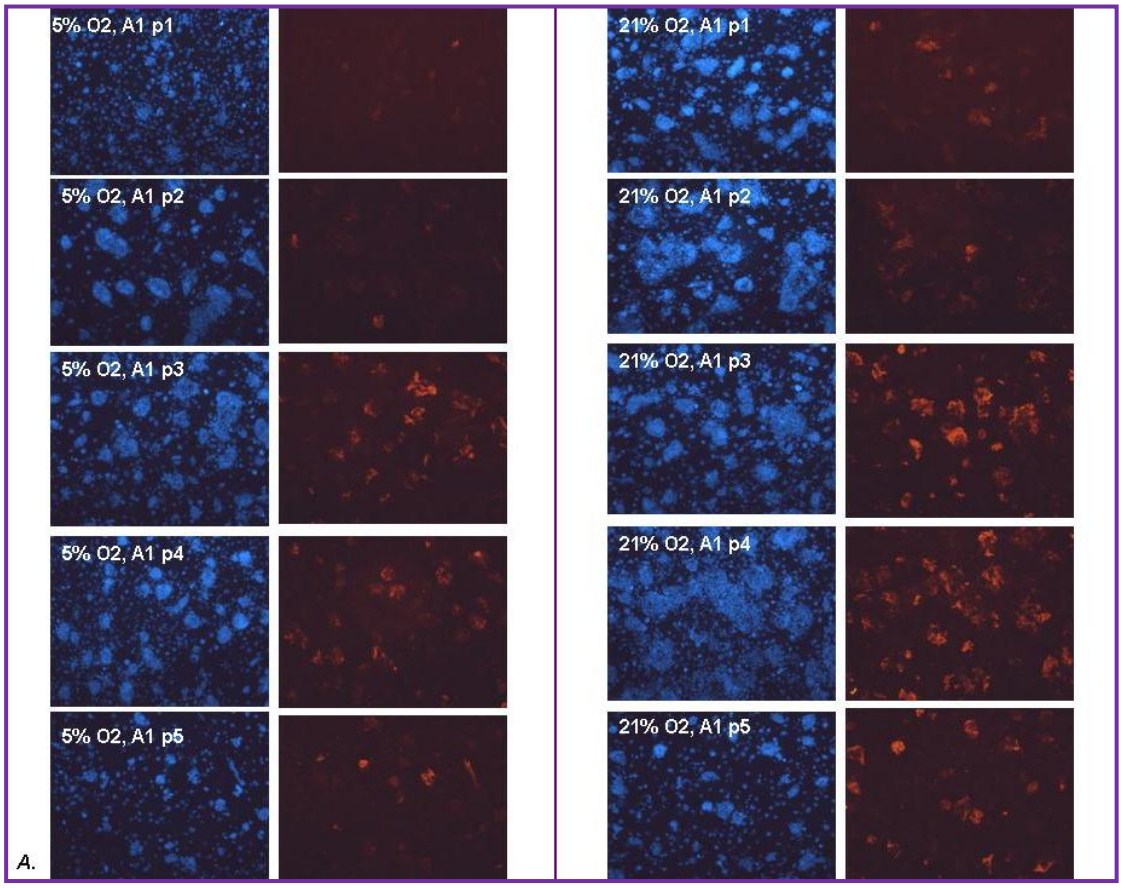
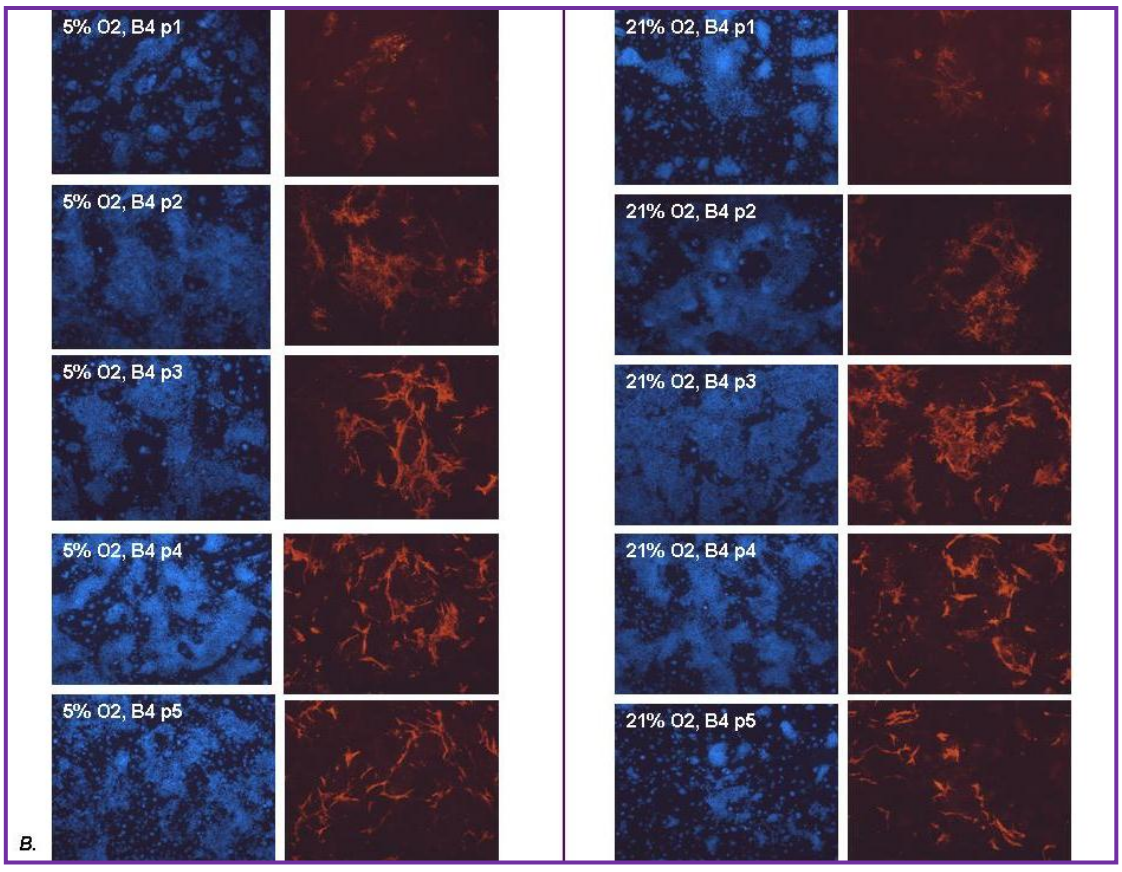
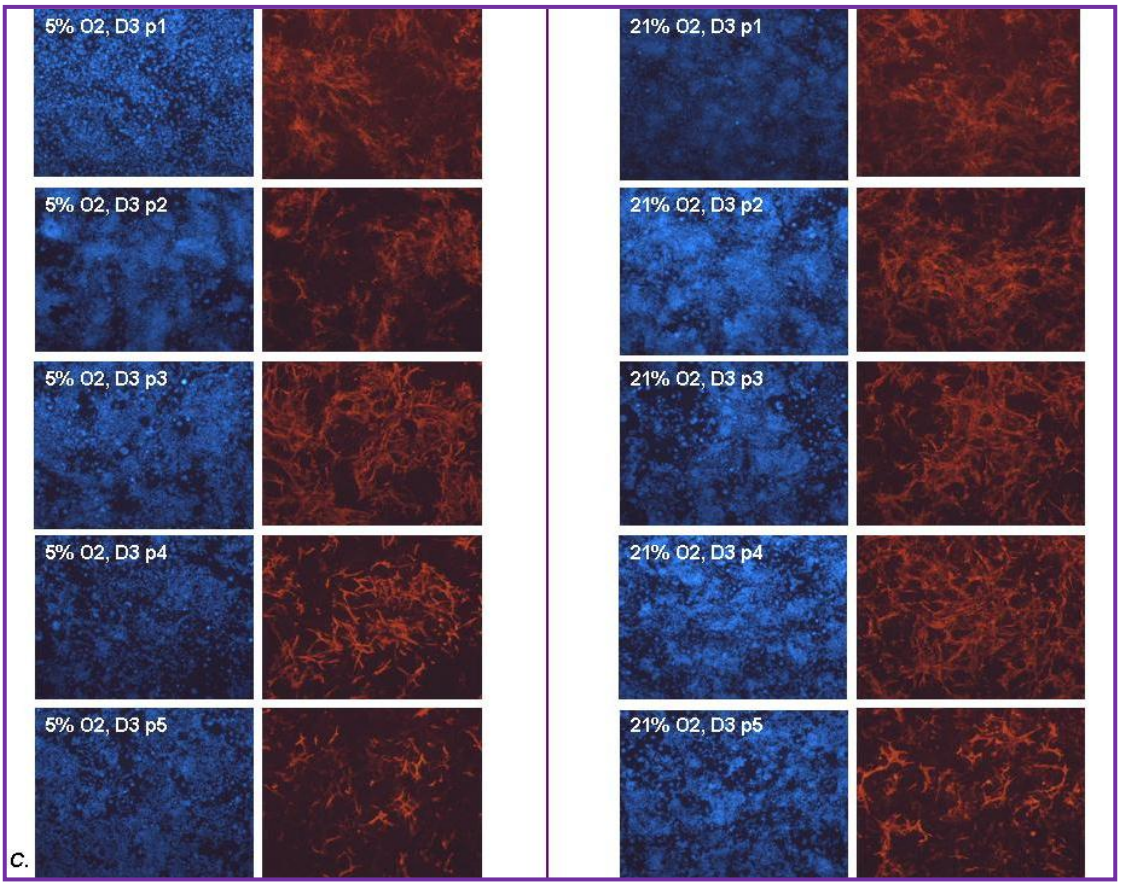


Figure 14.
A. **Overview of the results obtained from the Oct4 (green secondary antibody) and Nanog (red secondary antibody) staining in piPS cell line D3, kept in both conditions (5% and 21% O_2), over five passages, microscopic images, 10x objective;**
B. **Oct4- and Nanog-positive D3 colony, cultured at 21% O_2, microscopic images, 40x objective;**
C. **Oct4- and Nanog-positive D3 colony, cultured at 5% O_2, microscopic images, 40x objective.**

(Figure 15)


5% O2, A1 p1
5% O2, A1 p2
5% O2, A1 p3
5% O2, A1 p4
5% O2, A1 p5
21% O2, A1 p1
21% O2, A1 p2
21% O2, A1 p3
21% O2, A1 p4
21% O2, A1 p5
A.


5% O2, B4 p1
5% O2, B4 p2
5% O2, B4 p3
5% O2, B4 p4
5% O2, B4 p5
21% O2, B4 p1
21% O2, B4 p2
21% O2, B4 p3
21% O2, B4 p4
21% O2, B4 p5
B.



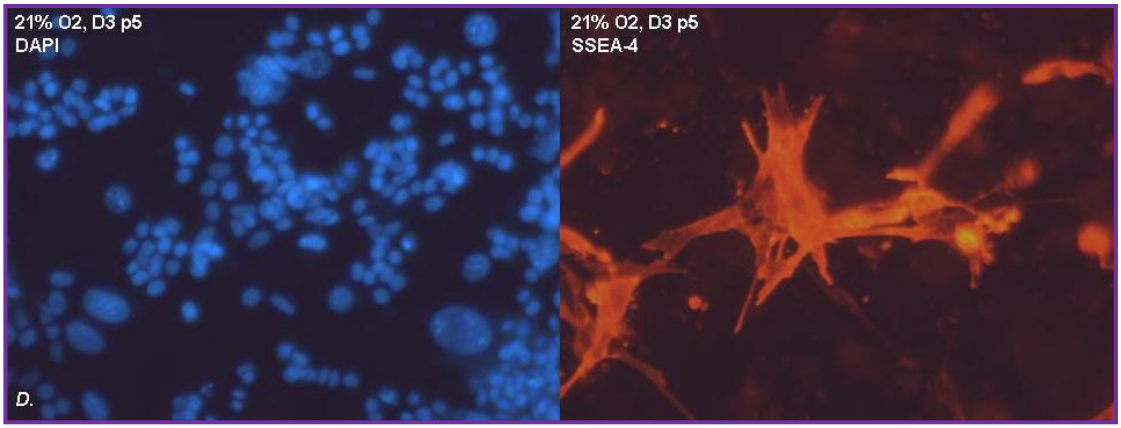


Figure 15. Overview of the results obtained from the SSEA-4 staining in piPS cell lines, kept in both conditions (5% and 21% O_2), over five passages.
A. Cell line A1, microscopic images, 10x objective
B. Cell line B4, microscopic images, 10x objective
C. Cell line D3, microscopic images, 10x objective
D. Cell line D3, microscopic images, 40x objective

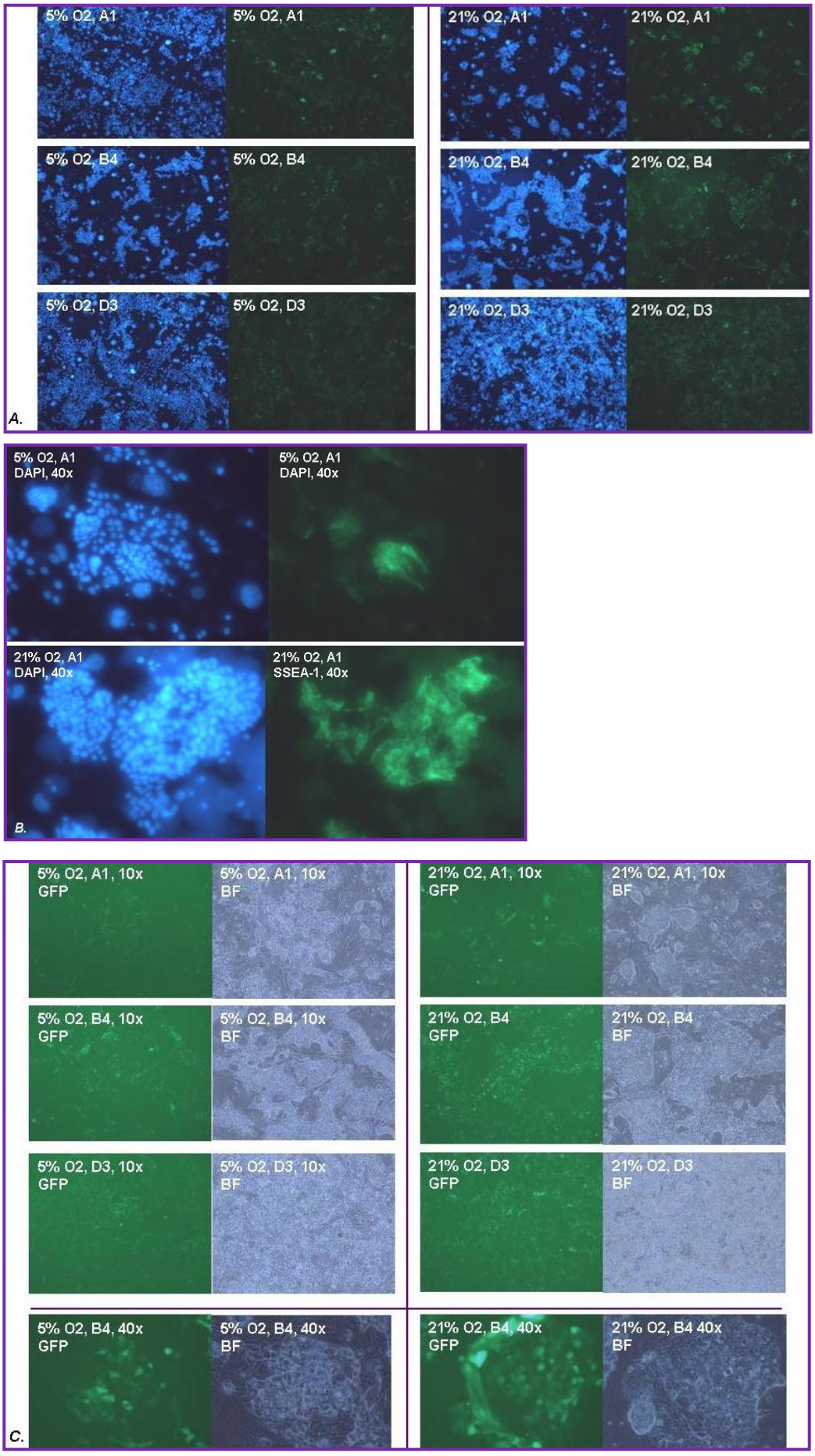
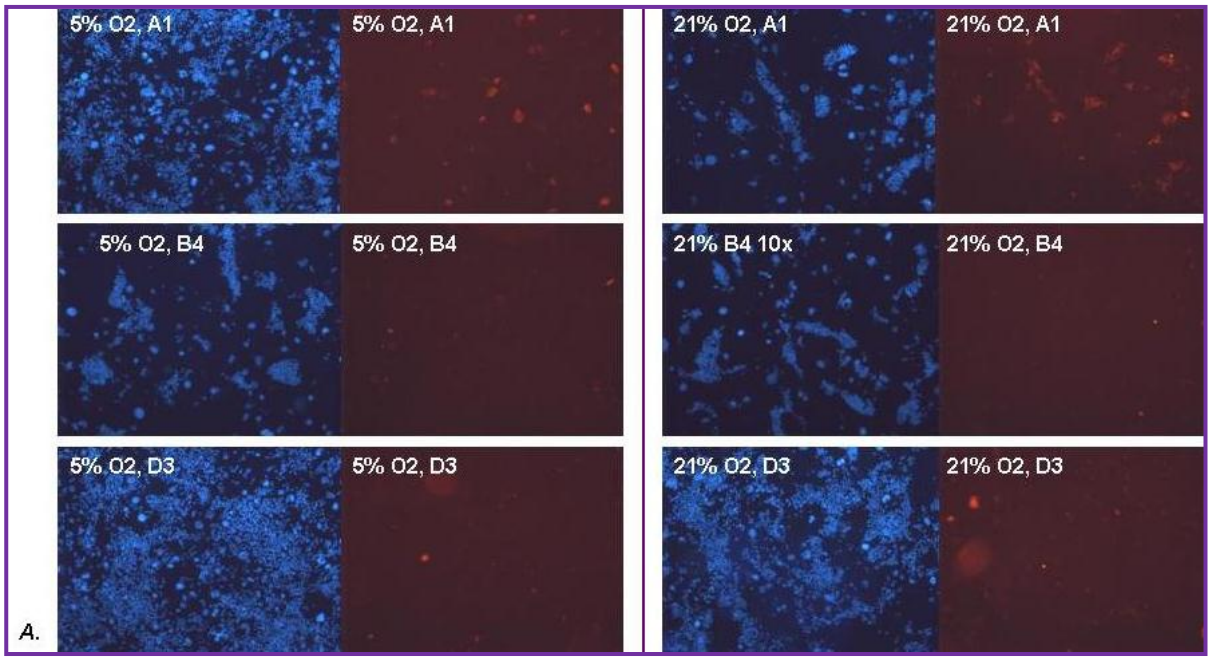


Figure 16. *A.* Microscopic images taken after SSEA-1 staining using green secondary antibody, 10x objective; *B.* SSEA-1 positive A1 colonies in 5% and 21% O_2 respectively, 40x objective; C. GFP-expression in all 3 cell lines.



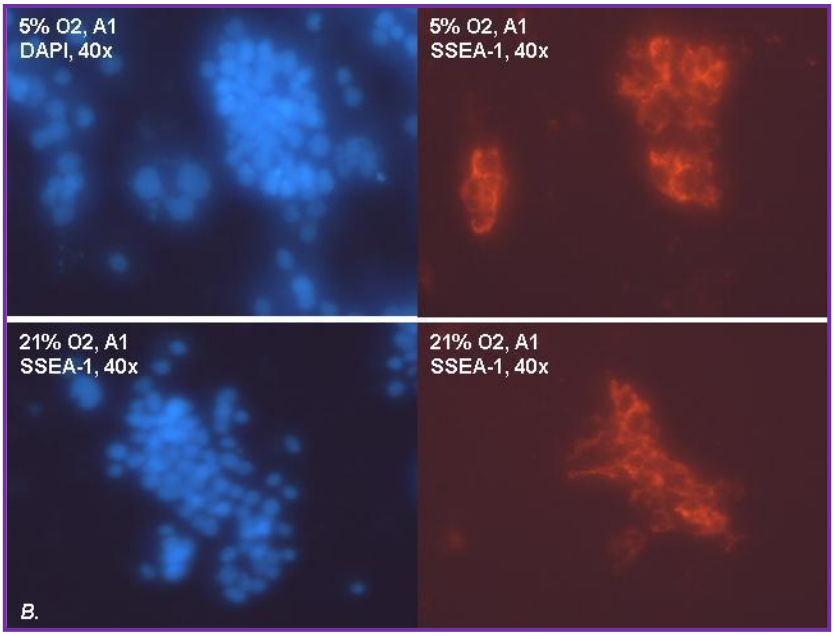


Figure 17.*A*. Microscopic images taken after SSEA-1 staining using red secondary antibody, 10 x objective. A1 is the only cell line in which SSEA-1 positive colonies were observed using the red secondary antibody; *B*. SSEA-1 positive A1 colonies in 5% and 21% O_2 respectively, 40 x objective.

3.6 Results from screening for pluripotency markers and level of transgene silencing by RT-PCR

The piPSC cDNA samples were screened for the pluripotency factors Rex1, endogenous Klf4, endogenous Oct4 and Nanog. For the analysis of the level of transgene silencing, RT-PCRs were performed to analyse the expression of transgenic Oct4, transgenic Klf4, transgenic Sox2 and transgenic c-Myc.

All RT-PCR's were performed on the following piPSC cDNA samples (from passage 1 and 5 of the first experiment):

- 5% O_2 A1, p.1
- 5% O_2 A1, p.5
- 5% O_2 B4, p.1
- 5% O_2 B4, p.5
- 5% O_2 D3, p.1
- 5% O_2 D3, p.5
- 21% O_2 A1, p.1
- 21% O_2 A1, p.5
- 21% O_2 B4, p.1
- 21% O_2 B4, p.5
- 21% O_2 D3, p.1
- 21% O_2 D3 p.5

In addition, no-RT samples of the piPSC were included in some of the RT-PCRs as well.

First, the piPSC cDNA was tested in two Actin RT-PCRs; one Actin RT-PCR included all piPSC cDNA samples plus the corresponding no-RT samples, the other Actin RT-PCR included all piPSC cDNA samples plus corresponding 1:5 dilution of each of the cDNA samples. The Actin RT-PCRs were included to confirm that the preparation of cDNA had been successful, and that equal amounts of product across the samples had been used (given the fact that the results showed a linear range).

For all RT-PCRs of the pluripotency factors, cDNA from a pig embryo (E38) was used as a positive control.

The positive controls for the transgene-expression included plasmid pMαs-Oct4, plasmid pMs-Klf4, plasmid pMs-Sox2 and plasmid pMs- c-Myc respectively. In all RT-PCRs, ddH_2O was used as a negative control. In all RT-PCRs, a sample of cDNA from mESCs (cell line E14) was included as well to confirm pig specificity.

The first Actin RT-PCR was done on 24 samples: all 12 piPSC cDNA samples and the corresponding –RT samples). The result is shown in *Figure 19.C*. The Actin RT-PCR was repeated with 24 samples including all 12 piPSC cDNA samples, and a 1:5 dilution of all of these samples. The result of this RT-PCR is shown in *Figure 20.D*.

With regards to the analysis of the pluripotency factors by RT-PCR, it can be concluded that the expression of Rex1 was not detected in any of the samples, while the other factors were expressed in all samples (*Figure 19.A.*). Some variation was seen in the brightness of the RT-PCR bands, but no obvious differences between the two oxygen conditions were seen. *Figure 19.B.* shows the results from the RT-PCRs performed to investigate the level of transgene silencing. No differences between the two oxygen conditions were found in the level of silencing of Sox2. However, for the other RT-PCRs it seemed that the transgenes were more expressed in the 5% O_2 condition than the 21% O_2 condition.

3.7 Results from EB formation, EB plating and immunohistochemistry

mESCS HM1 were used as a positive control. After analysing growth and pluripotency properties of the piPSCs, their differentiation capacity in vitro was investigated. For the EB-formation, -plating and -staining, mouse EBs (cell line HM1) were used as a positive control. The production of EBs was established in all 3 piPSC lines, but compared to the positive control (mECSs HM1) the EBs seemed to be of less quality (*Figure 20*). In particular cell line D3 formed poor EBs and their formation was accompanied by much cell death. Initially both the mouse and pig EBs were plated at a density of 20 EBs per well of a 6 well plate. After a few days, differentiation into cardiomyocytes was observed in the mouse EBs. However, for the pig EBs the density of 20 EBs per well appeared to be too low; many cells seemed to differentiate into fibroblasts. When the experiment was repeated, EBs were plated out at various densities (up to a maximum of 150 EBs per well), which seemed to result in differentiation into other cell types as well. The

plated EBs looked best in cell line A1 (5% O_2); dense, rounded EBs were visible with outgrows of piPSCs from the EB-structures (F*igure 21.A.*). Such a spherical shape of EBs was not observed in B4 nor in D3. In the lines B4 (21% O_2) and A1 (5% O_2) differentiation in neurons was clearly observed using bright field light microscopy, while in D3, much cell death was observed in both conditions (*Figure 21*). Plated EBs were fixed and stained for the ectodermal marker β-tubulin (β-TB). Details on the antibodies used can be found in the 'materials and methods' section. In all the lines (B4: 21% O_2, D3: 5%O_2, and A1:5% O_2) small patches of neuronal differentiation were found using UV-fluorescent microscopy after staining for β-TB. However, although staining of the plated EBs revealed the presence of neurons in D3 as well, only one small patch of neurons has been found in this cell line, and only in the 5% O_2 condition. The rest of the D3-cells stained weakly for the ectodermal marker in both conditions (*Figure 22*). B4 and A1 stained clearly positive for β-TB (*Figure 22*). No obvious differences between the two oxygen conditions were observed.

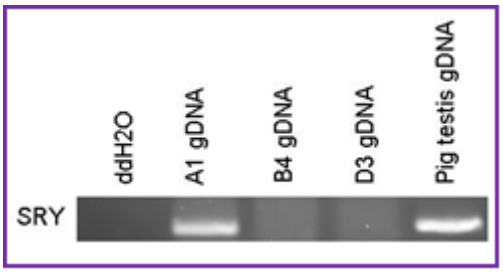
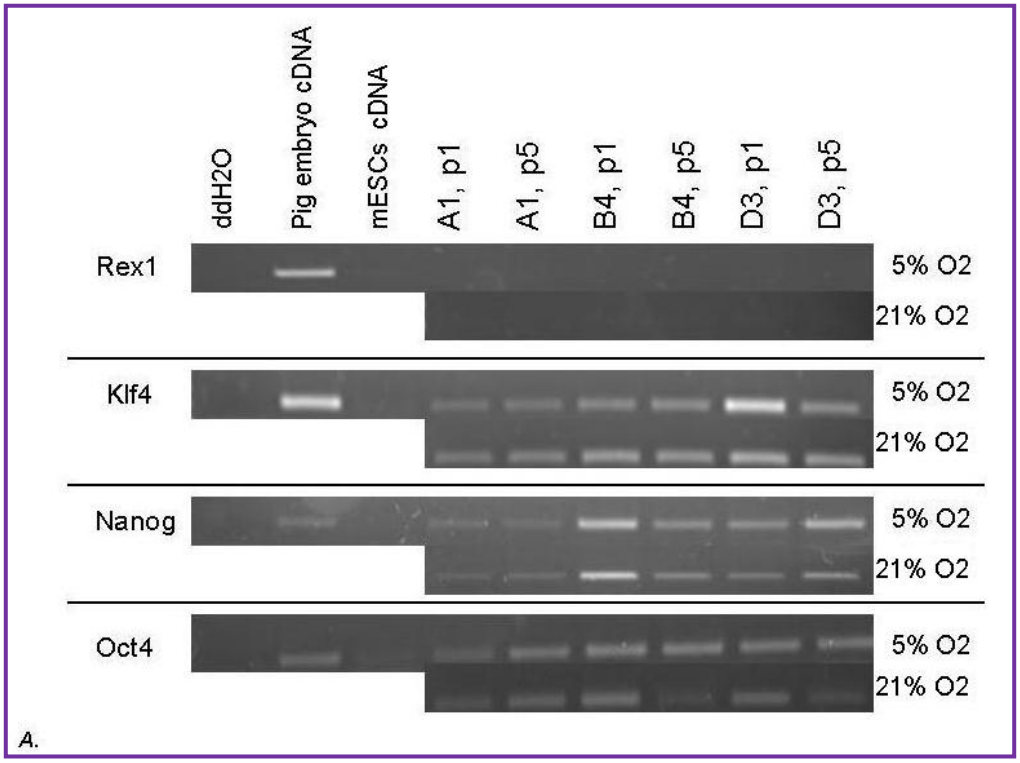


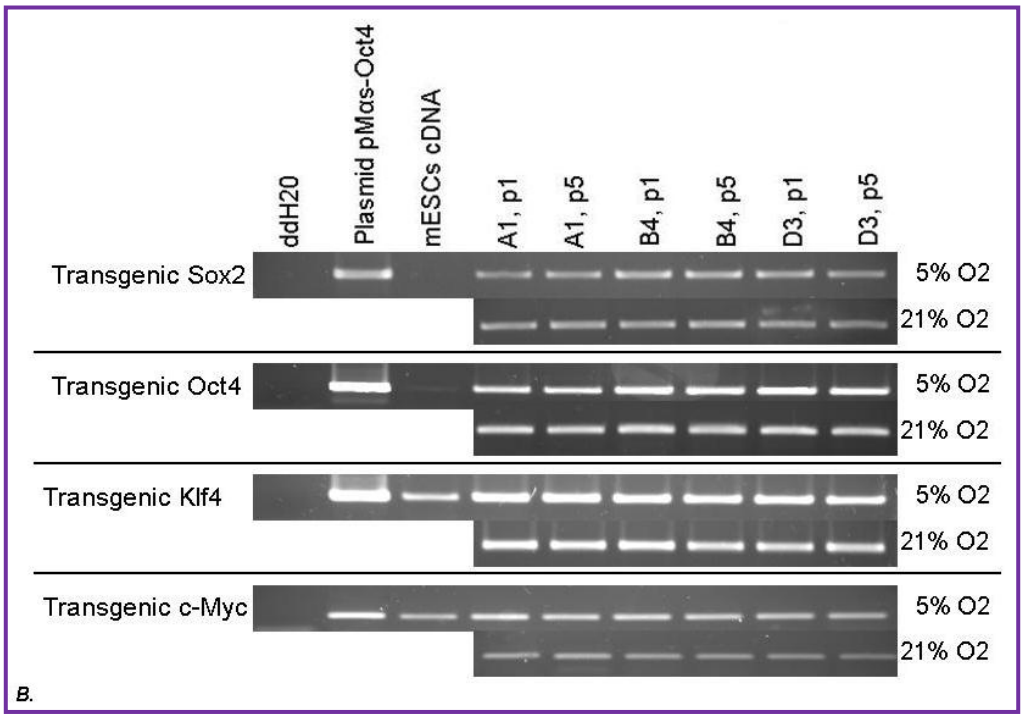
Figure 18. SRY-PCR in piPSCs

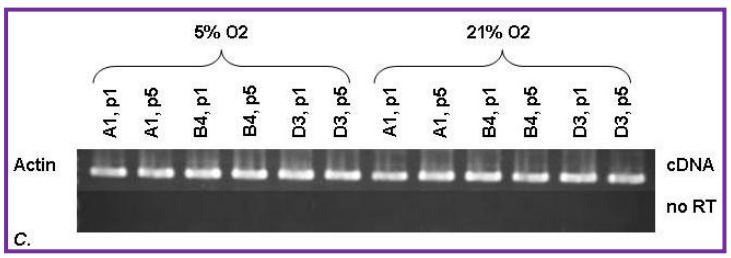
3.8 Results from SRY PCR

First, an actin PCR was performed on 5 samples:
- ddH_2O (negative control)
- gDNA piPSC line A1
- gDNA piPSC line B4
- gDNA piPSC line D3
- gDNA pig testis (positive control)

This was done to make sure that the gDNA of all samples used, was good. Then, an SRY PCR was performed on those 5 samples.
As can be concluded from *Figure 18*, B4 and D3 do not show a band, which indicates that these cell lines do not express the SRY gene. B4 and D3 are likely to be female cell lines . A1 on the contrary, shows a band similar to the male positive control (pig testis), which means that A1 has the SRY gene and is thus male.







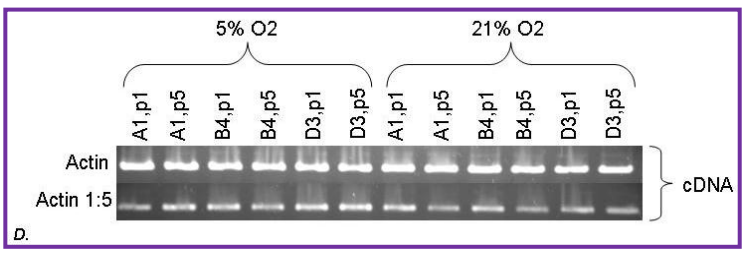


Figure 19.*A*. Screening for pluripotency markers; *B*. Level of transgene silencing; C. Actin RT-PCR on DNA and no RT samples of piPSCs; D. Actin RT-PCR on cDNA samples, a 1:5 dilution of the samples was included.

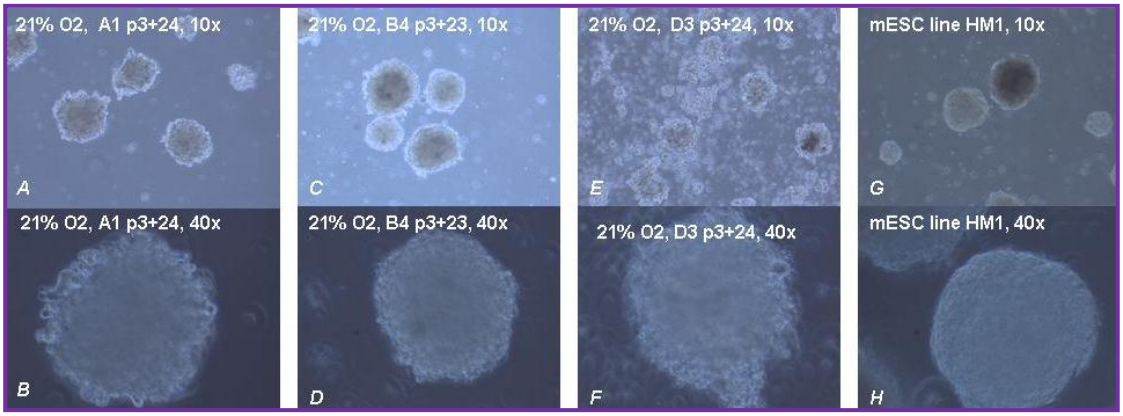
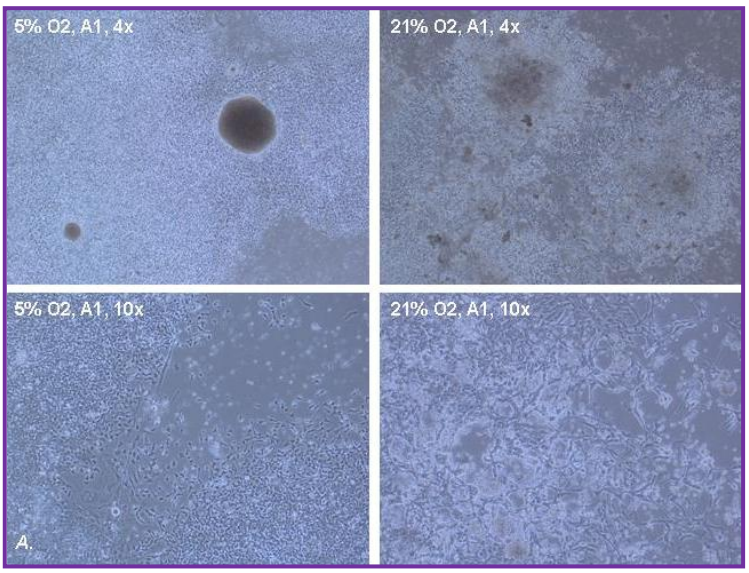
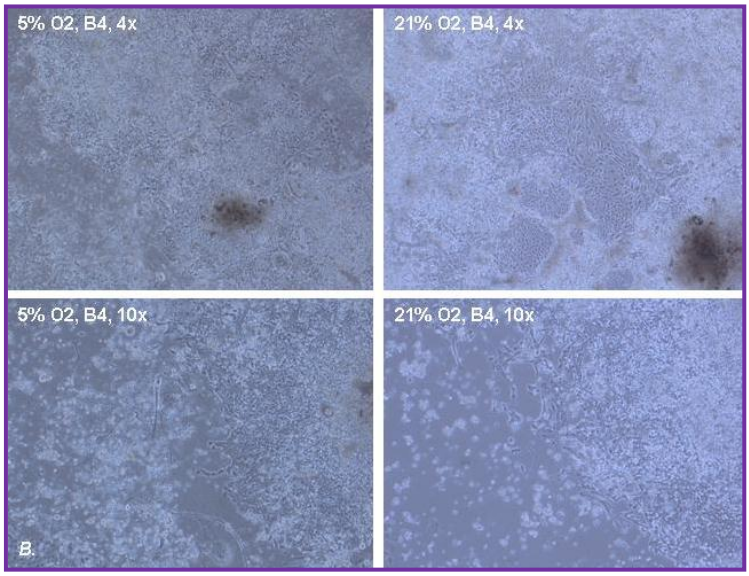


Figure 20. Bright field microscopic images (10x and 40x objective) of EBs in piPSC lines A1 (A and B), B4 (C and D) and D3 (E and F) and in mESC line HM1 (G and H).





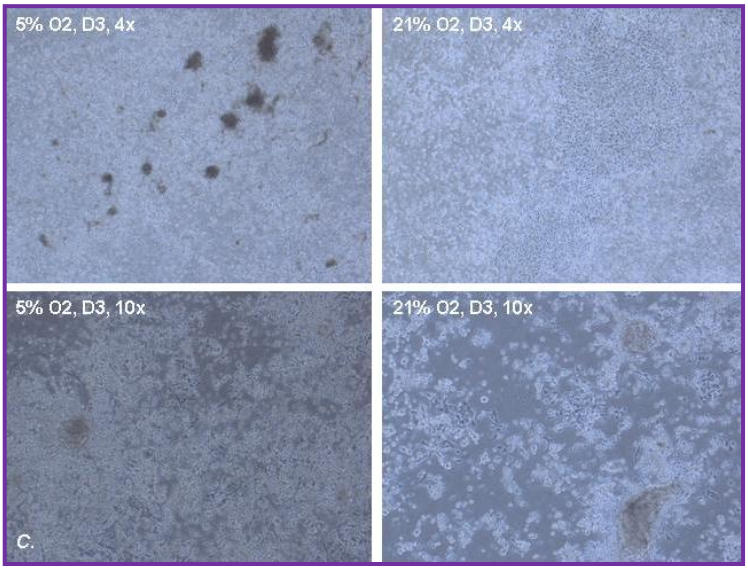


Figure 21. Bright field microscopic images (4x and 10x objective) of plated EBs of piPSC lines.
***A.* piPSC line A1;**
***B.* piPSC line B4;**
***C.* piPSC line D3.**

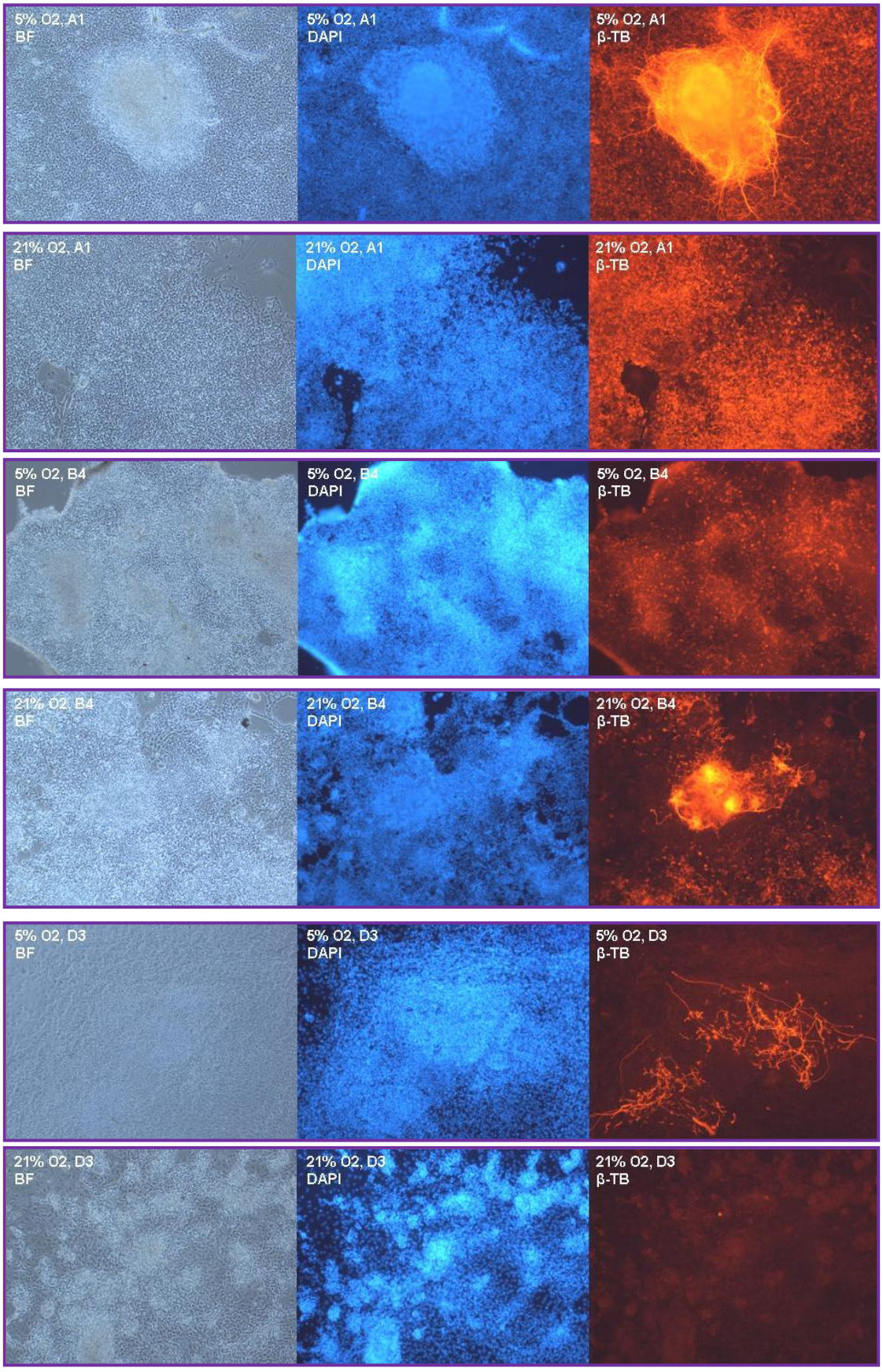


Figure 22. Immohistochemistry: β-Tubulin (β-TB). Overview of the staining results for the ectodermal marker β-TB in the piPSC lines, kept in 5% and 21% O_2. Microscopic images, 10x objective

4. DISCUSSION

The aim of this project was to investigate whether culturing piPSCs in 5% versus 21% O_2 enhances their reprogramming.

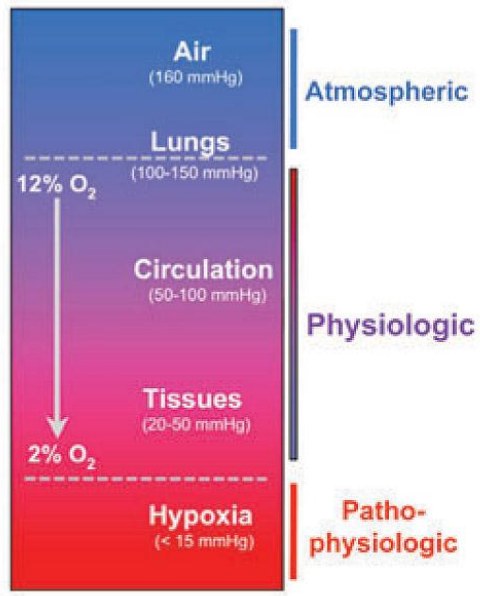


Figure 23. Physiologic oxygen state of adult and embryonic cells in vivo[58]

4.1 Why low oxygen?

As stated in the *Introduction* section of this report, mammalian embryos develop in a relatively hypoxic environment in comparison to the atmospheric environment[55]. The physiologic O_2 state of adult and embryonic cells in vivo is generally considered to range from 2-12 %, although it must be mentioned that different reports have described different ranges (*Figure 23*)[58]. Anyway, the physiologic oxygenation level is usually much lower than the atmospheric O_2 concentration[59]. Apart from the role of O_2 as an ATP supply, O_2 also seems to be of great importance in terms of regulation of cell fate in some developmental processes [59, 60]. O_2 levels can promote as well as inhibit the diffenrentiation of stem cells into certain cell types, and O_2 is thought to act in a concentration dependent manner[59]. For example, placental trophoblast stem cells in murine species, turn into spongiotrophoblast cell types instead of trophoblast giant cell types, when cultured at low O_2 instead of environmental O_2[59]. For several other examples of the influence of O_2 on cell fate, the reader is referred to a review published by Simon & Keith (2008) [59].

Earlier reports describe the detrimental effects of 21% O_2, and the advantages of a low O_2 concentration compared to an atmospheric O_2 concentration, on the in vitro development of porcine blastocysts[61-63].

Moreover, there is growing evidence for the positive effects of low O_2 concentration on reprogramming of somatic cells and the maintenance of pluripotency[60]. For instance, as already mentioned, Yoshida et al (2009) reported an increased reprogramming efficiency of MEFs and human dermal fibroblasts; in both cases iPSCs could be generated more quickly under 5% O_2 than under atmospheric O_2[50]. This suggests that the level of oxygenation has influence on the developmental state of pluripotent stem cells[59].

In this research project, a number of assays was developed and performed in order to analyse growth rate, colony morphology, pluripotency, in vitro differentiation and transgene silencing in three different porcine iPSC lines, which were all cultured in the two different O_2 conditions.

4.2 Growth rate

From the initial experiments performed to investigate the influence of the O_2 concentration on cell growth, the major finding was a significant difference in growth rate; at the end of each passage more cells were counted in the 21% than in the 5% O_2 condition. This was probably due to a higher proliferation rate of cells grown in 21% O_2 (*Figure 7 & 8*).

This observation is comparable with several studies performed on hESCs/hiPSCs as well as on mESCs. Abaci et al. (2010) described that the proliferation rate of hESCs and hiPSCs was slower in 5% O_2 in comparison to 21% O_2, although the results in hiPSCs were not found to be significant[60]. Chen et al (2009) also reported that the proliferation rate of hESCs grown in 5% O_2 was relatively slow in contrast with the growth rate of 21% O_2 cells[64]. In addition, Kurosawa et al. (2006) compared the

proliferation rate of mESCs cultured under 5% versus 21% O_2 and concluded that the proliferation rate of the cells was supressed upon cultivation in 5% O_2[57].
However, data regarding the proliferation rate of pluripotent cells and the influence of a low O_2 concentration are conflicting.
For example, Ezashi et al. (2005) found no difference in the cell growth rate of hESCs cultured at either 3-5% O_2 versus 21% O_2[56]. Only at a 1% O_2 level, the proliferation of cells was found to be reduced, but it remains unclear whether this difference is significant or not [56].
On the other hand, Yoshida et al. (2009) reported an increase in the number and percentage of GFP-positive colonies in the 5% O_2 condition in both hiPSCs and miPSCs, when compared to the 21% O_2 condition[50]. However, cultivation under 1% O_2 resulted in a decrease in the number of colonies of hiPSCs, whereas a 1% O_2 concentration yielded no effect on miPSCs in this study[50]. In contrast to that, Abaci et al. (2010) reported an increase in proliferation rate in hESCs grown in 1% O_2[60].
From the above, it can be stated that the experiments conducted so far, do not provide significant evidence for the pros or cons of a low O_2 tension on growth rate, at least in human and mouse derived cells.
In the current experiment, the difference between the number of piPSCs kept in 5% versus 21% O_2 gets smaller as time progresses, due to a decline in the amount of cells in the 21% O_2 condition (*Figure 7*). From the subsequent cell count experiment (1 day after plating), it was shown that plating efficiency on its own is not responsible for the observed difference in growth rate, since minor differences regarding the plating efficiency between the two O_2 conditions were found (*Figure 8*). This makes it highly likely that the difference in cell number between the two O_2 conditions is primarily caused by a higher proliferation rate of the cells in 21% O_2. The reason why the number of cells at the end of each passage decreases in the 21% O_2 condition, remains unclear for now. It might be possible that cells that grow fastest are somehow selected out, due to loss of stem cells or an increase in cell differentiation for example, but in order to further investigate the cell growth in both conditions, more accurate, quantitative measurement of cell proliferation would be needed.

4.3 Colony morphology

In addition to a difference in growth rate in all cell lines, a slight morphologic difference between cells cultured in 5% versus 21% O_2 was found in cell line A1 alone. This difference in colony morphology was found using light microscopy for unstained living cells and AP-stained cells (bright field) and fluorescence microscopy for the immuno-stained cells (UV). A1 cells grown in 21% O_2 resulted in rather big an flattened colonies with rough colony borders, while cultivation of A1 cells in 5% O_2 lead to the formation of well-bordered, compact dome-shaped colonies. In comparison to the 21% O_2 colonies, the 5% O_2 colonies were relatively small.
The observation that alteration of the O_2 is able to induce differences in colony morphology, is supported in the literature. Peura et al. (2007) studied the growth of hESCs in reduced (5%) versus atmospheric O_2, and found hESC colonies grown in low O_2 tension to be twice as big as hESC colonies cultured in 21% O_2[65]. According to Ezashi et al. (2005) a reduced O_2 concentration has been shown to prevent differentiation of hESCs, which was concluded after analysis of the morphology of hESCs colonies grown in both 5% and 21% O_2[56].
Cells from the other two piPSC lines (B4 and D3) appeared to be morphologically similar in the two O_2 concentrations. Similar results, whereby reduced O_2 levels do not seem to influence colony morphology, have previously been reported in the literature. Yoshida et al. (2009) reported that in their miPSCs nor in their hiPSCs, differences in colony size nor colony morphology were observed between low or ambient O_2 tensions [50]. Initially, the findings of Chen et al. (2009) were similar to those of Yoshida et al. (2009), however, after prolonged culture it was found that hESCs cultured in 5% O_2 were thinner to some extent, and more transparent and fragile as well, and the colonies grown in 5% O_2 were

thought to look better than their 21% O_2 counterparts[64].
Since the morphologic difference in the current study was consistently observed in cell line A1, it is supposed to be caused by the difference in O_2 conditions.

4.4 Pluripotency: alkaline phosphatase (AP)-staining

With regards to the AP-staining in the cell lines B4 and D3, no obvious differences were found between the two O_2-conditions (*Figure 9.A, B, D and E)*). However, as can be concluded from *Figure 7.B and C*, the number of cells in B4 and D3 wells grown in 5% O_2 is consistently lower at the end of each passage compared to the wells cultured in 21% O_2. Thus, when the amount of cells per well is taken into account, it is remarkable that wells cultured in 5% O_2 sometimes stain as good as, or even more strongly than wells cultured in 21% O_2. This could be explained by the possibility that although there are fewer cells present in the 5% O_2 wells, the cells stain more intense than cells grown in 21% O_2 (*Figure 9D and E*).
A more pronounced difference was found in cell line A1: there seemed to be more staining in the 21% O_2 condition compared to the 5% O_2 condition (*Figure 9.A,B and C*). But again, the number of cells per well must be taken into account, since – like in the other two cell lines – the number of cells in A1 wells grown in 5% O_2 is lower than that of wells grown in 21% O_2 (*Figure 7.A*) Therefore, the more intense staining observed in A1 cultured in 21% O_2 might be explained by the fact that there are more cells rather than there is more intense staining of the individual cells.
Besides, as mentioned, staining in the flattened 21% O_2 colonies was more widespread, whereas the staining in the compact 5% O_2 colonies seemed more intense, probably due to the higher cell density in the latter colonies. Thus a difference in colony morphology might explain the difference in staining results as well.
NB. The different passages will have been stained on different days and therefore there will be variation in the intensity of AP due to this. When analysing the AP staining, this is an issue if you are comparing between passages, not if you are comparing between the two O_2-concentrations at the same passage or between lines at the same passage.
Yoshida et al. (2009) demonstrated that the number of AP-positive colonies increased in the wells containing miPSCs cultured in 5% O_2 compared to the 21% O_2 wells[50]. Likewise, more AP-staining in mESCs cultured in 5% rather than in 21% O_2 was described by Gibbons et al. (2006)[66].
As opposed to those results, Chen et al. (2009) observed reduced AP-staining in the 5% O_2 hESC colonies, and this observation was in accordance with the thinner colonies in 5% O_2[64]. Additionally, Kurosawa et al. (2006) reported less AP-activity in mESCs grown in 5% O_2 compared to 21% O_2 cells [57].
As is the case with growth rate and colony morphology, it can be concluded that the data published so far, regarding the influence of O_2 tension on the AP-staining, are conflicting.

4.5 Pluripotency: immunohistochemistry

The Oct4, Nanog and SSEA-4 antibody staining results revealed no clear differences between the two O_2 conditions in the cell lines B4 and D3 (*Figure 11-14*). The lack of (significant) differences with regards to the expression of certain pluripotency genes, corresponds with the results obtained with hESCs[64]. On the contrary, Yoshida et al. (2009) reported a higher expression of Oct3 and-4 as well as of Nanog, in reprogrammed MEFs after three days of hypoxic treatment[50].
In cell line A1, the morphologic difference first found using light microscopy (bright field) was emphasized again using fluorescence microscopy after staining with Oct4 and Nanog (*Figure 12*).
Furthermore, the expression pattern of the Oct4 staining was not as homogenous in A1 as in the other two cell lines: cells in the periphery of A1 colonies stained brighter than cells in the middle of colonies. From the co-staining with Nanog, it was concluded that the bright Oct4 positive cells are also positive for Nanog, but a bright Oct4 positive signal was not necessarily accompanied by a strong Nanog

positive signal as well (*Figure 11*). What this means in terms of the level of reprogramming, is not entirely clear. One of the possible explanations is that these bright staining peripheral cells are in a less reprogrammed state than cells in the centre of colonies. In that case, the Oct4 staining does not only reflect the level of endogenous porcine Oct4, but is also caused by the persistent expression of transgenic Oct4, which all together results in strong Oct4 positive staining cells. If this hypothesis would be true, then cells in the centre of A1 colonies would represent the more developmentally naïve cells, which naturally would be very interesting. Further analysis of the staining in cell line A1 would be needed to either confirm or reject this explanation.

Apart from the Oct4 staining, cell line A1 distinguished itself also from B4 and D3 with respect to the SSEA-1 staining, since it was the only cell line found to stain positive for this cell surface marker.

As mentioned in the *Introduction,* SSEA-1 is known to be a pluripotency marker in mESCs/miPSCs (*Figure 10*). The fact that SSEA-1 was only expressed in cell line A1, suggests that this cell line resembles mESCs more than the other two piPSC lines do. This might subsequently indicate that A1 was in a more naïve state rather than a primed state. Regardless, no differences between the two O_2 conditions were found within this cell line.

4.6 Pluripotency: reverse transcriptase (RT)-PCR

Apart from the staining experiments, pluripotency of the cells was also measured using reverse transcriptase (RT)-PCR (*Figure 19.A*).

RT-PCR has been an important tool in the analysis and semi-quantification of mRNA expression in different samples for many years[67]. In short, RT-PCR is based on the generation of cDNA from mRNA samples using the enzyme reverse transcriptase, and the subsequent amplification of cDNA by running the RT-PCR. The amplified cDNA has been visualized according to the conventional method, i.e. after electrophoresis, staining with ethidium bromide, and analysis of the bands using UV-light[68] .

Although RT-PCR can point out some differences, the RT-PCR technique has serious limitations in terms of reliability and quantification. The accuracy of experimental procedures prior to running the RT-PCR can lead to variation in the amount and quality of starting material, which includes, the RNA itself, the reverse transcriptase and several reaction components, and this strongly influences the outcome of the RT-PCR[69]. Also, post-amplification procedures are involved in RT-PCR, which gives room for human error, for instance by getting cross-contamination of the samples during the loading on the gel[69]. Besides that, post-amplifications procures are time-consuming as well.

Since the conventional RT-PCR does not result in quantitative data, it could be more difficult to interpret the obtained results. Therefore, a Real-Time or Quantitative RT-PCR (Q-RT-PCR) would have been far more suited for the quantitative detection of any differences in mRNA expression between cells grown in 5% versus 21% O_2. With Q-RT-PCR it is possible to visualize the amount of product that is being formed using fluorescent dyes, during the Q-RT-PCR reaction[70]. The technique is extremely sensitive and differences in gene expression as small as 23% can be revealed[70]. The major objection to Q-RT-PCR are the high costs of equipment and reagents[69].

A final point of improvement involves the use of control genes. During this project Actin was used as a control gene and as a positive control for the reaction. Nevertheless, the reliability of Actin – which is commonly used, despite the evidence that its expression varies widely in porcine tissues – is questionable, since it has been shown to be affected by several biological and experimental processes[67, 69]. When normalization is established by using one single control gene, it is thought that the results can be misrepresented, and it is therefore advised to use more than just one control mRNA gene, or use ribosomal RNA as a reference, by some researchers [67, 69]. (*More information on this can be found in papers published by Vandesompele et al. (2002) and Gu et al. (2011)*) [75, 76].

In conclusion, RT-PCR might give an indication of existing differences, but a Q-RT-PCR analysis should be performed, in order to further analyse and quantify the observations. Any conclusions regarding the presence of (significant) differences between the two O_2 conditions can therefore not be made.
From the RT-PCR results it can only be concluded that Rex1 is not expressed in the cells, no matter in what O_2 concentration the cells have been cultured. Rex1 is known to be expressed in bona fide hiPSCs, and thus expression of this pluripotency marker could therefore probably have indicated a more developmentally naïve state[71].

4.7 In vitro differentiation

All three piPSCs in both O_2 conditions were tested for their in vitro differentiation capacity by the formation, maintenance, plating and staining of EBs.
Potonick et al. (1994) reported that a 5% O_2 concentration resulted in the formation of more mouse EBs than in an ambient O_2 concentration[72]. Similarly, Ezashi et al. (2005) reported that a significantly greater number of human EBs was formed from cells that had been cultured in 5% compared to the cultivation in 21% O_2[56]. Also, EBs cultured in 21% O_2 were found to attach at lower efficiency than 5% O_2 EBs upon plating[56]. On the contrary, other research groups concluded that O_2 tension does not seem to be play a role, after they observed no differences in formation, plating and staining of mouse or human EBs cultured under high versus low O_2 tension[57, 64].
When a comparison was made between the two O_2 conditions within each cell line in this project using porcine cells, no clear difference was found, apart from the interpretation that the EBs looked particularly well in cell line A1 in 5% O_2, compared to other cell lines in both conditions (*Figure 20.A*). This might correlate with other changes observed in cell line A1 at 5%. However, EBs were only stained for the ectodermal marker β-tubulin and hence no information is available on the differentiation into the other two germ layers. This makes it rather difficult to draw firm conclusions. Therefore, when this experiment would be repeated, staining with endodermal and mesodermal markers should be included as well.
To date, no reports have been published which describe the influence of O_2 tension on the characteristics of EBs formed out of porcine pluripotent cells, and the data published on human and mouse pluripotent cells seem to be adversary. Further studies are needed to clarify the role of O_2 on the in vitro differentiation capacity.

4.8 Transgene silencing & RT-PCR

Up till now, all research groups working on piPSCs, reported the persistent expression of the transcription genes used to reprogram the cells[3, 5, 13].
During this project, RT-PCR was used to investigate the level of transgene silencing and to detect any differences in transgene silencing in cells cultured in 5% versus 21% O_2. The limitations of the interpretation of RT-PCR results discussed above, apply to the analysis of transgene silencing as well. In the current research project, lack of transgene silencing was also observed, regardless of the O_2 level (*Figure 19.B*).
It might be possible that silencing develops gradually during the transition from a differentiated state towards an iPSC state[11]. Among the factors that might explain the lack of transgene silencing observed, are the possibilities of suboptimal culture conditions and permanent genome modification caused by retroviral integration – both subjects are discussed in the *Introduction* section –, lack of stem cell state in pig cells, or lack of mechanisms present in pig cells to induce transgene silencing.
Part of the research concerning piPSCs is currently focused on finding feasible ways to achieve transgene silencing. It has already been shown by other research groups that the degree of silencing is less when using lentiviruses versus retroviruses, although lentiviruses seem to increase the efficiency of iPSC generation[10]. Silencing could be established by various ways which includes the deletion of transgene sequences (for instance by using so-called

piggyBac insertions[50]), manual silencing, and avoidance of integrating vectors used to transduce the reprogramming factors[2, 5, 9].
The chosen cell type for reprogramming might also influence the degree of silencing[3].
In the report published by Wu et al. (2009), it has been described that the expression of exogenous reprogramming genes is under the control of doxycycline; upon withdrawal of this drug, the expression of reprogramming genes was immediately down-regulated, resulting in differentiation of the pig cells[13] .
This system resulted in the temporal control over transgenes[11]. When applying a system that relies on the withdrawal of suppression of transgene expression, one can automatically select the fully reprogrammed cells because cells relying on transgene expression will be eliminated[11].

4.9 Future directions

From the above discussion of the results, it can be concluded we have shown no advantage of a low O_2 concentration compared to a high O_2 concentration.
If t his study was to be repeated, it is useful to make some changes and possibly add some experiments, which would make the outcomes of the study more convincing, quantitative and reliable. (NB. Due to lack of time, the potential follow-up experiments mentioned below, could not be incorporated in this project.)
During this project, cells were incubated in standard tissue culture incubators, with CO_2 concentrations in the range of 5-7% and an standard 'air' O_2 concentration of 21% [73]. In order to achieve a 5% O_2 tension, the concerning plates were transferred to airtight pots, which were thereafter gassed for exactly two minutes. This procedure is probably not very accurate. Moreover, all laboratory operations – including the frequent medium changes and the passaging of cells – took place in 21% O_2, which made it necessary to switch the 5% O_2 cells back and forth between two different O_2 tensions repeatedly. This has probably led to fluctuations in the O_2 concentration for those cells cultured at 5% O_2[50]. The fluctuations may in turn have caused oxidative stress, which is known to result in DNA mutations and/or lesions and premature senescence in tissue culture[73]. When all laboratory operations would have been performed under the same O_2 level as the level in which the cells are grown, the more strict control could have led to more accurate conditions en thus more precise and reliable results[50].
Since the immortality of ESCs and iPSCs is partly due to the expression of telomerase, it would be interesting to measure the level of telomerase activity or telomere length as well, and compare the results between the two O_2 conditions[64, 73]. If low O_2 tensions would significantly improve immortality of cells, one could reasonably expect higher telomerase activity or longer telomeres in cells cultured at low versus ambient O_2 levels, for example.
Besides, when one would decide to repeat this experiment, it could be relevant to investigate the expression of hypoxia-related genes, like hypoxia-inducible factor (HIF)-1-α [59, 60, 64].
True hypoxia occurs when the O_2 concentration gets below the physiological O_2 tensions[60]. The transcription factor HIF-1-α is one of the indispensable factors which are crucial for the maintenance of O_2 homeostasis in cells and whole tissues, and it has been demonstrated that HIF-1-α protein accumulates in response to hypoxia[59, 60].
However, extreme hypoxia does not have any beneficial effects at all; when O_2 levels are extremely reduced, cytotoxic effects are induced and growth arrest and even apoptosis may occur[50, 50, 64, 74]. Although it must be mentioned that the susceptibility for hypoxic conditions varies among different cell types[50].
Also, when cells are cultured under reduced O2 levels, it is highly likely that apart from the pluripotent cells, the feeder cells get influenced by the hypoxic conditions as well. To exclude the possibitlity that the effects observed in piPSCs are in fact established by stimulating effects on the feeder cells – either positive or negative –, one might want to set up a feeder-free culture of the piPSCs [50]. In that case, the results obtained from experiments with piPSCs on feeders and feeder-free piPSCS could be compared.
In the context of epigenetics, it is useful to

study the state of X-chromosome inactivation, since the presence of two active X-chromosomes is thought to be an important hallmark of full pluripotency in female cells[27, 52]. The results obtained after screening for the SRY gene in the piPSC lines A1, B4 and D3, indicate that cell line A1 is the only male cell line, since it is the only cell line expressing the SRY gene after analysis using PCR (*Figure 18*). However, the three piPSC lines used in this project have originally been derived from the same male foetus. This means that the other two cell lines B4 and D3 cannot possess two X-chromosomes, and the lack of SRY-expression in those cell lines is probably due to loss of their SRY-gene over time. In future studies, the use of female-derived cells might be advantageous over the use of male cell lines, because of the extra useful information on the state of both X-chromosomes.

In literature, a distinction is made between derivation and culturing of iPSCs under certain oxygen conditions. Several research groups reported the derivation of pluripotent murine and human cells under physiologic oxygen, as well as under atmospheric oxygen[27, 50, 56, 57, 60, 64, 66, 73]. Also, several groups reported the culturing of pluripotent cells under hypoxic (1-2% O_2), physiologic (5% O_2), atmospheric (21% O_2) and hyperoxic (40% O_2) concentration [27, 50, 56, 57, 60, 64, 66, 73]. For any future studies, it is necessary to investigate which O_2 – concentration is optimal for the derivation and culturing of piPSCs. Lengner et al. (2010) described the derivation of hESCs in 5% and 20% O_2 from embryos which had been cultured in 5% O_2 prior to derivation of the cells. This group also showed that exposure to 20% O_2 initiated the inactivation of one X-chromosome in female cells, and that switching cells cultured under 20% O_2 back to 5% O_2, does not reverse the inactivation of the X-chromosome – which characterizes incomplete pluripotency – indicating that the X-chromosome inactivation is irreversible. Cells that were cultured under 5% O_2 maintained two active X-chromosomes and inactivation was immediately initiated upon switching of those cells to 20% O_2 conditions. Given those results, it seems impossible to generate fully reprogrammed cells when those cells have previously been cultured under 21% O_2. Conversely, later is was concluded that the process of X-chromosome inactivation in hESCs is indeed reversible; upon using the 2i-medium plus LIF, naïve hESC lines could be established, which – in contrast to primed hESCs – exhibited two active X-chromosomes[29].

All steps in the process of the derivation of the three porcine iPSC lines in the Burdon lab, were performed under 21% O_2 (unpublished data). Thus for a future experiment, it might be useful to analyse the effects of low O_2 on cells that also have been derived under low O_2. This would circumvent the issue of oxidative stress and its consequences and potential influence on the research outcomes.

Finally, as already discussed, it is recommended to apply more quantitative measurements in future experiments.

5. CONCLUDING WORDS

In summary, it can be concluded that culturing piPSCs in 5% O_2 does not seem to be clearly advantageous over culturing cells in 21% O_2. The major finding from this project is a consistent difference in the growth rate between the two O_2 concentrations. Additional differences between 5% and 21% O_2 – which were likely caused by the O_2 concentrations – were exclusively found in cell line A1 and included a difference in colony morphology, AP-staining and the appearance of the plated EBs. Furthermore, differences were found between the three different cell lines, whereby A1 showed characteristics that most closely resemble mESCs, when compared to B4 and D3. These characteristics include a typical colony morphology (tightly packed, well-defined borders and the three-dimensional dome-shaped appearance), as well as the expression of pluripotency markers Oct4, Nanog, SSEA-4 and in particular: SSEA-1.

All results observed did not seem to change significantly over time.

Further studies are needed to accurately analyse the influence of low O_2 on the developmental state of porcine induced pluripotent stem cells.

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7. APPENDIX: PRIMER DETAILS

	Annealing temperature ^{o}C	Primer sequence
Pig Nanog	55	F 5'-CCTCCATGGATCTGCTTATTC-3' R 5'-CATCTGCTGGAGGCTGAGGT-3'
Pig OCT4	67	F 5'-CAAACTGAGGTGCCTGCCCTTC-3' R 5'-ATTGAACTTCACCTTCCCTCCAACC-3'
Pig SOX2	60	F 5'-AATGCGCACAGCGCGGCT-3' R 5'-GCCCATGGAACCGAGCGT-3'
Pig KLF4	60	F 5'-GCAAAGACTTCCCCCGGTGCTT-3' R 5'-CTCCTGATGAGACAGTGAGTTGGAG-3'
Pig ZFP42	60	F 5'-GCATCTCTCCGTTCACAGTCC-3' R 5'-CAGTCTTCTTTCACTGATTTGTATTGGC-3'
Pig β-ACTIN	63	F 5'-CACGCCATCCTGCGTCTGGA-3' R 5'-AGCACCGTGTTGGCGTAGAG-3'
Mouse OCT4 transgene	56	F 5'-GGGGTGGACCATCCTCTA-3' R 5'-CCTCCGCAGAACTCGTAT-3'
Mouse KLF4 transgene	56	F 5'-CCCACCGCCCTCAAAGTA-3' R 5'-GCTGGACGCAGTGTCTTCT-3'
Mouse SOX2 transgene	56	F 5'-CCCACCGCCCTCAAAGTA-3' R 5'-GGACCATACCATGAAGGCGTT
Mouse C-MYC transgene	65	F 5'-CAGAGGAGGAACGAGCTGAAGCGC-3' R 5'- TTATGCACCAGAGTTTCGAAGCTGTTCG-3'

Appendix 1. Primer details

8. REFERENCES

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