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Derivation of porcine induced pluripotent stem cells and somatic cells gene targeting in pig

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Abstract

Large animal models are important for medical and development researches, in particular porcine models, due to their anatomical and physiological similarity to humans. However, the derivation of large animal models with precise genetic modifications has proven difficult due to the lack of pluripotent stem cells. Because of the failure to isolate porcine embryonic stem cells (ESCs), porcine induced pluripotent stem cells (iPSCs) were considered as a possible alternative choice for the generation of gene targeted pigs.

In this study, the isolation of porcine iPSCs was attempted by using several different methods. Porcine somatic cells were transfect with combinations of the reprogramming factors Oct4, Sox2, Klf4, cMyc, Nanog, N-Myc, and Lin28. Site-specific recombination or episomal vectors were used to deliver the factors into cells. Embryonic stem cell specific microRNAs were also tried to generate the iPSCs with the help of valproic acid. Putative porcine iPSCs were tested for pluripotency. Cell populations which differed in the expression of embryonic specific markers were identified, separated and assessed for their differentiation potential. The results showed that these putative iPSCs were partially reprogrammed with limited pluripotency. They could be useful for gene targeting because of their fast and extended proliferation. The achievements of this study provide a basis to develop genuine porcine pluripotent stem cells.

As an alternative approach to gene targeting in pluripotent stem cells, somatic stem cells were assessed. To improve targeting efficiency, synchronized and unsynchronized mesenchymal stem cells (MSCs) were compared, and two different gene loci were targeted, *Adenomatous Polyposis Coli* (APC) and the tumour suppressor protein p53 (TP53). Correctly targeted cell clones were identified. APC targeted cells were then used for somatic cell nuclear transfer (SCNT) and three piglets were born. Comparison of targeting efficiency showed that the synchronization did not lead to an improvement. Other synchronization methods should be tested in the future.

Zusammenfassung

Großtiermodelle sind wichtige Werkzeuge für die medizinische Forschung und Entwicklung. Von besonderer Wichtigkeit sind hierbei aufgrund ihrer anatomischen und physiologischen Ähnlichkeit zum Menschen Schweinemodelle. Allerdings gestaltet sich die Gewinnung von Großtiermodellen mit genau definierten genetischen Modifikationen wegen des Fehlens pluripotenter Stammzellen schwierig. Da es nicht möglich ist, porcine embryonale Stammzellen (ESCs) zu isolieren, wurden porcine induzierte pluripotente Stammzellen (iPSCs) als mögliche Alternative für die Generierung von genmodifizierten Schweinen in Betracht gezogen.

In der vorliegenden Studie wurde die Isolierung von porcinen iPSCs mit verschiedenen Methoden getestet. Porcine somatische Zellen wurden mit einer Kombination der Reprogrammierungsfaktoren Oct4, Sox2, Klf4, cMyc, Nanog, Lin28 und N-Myc transfiziert. Site-spezifische Rekombination oder episomale Vektoren wurden verwendet, um die Faktoren in die Zellen einzubringen. Weiterhin wurde miRNAs, die spezifisch für embryonale Stammzellen sind, mit Hilfe von auf ihre Fähigkeit zur Generierung von iPSCs getestet. Putative porcine iPSCs wurden auf ihre Pluripotenz hin untersucht. Zellpopulation, deren Expression von spezifischen embryonalen Markern auffällig war, wurden identifiziert, abgetrennt und ihr Differenzierungspotential getestet. Die Ergebnisse zeigten, dass diese putative iPSCs teilweise reprogrammiert waren und eine eingeschränkte Pluripotenz aufwiesen. Sie könnten aufgrund ihrer schnellen und anhaltenden Prolifierung nützlich für Gene Targeting-Experimente sein. Das in dieser Arbeit Erreichte kann als Grundlage für die Entwicklung echter porciner iPSCs dienen.

Als Alternative zu Gene Targeting in pluripotenten Stammzellen wurden somatische Zellen untersucht. Um die Targeting-Effizienz zu verbessern, wurden synchronisierte und unsynchronisierte mesenchymale Stammzellen (MSCs) miteinander verglichen und zwei verschiedene Loci getargetet, Adenomatous Polyposis Coli (APC) und das Tumorsuppresor-Protein p53 (TP53). Korrekt getargete Zellklone wurden identifiziert und APC-getargete Zellen anschließend für den Kerntransfer (SCNT) verwendet. Daraus entstanden drei Ferkel. Vergleich der Targeting-Effizienzen zeigte, dass die Synchronisierung nicht zu einer Verbesserung führte. Weitere Synchronisierungsmethoden können in der Zukunft getestet werden.

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1 Introduction

1.1 Genetically modified animal

Genetically modified animal, also known as transgenic animal, is an animal whose genetic material has been altered. To do so, a number of different methods are available. For example, a foreigner DNA sequence from different sources can be introduced by homologous recombination into the host's genome (Thomas and Capecchi, 1987).

Mouse has some good points to be used as an animal model, including defined genetic background, easy to handle and control, high reproduction rate. As a popular animal model, it has been used in human disease research for a long time and the techniques are well developed and routine. Besides, the established mouse embryonic stem cells also induced pluripotent stem cells have been used in biomedical research. However, due to its distant genetic relationship with human and difference in body size, the limitations of mouse model are obvious. More and more large animals have been used in transgenic animal's researches (Zawada *et al*., 1998; Imaizumi *et al*., 2000; Lai *et al*., 2002; Flisikowska et al., 2012). Compared to mouse, the large animal models have several potential advantages: Pigs are more closely related to human in genetic background and physiology, and have similar organ size with human. It is an optimal choice to be used in human disease research as an animal model and organ donor (Rudolph and Mohler, 1999). Up to now, pigs are already used as models for human diabetes, arteriosclerosis, myocardial infarction and familial adenomatous polyposis (FAP) (Turk and Laughlin, 2004; Larsen and Rolin, 2004; Bellinger *et al*., 2006; Granada *et al*., 2009; Flisikowska et al., 2012).

During the continuous studies in last decades, several methods were used to generate genetically modified pigs: pronuclear microinjection of DNA, sperm mediated gene transfer, retrolviral and lentiviral transgenesis, somatic cell nuclear transfer (SCNT) with genetically modified cells. In 1974, scientists created the first transgenic mouse by injecting DNA into the blastocyst (Jaenisch and Mintz, 1974). The pronucleus of fertilized eggs was chose to be injected with DNA. The first

transgenic large animals were also derived by the DNA microinjection method (Hammer *et al*., 1985). Sperm mediated gene transfer was used a few years later (Lavitrano *et al*., 1989). Using these two methods, many different transgenic pigs have been reported (Hirabayashi *et al*., 2001; Niemann *et al*., 2001; Uchida *et al*., 2001; Lavitrano *et al*., 2006; Manzini *et al*., 2006). But, these methods caused random integration in most cases, and the copy number of integrated gene cannot be controlled (Robl *et al*., 2007). In parallel, retroviral and lentviral transgenesis was applied to generate transgenic animals. After successful transgenic mouse was created with retroviral vector (Jaenisch, 1976), the transgenic mouse was also generated by lentiviral transgenesis (Lois *et al*., 2002). Compared to retroviral vectors, the lentiviral can infect non-dividing cells vectors and cannot be silenced during embryo development (Pfeifer, 2004; Robl *et al*., 2007). However, the maximum DNA capacity of the lentiviral vectors is about 10 kb and random integration of vector also happens (Robl *et al*., 2007). To modify the large animals more precisely, somatic cell nuclear transfer was established using *in vitro* modified cells. A sheep was successfully cloned by somatic cell nuclear transfer (SCNT) (Wilmut *et al*., 1997). Together with genetically modified cells, a transgenic sheep was generated with SCNT (Schnieke *et al*., 1997). In the following several years, cloned pigs was generated with SCNT (Polejaeva *et al*., 2000; Onishi *et al*., 2000; Betthauser *et al*., 2000). Transgenic pigs were also derived by using the same method (Hyun *et al*., 2003; Lee *et al*., 2005; Kurome *et al*., 2006; Brunetti *et al*., 2008; Cho *et al*., 2009; Umeyama *et al*., 2009). Those achievements lead a common research approach: By modifying cells with different methods, such as homologous recombination, a specific fragment of genomic DNA can be added, deleted or replaced, resulting in knock-in, knock-out or precise mutation of the genome.

1.2 Gene targeting

1.2.1 Progress in gene targeting

Gene targeting, used to change the endogenous gene, is a genetic technique in modern scientific research. It presents a precise way to manipulate the genome. Genes can be deleted, added, mutated or silenced by different methods, such as traditional homologous recombination, the new more efficient zinc-finger nuclease and transcription activator-like effector nucleases (TALENs) mediated methods

(Bibikova *et al*., 2003; Christian *et al*., 2010). Classified by the function, they are also called gene knock-out, gene knock-in, gene knock-down. Scientists can design the targeting vector using known sequences, which gives full control of the target gene locus (Capecchi, 2005) (Figure 1). Compared with other research methods, gene targeting is more accurate and almost unlimited of the gene size. Due to its advantages, gene targeting has been used widely in the researches of gene functions and human disease models.

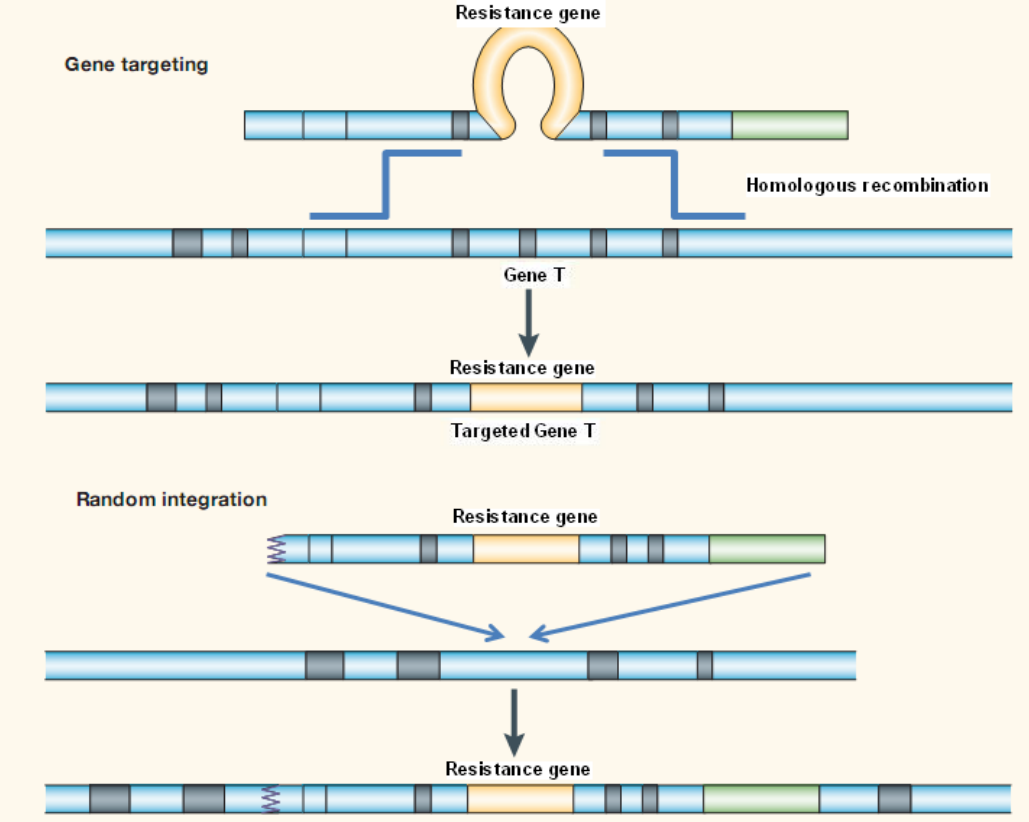

Figure 1: Positive-negative selection in gene targeting.
A selection protocol used to enrich for embryonic stem cell lines that contain a targeted disruption of any chosen gene (gene T), regardless of its function or expression in ES cells. (Adapted from Capecchi, 2005).

Homologous recombination was applied for gene targeting first in 1985 (Smithies *et al*., 1985). Its mechanism is animating the DNA repair to form a Holiday junction. By using this approach, genetically modified mouse, rabbit and pig were created

successfully (Fodor *et al*., 1994; Lai *et al*., 2002; Bosze *et al*., 2003). Recently, nucleases which can bind to specific DNA sequences have been used to improve the efficiency of gene targeting. Zinc-finger nucleases and TALENs are reported (Bibikova *et al*., 2003; Christian *et al*., 2010). Combined with Cre-LoxP or other System, conditional gene targeting provides accurate disease model for medicine and development (Wirth *et al*., 2007).

1.2.2 Different cell types used for gene targeting

The efficiency of gene targeting depends on many things, main factors are the targeting methods, the cell types and the properties of the DNA sequence. Targeting of embryonic stem cells, followed by production of chimeric animals with germline transmission, became the routine method for the mouse. The ability to clone animals by nuclear transfer from cultured somatic cells (Campbell *et al*., 1996; Wilmut *et al*., 1997; Polejaeva *et al*., 2000), offered an alternative route to germline modification applicable to many species (Clark *et al*., 2000). However, up to date, the targeting efficiency is insufficient for the method to be wildly applicable. Differences of cell types have an influence on the gene targeting efficiency. Due to the requirement of long time expansion, highly efficient transfection and selection, the cells capable for gene targeting should have the ability to be passsaged more than 45 times *in vitro* (Clark *et al*., 2000). Genetic modification and subsequent preparation for NT must be accomplished before the cells senesce or enter crisis and transform (Denning *et al*., 2001).

In previous studies, fetal fibroblasts, mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells were used for gene targeting. In mouse, embryonic stem cells were targeted directly and used for blastocyst injection subsequently. This method is routinely used in mouse gene targeting research. However, since the lack of ESC and iPSC in livestock, only primary type cells, like fetal fibroblasts and mesenchymal stem cells (MSCs), were used for targeting with the following SCNT (Denning and Priddle 2003; Flisikowska *et al*., 2012).

Fetal fibroblasts are isolated from a fetus and commonly used for gene targeting (McCreath *et al*., 2000; Denning *et al*., 2001). Adult fibroblasts were also tried for

gene targeting (Kubota *et al*., 2000), but their lifespan is limited to about 40 population doublings (Denning *et al*., 2001).

Mesenchymal stem cells are multipotent stromal cells, which can differentiate into myoblasts, fibroblasts, osteoblasts, chondrocytes, and adipocytes. MSCs can be isolated from bone marrow, adipose tissue, muscle, and umbilical cord. It was considered as donor for gene targeting in large animals (Bosch *et al*., 2006). Cultured with basic fibroblast growth factor, the lifespan of MSCs could increase up to more than 70 population doublings (Bianchi *et al*., 2003). In addition the efficiency of transfection can be as high as 67% in porcine MSCs (Colleoni *et al*., 2005).

To generate transgenic animals, embryonic stem cells (ESCs) are the best choice for gene targeting (Suzuki *et al*., 2008). Because of the rapid proliferation *in vitro*, ESCs provide an inexhaustible supply of cells capable of homologous recombination with a newly introduced mutated DNA sequence (Capecchi, 1989). Pure targeted ESCs can be injected into blastocysts directly and contribute to the germ cells in the chimeric animal. In the next generation, pure transgenic animal inherited the modified genome from the parents. The first successfully targeted gene in ESCs is hypoxanthine phosphoribosyl transferase (Hprt). In this study, a specialized construct of the neomycin resistance (neo^r) gene was introduced into an exon of a cloned fragment of the Hprt gene and used to transfect mouse ESCs (mESCs) (Thomas and Capecchi, 1987).

Since the first induced pluripotent stem cells (iPSCs) were generated, they were considered as a replacement of ESCs, though they are not considered to be fully identical. They share the advantages with ESCs of fast proliferation, long lifespan, and germline contribution. Hence, they brought a new possibility for targeting in those species where ESCs isolation was failed. In human, iPSCs could be generated directly from patient, made them potential donors for gene therapy by precise modification through gene targeting (Ye *et al*., 2009).

1.3 Pluripotent stem cells

1.3.1 Embryonic stem cells (ESCs)

Embryonic stem cells are a kind of pluripotent stem cells, which can differentiate into all kinds of somatic cells under proper conditions and have the ability of self-renewal (Rossant, 2008; Buecker *et al*., 2010). The fertilized eggs proliferate quickly into morula cell cluster, then the outer layer of the cluster differentiates to trophectoderm while the inner layer forms the inner cell mass. The ESCs were first isolated from mouse inner cell mass of the blastocyst at day 3.5 (Evans and Kaufman, 1981). In theory, they can be induced to differentiate into all lineages of cells which can be used to rebuild the organism. If cultivated in conditioned medium, the cells may proliferate forever without differentiation. These properties make them to be a potential source for regenerative medicine and a powerful tool for developmental research (Chen *et al*., 2008).

Later on, the ESCs were also isolated successfully from human, monkey and rat (Thomson *et al*., 1995; Thomson *et al*., 1998; Ueda *et al*., 2008). In previous studies, isolation from different species, such as hamster (Doetschman *et al*., 1988), rabbit (Schoonjans *et al*., 1996), ovine (Piedrahita *et al*., 1990a), porcine (Evans *et al*., 1990), bovine (Evans *et al*., 1990; Strelchenko *et al*., 1996), dog and cat (Hatoya *et al*., 2006; Yu *et al*., 2008) were also attempted. Unfortunately, all of them can't be verified as pluripotent stem cells entirely. Only rat, the relative of the mouse, was another animal from which fully pluripotent stem cells were recently established (Li *et al*., 2008).

1.3.2 Induced pluripotent stem cells (iPSCs)

Due to the limited source of ESCs and ethical reasons, scientists had been trying to find another way to derive pluripotent stem cells. Three approaches for reprogramming to pluripotency were established (Figure 2).

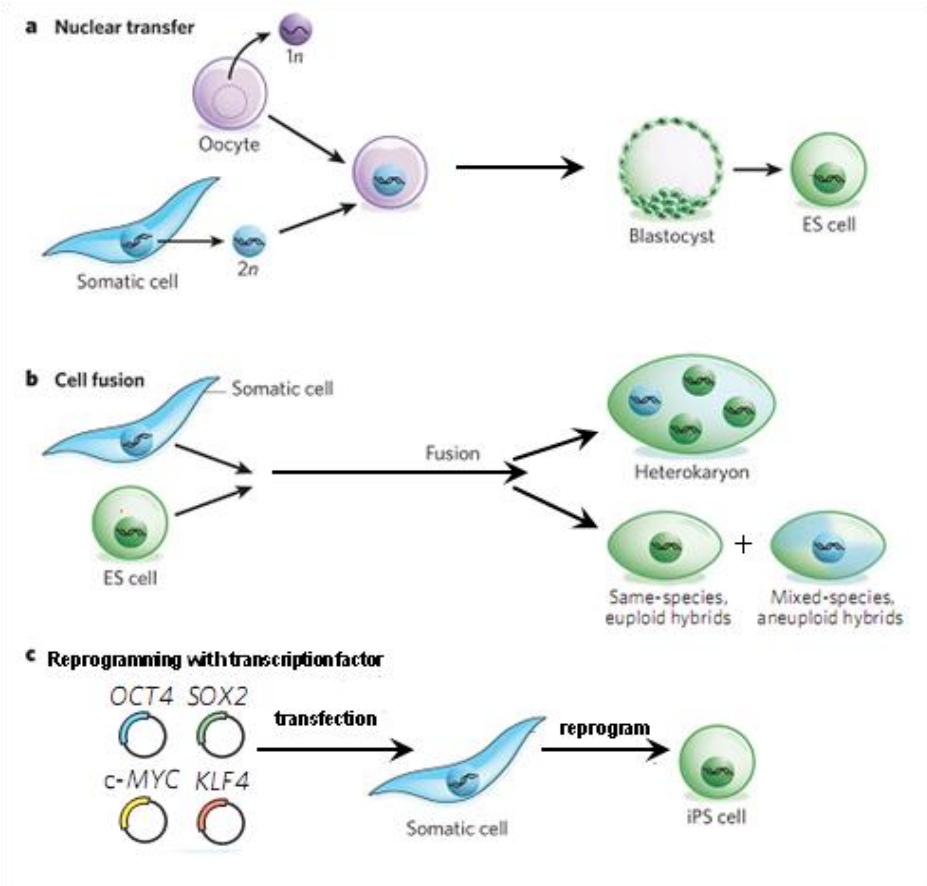

Figure 2: Three methods to nuclear reprogramming to pluripotent state.
a, Nuclear transfer. The nucleus of somatic cell can be transplanted into an enucleated oocyte. b, Cell fusion. Two different cell types are fused to form a single entity. The resultant fused cells can be heterokaryons or hybrids. c, Reprogramming with transcription factors. This approach can be used to generate induced pluripotent stem (iPS) cells, from almost any cell type in the body through the introduction of four genes (Oct4, Sox2, Klf4 and c-Myc) by using retroviruses (Adapted from Yamanaka and Blau, 2010).

Half a century ago, scientists transferred the frog intestinal cell nucleus to an enucleated egg and were successful in obtaining a new frog (Gurdon, 1962). Almost 30 years later, a sheep was generated by somatic cloning of epithelial cells (Wilmut *et al*., 1997). These progresses made it promising that somatic cells could be reprogrammed to a pluripotent state. Along with the development of ESCs study, the culture conditions and maintenance factors had been established (Smith *et al*., 1988; Thomson *et al*., 1998). The hypothesis that multiple endogenous factors control the ESCs state was developed. In 2006, Yamanaka's group tested 24 factors to reprogram mouse somatic cells to a kind of stem cell whose properties were similar

to ESCs (Takahashi and Yamanaka, 2006). These cells were defined as induced pluripotent stem cells and the 4 important reprogramming factors were: Oct4, Sox2, Klf4 and cMyc. One year later, two different groups successfully reprogramed human fibroblasts into iPSCs by the 4 factors respectively (Takahashi *et al*., 2007, Yu *et al*., 2007). Soon after these reports, many achievements about iPSCs were published (Maherali *et al*., 2007; Wernig *et al*., 2007; Lowry *et al*., 2008). Later on many different types of cells, including terminally differentiated cells, were used to generate iPSCs (Loh *et al*., 2009; Kim *et al*., 2011). But, the mechanisms of reprogramming were not clear and the efficiency was low.

In initial iPSC researches, the reprogramming factors were delivered by retroviral or lentiviral vectors, which may lead to insertional mutagenesis by integrating into the genome. This side effect limits the application of iPSCs in gene therapy (Hacein-Bey-Abina *et al*., 2003). Soon after new methods had been attempted to avoid the integration problem, adenoviral vector, plasmids, RNAs, and proteins were successfully used to generate iPSCs (Stadtfeld *et al*., 2008b; Okita *et al*. 2008; Kim *et al*., 2009a; Warren *et al*., 2010). The episomal plasmid vector, which exists independently from the genome and replicates during cell division, is widely used now.

Although the iPSCs are similar to ESCs in morphology, gene expression, and differentiation ability (Guenther *et al*., 2010; Hu *et al*., 2010; Newman *et al*., 2010), they still have differences, for example in the DNA methylation pattern (Deng *et al*., 2009; Doi *et al*., 2009). Microarray analysis also showed that a lot of genes were differently expressed between human ESCs and iPSCs lines (Chin *et al*., 2009). Furthermore, scientists found that the iPSCs had epigenetic memories from parent cells (Kim *et al*., 2011; Lister *et al*., 2011; Ohi *et al*., 2011). Hence, different methods for generating iPSCs were developed in the last several years (Table1). Except the adenovirus, all the viral delivery methods can cause permanent transgenes integration or vector fragments in genome. Non-viral methods were developed for transgene free iPSCs (Figure3).

Table 1: Comparison of different methods for generating iPSCs.

(Adapted from Parameswaran *et al*, 2011).

Spiece	Cell type	Methods, factors	Efficiency (%)	Time (d)	Reference
Mouse	EF	Retroviral, 24 factors	NA	16	Takahashi *et al*., 2006
Mouse	EF	Retroviral, OKSM	0.001-0.03	12	Okita *et al*., 2007
Mouse	EF,AF	Retroviral, OKSM	0.05-0.1	20	Wernig *et al*., 2007
Human	AF	Retroviral, OKSM	0.02	30	Takahashi *et al*., 2007
Mouse	EF	Retroviral, OKSM	NA	21	Maherali *et al*., 2007
Human	AF	Retroviral, OS	1	30	Huangfu *et al*., 2008a
Mouse	EF	Retroviral, OKSM	0.5-11.8	14	Huangfu *et al*., 2008b
Human	Oral mucosa	Retroviral, OKSM	0.022	25	Miyoshi *et al*., 2010
Human	Cord blood cells	Retroviral, OKSM	0.06-0.2	21-28	Zaehres *et al*., 2010
Human	T cells	Retroviral, OKSM	0.002	25-25	Loh *et al*., 2010
Human	T cells	Retroviral, OKSM	0.01	25-30	Brown *et al*., 2010
Human	Molar stromal cells	Retroviral, OKS	0.0026-0.0302	25	Oda *et al*., 2010
Rat	Neural precursor	Retroviral, OKSMN	0.2	21	Chang *et al*., 2010
Human, Mouse	Adipose cells	Retroviral, OKSM	0.25-0.42	24	Sugii *et al*., 2010
Mouse	Neural progenitors	Retroviral, OKSM+2i	NA	12	Li *et al*., 2009
Human	Keratinocytes	Retroviral, OKSM	1	10	Aasen *et al*., 2008
Mouse	Neural stem cells	Retroviral, O	0.014	28-35	Kim *et al*., 2009b
Mouse	Meningiocytes	Retroviral, OKSM	0.8	14	Qin *et al*., 2008
Mouse	Neural stem cells	Retroviral, OK	0.11	14-21	Kim *et al*., 2009a
Mouse	Secondary EF	mc lentiviral, OKSM	4	9-13	Wernig *et al*., 2008
Human	Adipose cells	mc lentiviral, OKSM	0.2	18	Sun *et al*., 2009
Rabbit	Hepatocyte	mc lentiviral, OKSM	0.25-0.55	12-18	Honda *et al*., 2010
Human	FF, AF	mc lentiviral, OSNL	NA	12	Mali *et al*., 2008
Mouse	Pancreatic beta	mc lentiviral, OKSM	0.1-0.2	24	Stadtfeld *et al*., 2008a
Human	AF	mc lentiviral, OKSM	0.005	21-35	Soldner *et al*., 2009
Mouse	Neural progenitors	mc lentiviral, OKM	0.001-0.002	11	Eminli *et al*., 2008

Human	AF	mc lentiviral, OKSMNL	0.166	17	Liao *et al*., 2008
Human	AF	mc lentiviral, OKSM	2	28	Maherali *et al*., 2008a
Human	Keratinocytes	mc lentiviral, OKSM	0.002	18	Hockemeyer *et al*., 2008
Mouse	EF	mc lentiviral, OKSM	0.0827	11	Maherali *et al*., 2008b
Human	IMR90	mc lentiviral, OSNL	0.0095-0.022	20	Yu *et al*., 2007
Human	Peripheral blood	pc lentiviral, OKSM	0.001-0.002	25-40	Staerk *et al*., 2010
Mouse	EF	Si-pc lentiviral, OKSM	1.04	15	Shao *et al*., 2009
Mouse	AF	lentiviral, OKSM	0.5	20-25	Sommer *et al*., 2009
Mouse	Fetal hepatocytes	Adenoviral, OKSM	0.0001-0.001	24-30	Stadtfeld *et al*., 2008b
Human	T cells	Sendai virus, OKSM	0.1	25	Seki *et al*., 2010
Mouse	EF	PiggyBac, OKSM	1	14	Yusa *et al*., 2009
Human, Mouse	EF	PiggyBac, OKSM	2.5	20-30	Kaji *et al*., 2009
Human, Mouse	EF	PiggyBac, OKSM	NA	10-14	Woltjen *et al*., 2009
Mouse	EF	Transient transfection, OKSM	0.0001-0.0029	24-25	Okita *et al*., 2008
Human	AF	Transient transfection, OKSM	0.0002	29	Si-Tayeb *et al*., 2010
Human	AF	Transfection-polyBamino esters	0.001	20-28	Montserrat *et al*., 2011
Human	Adipose cells	Nucleofection, OSNL	0.005	14-16	Jia *et al*., 2010
Human	AF	Episomal, OKSMNL	1	30-35	Yu *et al*., 2009
Human	AF, keratinocytes	Synthetiv mRNA, OKSML	4.4	21	Warren *et al*., 2010
Non-nucleic acid based					
Mouse	EF	Protein, OKSM+VPA	0.006	30-35	Zhou *et al*., 2009
Human	FF	Protein, OKSM, HIV-TAT	0.001	56	Kim *et al*., 2009a
Mouse	Cardiac fibroblasts	ESC extract	NA	25	Cho *et al*., 2010
Rat	Limbal cells	ESC conditioned medium	0.0025	15	Balasubramanian *et al*., 2009

Abbreviations: AF, adult fibroblast; EF, embryonic fibroblasts; ESC, embryonic stem cell; FF, fetal fibroblast; HIV-TAT, human immunodeficiency virus-transactivator of transcription; 2i, 2 inhibitors; K, Klf4; L, Lin28; M, cmyc; mc, monocistronic; N, Nanog; NA, not applicable; O, Oct4; pc, polycistronic; S, Sox2; si, self-inactivating; VPA, valproic acid.

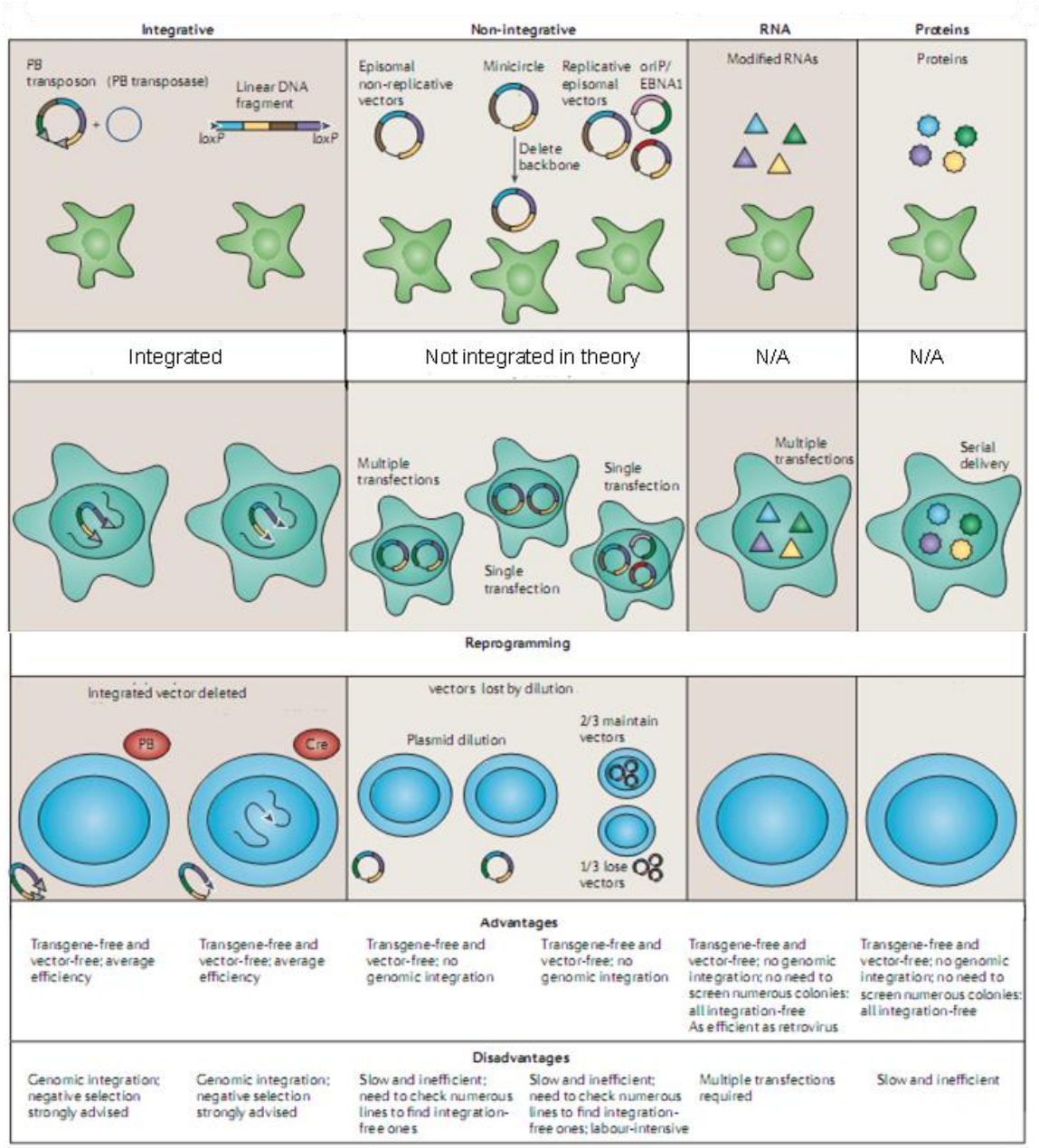

Figure 3: Non-viral delivery methods.

A flow diagram summarizing the main non-viral delivery methods, with their advantages described below. DNA-based delivery methods include those that do or do not involve integration into the genome. For each of the methods, the design of the vector is shown at the top, followed by the status of the cell after initial delivery of the vector. The coloured bars represent the transgenes. The blue cells show the status of the vector in reprogrammed cells. The orange cells show transgene-free cells after differentiation (Adapted from González *et al.*, 2011).

1.4 The molecular mechanisms of pluripotency

1.4.1 The transcriptional network of pluripotency and reprogramming factors

The transcriptional factors Oct4 (Octamer-binding transcription factor 4), Sox2 (Sry-box 2), and Nanog are key factors in the regulatory network of pluripotency. The gene Oct4, also known as POU5F1, is a member of Octamer family and recognizes the 8-bp DNA sequence ATGCAAAT (Falkner *et al*., 1984; Parslow *et al*., 1984). Oct4 plays a crucial role in regulating the pluripotency network. Loss of Oct4 can cease the development to blastocyst (Nicholes *et al*., 1998). Silencing of Oct4 triggered ESCs into trophectoderm differentiation. A slight increase of Oct4 induced mESCs into endoderm and mesoderm differentiation, and into endoderm differentiation for human ESCs (hESCs) (Niwa *et al*., 2000; Rodriguez *et al*., 2007). This means the level of Oct4 expression should be controlled in a precise manner. It functions by forming a complex with Sox2 and binds DNA cooperatively (Ambrosetti *et al*., 2000).

Sox2 is a member of Sry (Sex determining region Y) high mobility group (HMG) box family, in which all members have a HMG box DNA binding domain (Bowles *et al*., 2000). Silencing or deletion of Sox2 can also cause the differentiation to trophectoderm (Masui *et al*., 2007). Over expression of Sox2 resulted in neural differentiation (Kopp *et al*., 2008). Besides, Sox2 is considered not so curial in human ESCs because its function could be replaced by other Sox family members such as Sox4, Sox11 and Sox15 (Masui *et al*., 2007). Generally speaking, Oct4 and Sox2 are important in pluripotent stem cells and indispensable in reprogramming of iPSCs. But, it has been reported that Oct4 is able to reprogram the human somatic cells to iPSCs alone with the help of chemical compounds (Zhu *et al*., 2010). This means that Oct4 may be the only unique core factor needed for reprogramming.

Nanog is identified as ESC specific gene at first (Mitsui *et al*., 2003). It is also expressed in the inner cell mass and early germ cells (Chembers *et al*., 2003; Adjaye *et al*., 2005). The decrease of its expression level can induce ESCs to differentiate and upregulation of Nanog seems to have no effect on pluripotency (Darr *et al*., 2006; Wang *et al*., 2008a). Nanog-null ESCs still have the ability of self-renewal, which makes Nanog indispensable for ESCs (Chambers *et al*., 2007). By chromatin

immunoprecipitation (ChIP), Oct4, Sox2 and Nanog were found closely on the binding sites, which may indicate that they usually combine and bind to the target gene together (Boyer *et al*., 2005; Loh *et al*., 2006). Later on, other factors were found to share the binding sites with Oct4 and Sox2. These results support the Oct4-centric model, which includes Smad1, Stat3, and Tcf3 (Chen *et al*., 2008; Cole *et al*., 2008; Kim *et al*., 2008). Through Smad1, Stat3, and Tcf3, which are involved in bone morphogenetic protein (BMP4), leukemia inhibitory factor (LIF) and Wnt (Wingless/Int) pathway respectively, the transcriptional network is connected with the extracellular signals (Ng *et al*., 2011). Besides, the transcription factors interact and cross-regulate with each other (Kim *et al*., 2010).

Kruppel-like factor 4 (Klf4) was considered dispensable for self-renewal maintenance of ESCs (Nakatate *et al*., 2006). Depletion of Klf2, Klf4, and Klf5 lead ESC to differentiation, which may indicate (Jiang *et al*., 2008). Klf4 is included in the core factors for iPSCs. It interacts directly with Oct4 and Sox2 during reprogramming and is required to active Nanog in mouse (Wei *et al*., 2009).

CMyc is a member of the myelocytomatosis oncogene (Myc) family which also includes L-Myc and N-Myc (Brodeur *et al*., 1984). All three of them were shown to promote cell proliferation. The Oct4 binding sites are often not near to the transcription start site, compared to the cMyc binding site which is closer to the transcription start site. This may indicate the Oct4-centric group works as the enhancer. Three functionally separable modules were defined: core, polycomb and Myc. It is assumed that the Myc module is the shared signature of embryonic stem and cancer cells (Kim *et al*., 2010).

Constitutive targeted disruption of cMyc and N-Myc certified that both of them were indispensable during embryogenesis (Stanton *et al*; 1992; Davis *et al*., 1993). Conditionally knock-out of both cMyc and N-Myc in mouse ESCs showed that ESCs lost the ability of self-renewal and pluripotency (Varlakhanova *et al*., 2010). CMyc is not included in the core pluripotency network (Kim *et al*., 2010). Without cMyc, three factors (Oct4, Sox2, and Klf4 or Nanog) also can generate iPSCs with or without Lin28 (Nakagawa *et al*., 2007; Yu *et al*., 2007; Wernig *et al*., 2008). But, it is not as efficient or fast as with cMyc. In some experiments it was even impossible to obtain iPSCs without the participation of cMyc. A possible reason is that cMyc can repress

fibroblast specific gene expression which is crucial at the beginning of the reprogramming process (Sridharan *et al*., 2009).

1.4.2 The signalling pathway of pluripotency

Induced pluripotency depends on cooperation between expression of defined factors and the culture environment. The latter also determines the pluripotent state, that is, naïve or primed (Van Oosten *et al*., 2012). The extracellular factors cooperate with the intercellular networks by different signalling pathways to determine the cell fate (Figure 4), which makes signalling pathways play diverse, context-dependent roles in vertebrate development.

Leukaemia inhibitory factor (LIF) can activate JAK-STAT (Janus kinase, signal transducer and activator of transcription) signalling in mESCs and hESCs, and later activate pluripotency genes through PI3K (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase). The self renewal of mouse ESCs depends on the LIF pathway, but this pathway does not maintain pluripotency of human ESCs. In mouse ESCs, LIF plus serum defines the classic culture environment that enables the infinite self-renewal of ESCs (Smith *et al*., 1988; Williams *et al*., 1988). LIF contributes to this via the LIFRβ-GP130 signal transducer receptor complex that activates JAK, which then phosphorylate latent transcription factor Stat3 (Niwa *et al*., 1998; Matsuda *et al*., 1999). It also regulates Nanog activity by activating the T-box transcription factor Tbx3 (Niwa *et al*., 2009). The action of LIF requires the presence of serum, which can be replaced by BMPs (Silva and Smith, 2008).

Wnt/β-catenin signalling has been implicated in the maintenance of both mouse and human ESCs *in vitro* (Sato *et al*., 2004). By activating Wnt signalling, β-catenin accumulates in the cells in the nucleus, and binds to Tcf3 and other targets to mediate the maintenance of self-renewal. Many studies showed that activating Wnt signalling promotes self-renewal of mouse ESCs (Hao *et al*., 2006; Sato *et al*., 2004; Miyabayashi *et al*., 2007; Berge *et al*., 2011). But some studies indicated that β-catenin was required for multilineage differentiation and was dispensable for self-renewal (Wagner *et al*., 2010, Soncin *et al*., 2009; Lyashenko *et al*., 2011). In research of human ESCs, conflicting reports demonstrated that Wnt/β-catenin signalling promotes either self-renewal or differentiation. Wnt3A and GSK3 inhibitor

could activate Wnt signalling and maintain the self-renewal of human ESCs (Sato *et al*., 2004). However, Wnt3A or GSK3 inhibitors leaded hESCs to differentiate to primitive streak and definitive endoderm lineages (Nakanishi *et al*., 2009; Bone *et al*., 2011). It was also reported that Wnt signalling promoted reprogramming of somatic cells to induced pluripotent stem cells (Marson *et al*., 2008; Luis *et al*., 2008). Recent research described a hypothesis that Wnt/β-catenin signalling was actively repressed by Oct4 during self-renewal (Davidson *et al*., 2012). Whether Wnt signalling promotes differentiation remains controversial.

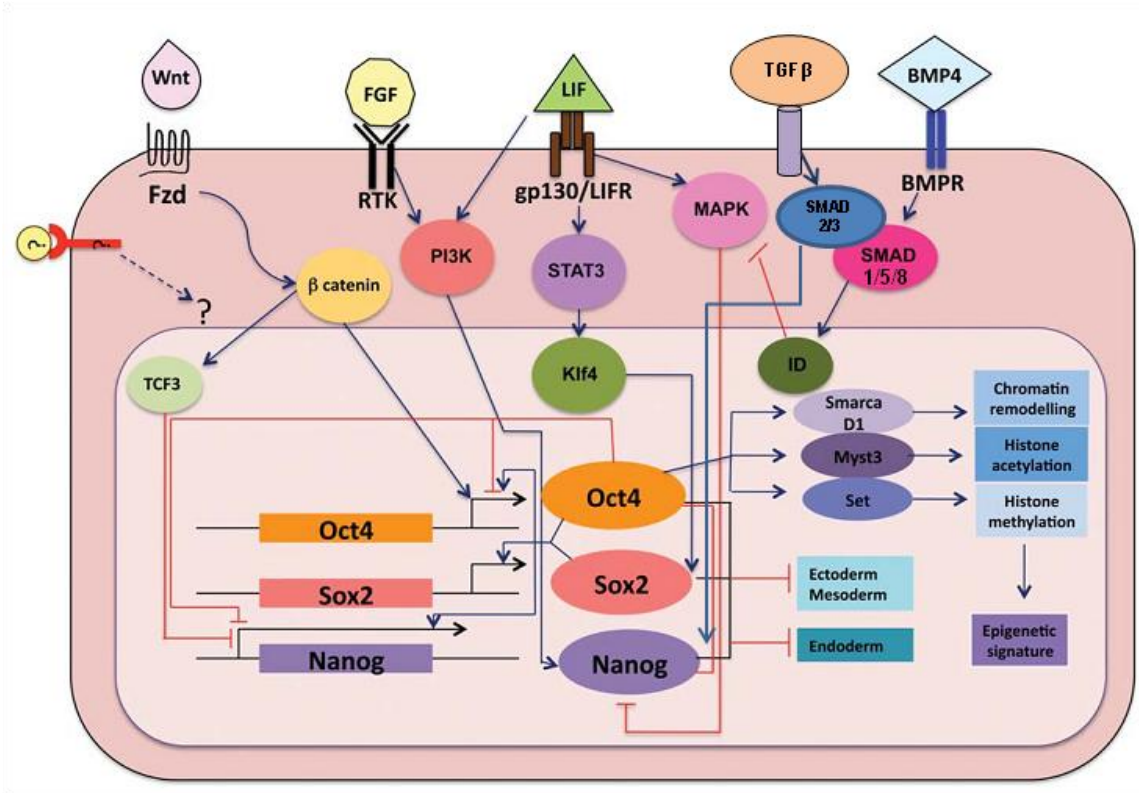

Figure 4: Putative signalling pathways underlying non-cell autonomous reprogramming. Different intracellular signalling pathways, mediated by known (Wnts, FGFs, LIF, and BMPs) and/or unknown (?) diffusible factors and through the recruitment of different intercellular effectors, alter the expression of Oct4, Sox2, and Nanog, which underpins the regulatory feedback circuit that initiates and maintains self-renewal and pluripotency. Cross-talks between these pathways influence the levels and efficiency of reprogramming (Adapted from Parameswaran *et al*., 2011).

TGF-β family, including TGF-β, Activin, Nodal and BMPs, is related with ESCs self-renewal maintenance. Some studies showed that activin or Nodal can synergize with

several other extracellular signalling proteins to promote self-renewal maintenance of ESCs (James *et al*., 2005; Vallier *et al*., 2005; Greber *et al*., 2007). Activin could cause the bFGF production when was added to hESCs in serum-free medium (Xiao *et al*., 2006). This may indicate that activin does not directly maintain hESCs undifferentiated. TGF-β can substitute for activin and/or nodal in hESCs maintenance, and blockade of the protein kinase activity of the TGF-β receptor induces more rapid differentiation of human ESCs than removal of exogenous TGF-β (Xu *et al*., 2008).

Besides, more factors were found related with the self-renewal. Shp2 promotes ES cell differentiation, mainly through bi-directional modulation of Erk and Stat3 pathways. Deletion of Shp2 in mouse ES cells results in more efficient self-renewal (Feng, 2007).

As described above, self-renewals of mouse ESCs and human ESCs depend on different signalling pathways. In general, mouse ESCs work with LIF pathway whereas human ESCs maintenance need bFGF and TGF-β/Activin/Nodal pathway (Figure 5). The discovery of murine epiblast stem cells (EpiSCs) recently gave rise to a new view of human ESCs (Brons *et al*., 2007; Tesar *et al*., 2007). EpiSCs are derived from post implantation murine epiblast embryos under culture conditions similar to hESC culture conditions. The mouse EpiSCs are similar to human ESCs. They share not only the similar bFGF/Activin A signalling pathways, but also flattened colony morphology, slower proliferation rate, and X-inactivation status (Buecker *et al*., 2010) So some scientist suspected that the hESCs may be from the EpiSCs and they proposed that the naïve hESCs should be like mESCs (Nichols and Smith, 2009; Hall *et al*., 2009). Wnt signalling and inhibition of MEK/ERK signalling were shown to promote the reprogramming to the naïve pluripotent state, while the FGF and Activin signalling promote the reprogramming to a EpiSC-like state described as primed pluripotency (Han *et al*., 2011). Primed and naïve pluripotent cells share some core transcriptional regulators but are clearly distinct from each other in aspects including epigenetic status, developmental capacity and culture requirements. It was reported that JAK/STAT3 pathway was sufficient for reprogramming and dominant for the establishment of naïve pluripotent state. In the presence of FGF and Activin, JAK/STAT3 enforced naïve pluripotency in EpiSCs (van Oosten *et al*., 2012).

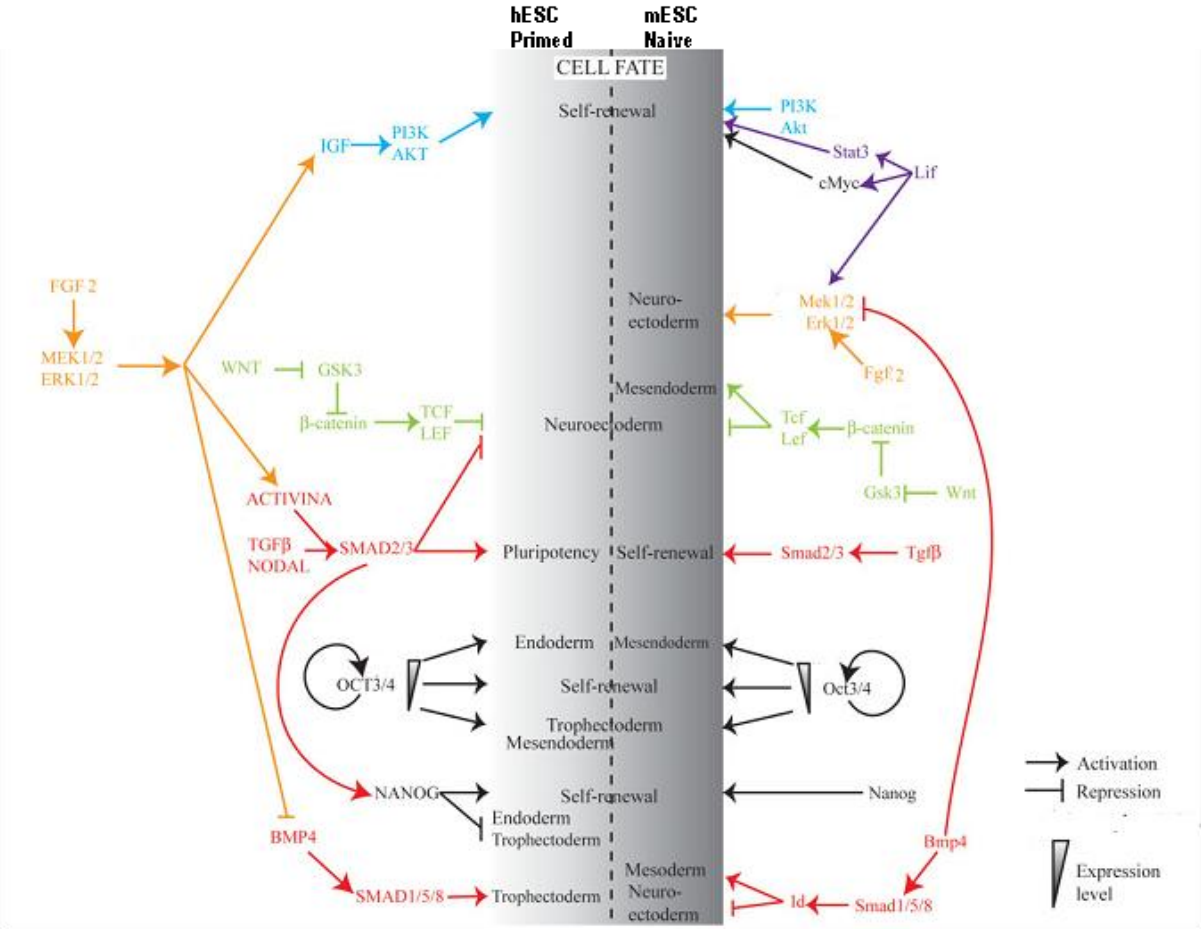

Figure 5: Human and mouse embryonic stem cell (ESC) identity is sustained by mainly distinct signalling networks.

BFGF (FGF2) is central mediators in the maintenance of undifferentiated hESCs, likely through MEK/ERK and PI3K/Akt activation. BFGF was reported to induce the expression of hESC maintenance factors such as TGF-β, SMAD2/3 indirectly. In contrast, LIF/Stat3 is required for maintaining the undifferentiated state in mESCs. As long as the balance remains in favour of Stat3, self-renewal is promoted at the expense of differentiation (MEK/ERK signalling pathway). BMP4 can inhibit the MEK/ERK differentiation pathway resulting in mESC self-renewal. (Adapted from Schnerch *et al*., 2010).

Thus, the differences between human and mouse ESCs could be a consequence of species-specific differences in development, or it is possible that human and mouse ESCs represent different stages of development (Pera and Tam, 2010).

1.4.3 MicroRNA (miRNA) and pluripotency

MicroRNAs are post-transcriptional non-coding RNA regulators. By binding to target mRNAs, they control the expression of downstream targets. One miRNA can suppress hundreds of mRNAs, so they are very efficient to regulate the expressions of cells thus the fate of cells (Subramanyam *et al*., 2011).

The transcripts are firstly cut by RNase Drosha and fold automatically into hairpin structures that are cut precisely by another RNase Dicer in the next step. Then Ago2 binds to the miRNA. The mature miRNAs are about 22nt long (Figure 6). It combines with the silencing complex and silences the mRNAs (Bartel, 2009).

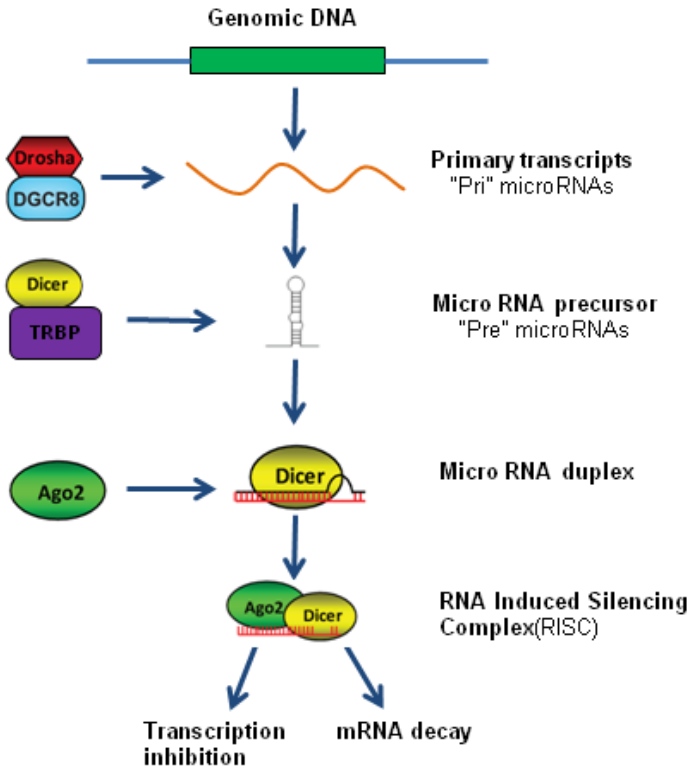

Figure 6: Schematic diagram of microRNA biogenesis.
(Adapted from Lakshmipathy *et al*., 2010).

MiRNA can regulate the pluripotency by suppressing gene expression. Let-7 is a miRNA expressed in differentiated cells. It suppresses several ESC specific genes including Lin28 which suppresses Let-7 in another way (Rybak *et al*., 2008). Myc can be negatively regulated by Let-7. MiR-145 suppresses the expression of Oct4, Sox2,

and Klf4 in human ESCs thus causes the loss of self-renewal (Xu *et al*., 2009). Some miRNAs were reported to be express specifically in ESCs, but their targets are often unknown (Morin *et al*., 2008). By analyzing the expression of ESC specific miRNAs, ESCs can be identified from the differentiated cells (Wang *et al*., 2008b). The miRNAs can also be regulated by Oct4, Sox2, and Nanog, such as miR-302 and miR-290 (Marson *et al*., 2008). Their promoters can be occupied by the core pluripotency factors in mouse and human ESCs (Boyer *et al*., 2005; Loh *et al*., 2006; Marson *et al*., 2008).

The miR-302-367 cluster is highly and specifically expressed in ESCs. The sequence of this cluster in different species is highly conserved. It is a direct target of Oct4 and Sox2. Five miRNAs are included in this cluster (miR-302a/b/c/d and miR-367) and transcribed as a single polycistronic primary transcript (Card *et al*., 2008; Rosa *et al*., 2009). In the presence of valproic acid, miR-302-367 cluster was reported to reprogram the mouse fibroblasts into iPSCs (Anokye-Danso *et al*., 2011). It showed that miR-367 was critical during the reprogramming. In this experiment, valproic acid was indispensable.

1.5 The developmental potential of iPSCs

ESCs carry balanced parental imprints that are critical for normal development, so they can contribute to the germline (Hochedlinger and Jaenisch, 2006). Although embryonal carcinoma cells (ECCs), germline stem cells (GSCs), and embryonic germ cells (EGCs) are pluripotent, only ESCs pass the most stringent developmental assay: tetraploid embryo complementation (Stastfeld and Hochedlinger, 2010). To confirm the pluripotency of pluripotent stem cells, full-scale identification methods are necessary (Table 2).

Morphology is the first direct standard but by far not sufficient. The iPSCs share the same assays with ESCs to evaluate their developmental potency. As iPSCs can be generated from different cell sources by different methods, they require also some special assays, like testing for retroviral silencing.

Table 2: Assays to evaluate the developmental potential of iPSCs.

(Adapted from Stastfeld and Hochedlinger, 2010).

	Assay	Time	Advantages	Disadvantages
Molecular	Morphology AP staining Pluripotency markers	Mintutes-1h 1-2 days	Rapid and simple Straightforward colorimetric assay	Not specific to pluripotent cells
	Retroviral silencing	1-2 days	Hall mark of pluripotent state	Require retroviral
	DNA demethylation	1-2 weeks	Indicator of epigenetic remodeling	Somatic cells also show demethylation
	Factor independency	4-7 days	Indicator of fully reprogramming	Requires inducible system
Functional	*In vitro* differentiation	weeks	Specific differentiation	Limited cell types
	Teratoma formation	Weeks-months	Give informations of *In vivo* differentiation potential of three germ layers	Not quantitative, Cannot detect abnormal cells
	Chimeric development	weeks	Tests potential to contribute to normal tissues	Subtle abnormalities may be masked
	Germline transmission	months	iPSC-derived offspring to form functional germ cells	Readout for single, very specialized
	Tetraploid complementation	weeks	Measures potential to direct normal development of an entire mouse, including all cell types	Subtle development or postnatal phenotypes may be missed; does not assess the capacity of cells to form extraembryonic tissues

1.6 Porcine pluripotent stem cells

1.6.1 Porcine embryogenesis and ESCs

Mouse and human pluripotent stem cells are most commonly used in research, not only due to the clearer genetic background compared to other species and known genomic sequences, but also based on the established standardized research methods and cell lines. For other species there are only few successful experiments reported. However, the gap in ungulate is needed to be filled to help understand the early embryo development of all mammalians. The large animal model also calls for pluripotent stem cell for gene targeting and generation of transgenic animals. The application of iPSCs and their transplantation needs reliable pre-clinical large animal models.

The development of porcine pluripotent stem cells is full of hardships. Two decades ago, scientists already started to isolate ESCs from porcine inner cell mass, but the isolated cells couldn't be passaged and had no pluripotency (Evans *et al*., 1990; Piedrahita *et al*., 1990a; Piedrahita *et al*., 1990b; Notarianni *et al*., 1990; Notarianni *et al*., 1991). Two groups carrying out the early work obtained some ESC-like cells from day 7-9 blastocyst. However, no cells survived more than passage 10 (Evans *et al*., 1990; Piedrahita *et al*., 1990a; Piedrahita *et al*., 1990b). Then other scientists tried to isolate cells from, day 9-12 (Strojek *et al*., 1990), day 5-6 (Hochereau-de and Perreau, 1993); day 6-10 (Anderson *et al*., 1994), and morula (Chen *et al*., 1999). Some of the cell lines could be passaged more than 10 passages and showed ES-like morphology. Some putative porcine ESCs, though they couldn't be cultivated for a long time, could form embryoid bodies, teratomas (Hochereau-de and Perreau, 1993), and even chimera (Chen *et al*., 1999). Cells obtained from day 7 were SSEA1 positive (Wianny *et al*., 1997). Cells isolated on day 7-9 from minipigs showed mouse ESC-like morphology and differentiated to neuron-like, smooth muscle, and epithelium-like cells (Li *et al*., 2003). Oct4, Sox2, and Nanog were found in cells isolated from day 6-8 (Blomberg *et al*., 2008). In recent years one group (Vassiliev *et al*., 2010) isolated putative porcine ESCs from *in vivo* and *in vitro* embryos by using a new method. They could passage some cell lines up to 14 passages. Oct4 and Nanog were detected in these cells. The results showed that these cells could form embryoid bodies, three germ layers and contribute to chimeric pigs.

Besides some progress with porcine ESCs, none of the isolated cells can be maintained over longer periods in an undifferentiated state. The reasons for this are still unclear. Special early development process of porcine embryo and the elusive proper culture medium might be the explanation (Kuijk *et al*., 2008) (Figure 7).

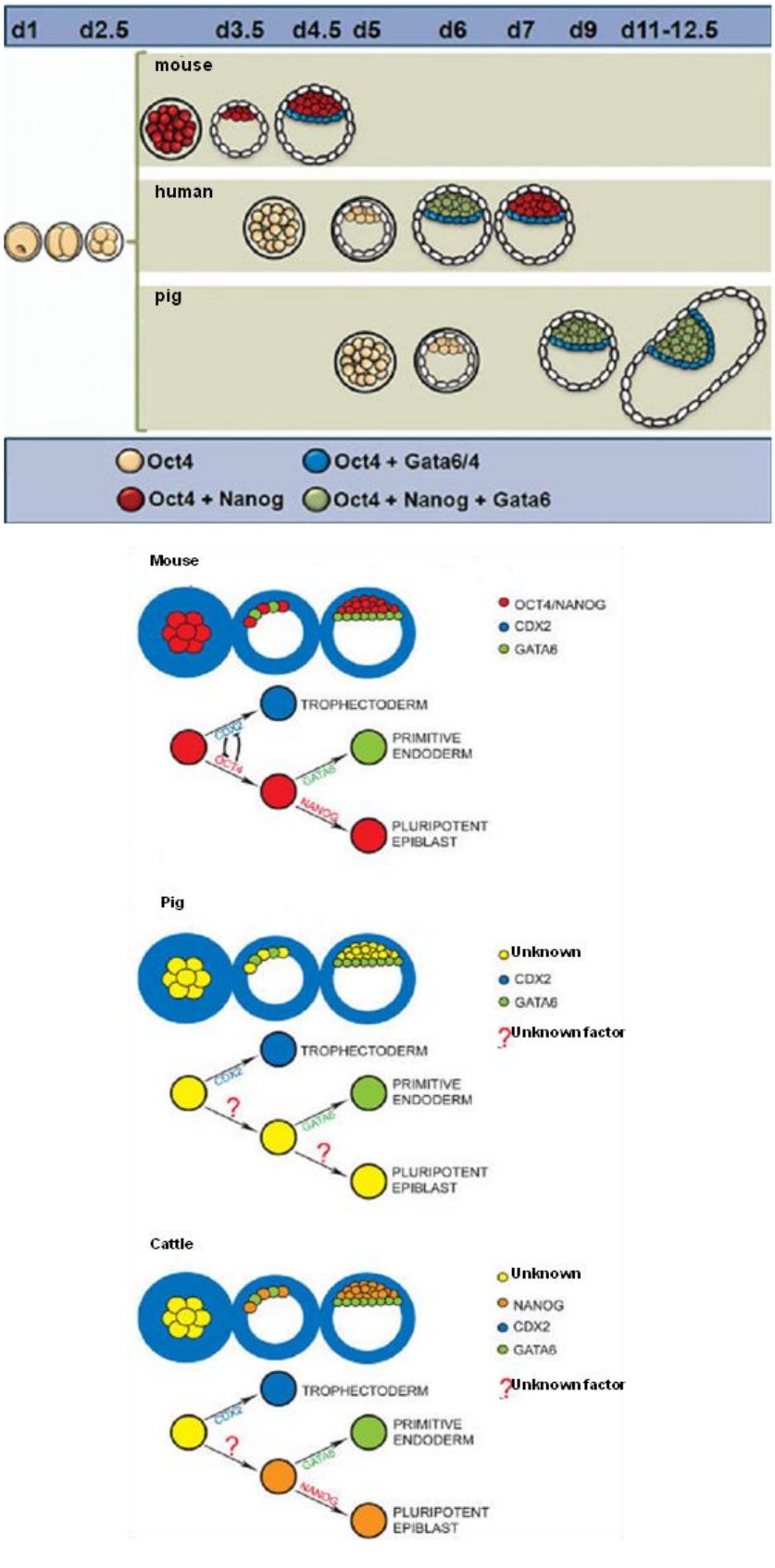

Figure 7: Early lineage segregation in mouse, human, pig, and cattle.
(Adapted from Kuijk *et al*., 2008; Alberio and Perez, 2012).

In the early embryo development, the mouse embryo finishes the formation of early blastocyst and forms trophectoderm and inner cell mass by day 3.5. By day 4.5, the primitive endoderm has been formed and the inner cell mass becomes the early epiblast and grows quite fast to create the real epiblast. Soon after this process, the embryo differentiates forms the three germ layers. This process takes longer in human and pig. In human, blastocyst forms by day 5, and the epiblast occurs by day 8-9. In pig, the embryo starts to hatch at days 7 to 8 and stays at epiblast stage for longer time than mouse and human. Both human and mouse embryos start to implant to the uterine walls invasively and part of their trophectoderms forms the placenta. But the porcine embryo keeps the blastocyst for a longer time and transforms to a filament before the non-invasive implantation (Enders and Carter, 2004) (Figure 7).

Given the differences of early embryogenesis, it is reasonable that the expressions of Oct4, Sox2, and Nanog in porcine blastocyst are distinct from mouse and human (Hall, 2008). In mouse morula stage, Oct4 and Cdx2 inhibit the expression of each other, subsequently, the Cdx2 positive outer cells differentiate to trophectoderm whereas the Oct4 expressing cells become the inner cell mass (Niwa *et al*., 2005). In the porcine blastocyst, Oct4 was found not only in the inner cell mass but also in the trophectoderm. In mouse, Nanog expression promotes some inner cell mass to become epiblast and the cells which express Gata6 become primitive endoderm (Chazaud *et al*., 2006). However, Nanog is difficult to detect in pig. The bovine inner cell mass was reported to express Nanog. These previous results may indicate that the procedure of porcine embryogenesis is neither like human nor like mouse (Keefer *et al*., 2007; Blomberg *et al*., 2008). Recently, a study (du Puy *et al*., 2010) showed some new discoveries of expression of porcine factors in the embryo. They used whole mount in situ hybridization, qRT-PCR and whole mount immunofluorescence to test the expression pattern of key factors at blastocyst stage by day 6.5-10.5. They found the inner cell mass and the epiblast express Sox2 and Nanog. Oct4 was detected and restricted in the epiblast by day 9.5. The *in vitro* undifferentiated colonies expressed Oct4, Sox2, Nanog, and CK18 (Cytokeratin 18) which indicated the cells were more like human ESCs and mouse EpiSCs than mouse ESCs (Figure7).

1.6.2 Porcine iPSCs

Because of the failure in isolating porcine ESCs from inner cell mass, other possibilities have been tried in past. After the success of mouse and human iPSCs, scientists also are focusing on porcine iPSCs. Several groups tried to generate porcine iPSCs by using different methods (Esteban *et al*., 2009; Ezashi *et al*., 2009; Wu *et al*., 2009; West *et al*., 2010). The first three reports described the generation of porcine iPSCs from fetal fibroblasts, ear fibroblasts and primary bone marrow cells. These porcine iPSCs expressed pluripotency markers and specific surface markers. All of them could form teratoma and generated three germ layers and had high level of telomerase activity. One group showed their cells shared hESC morphology and were positive for hESC surface markers, SSEA3, SSEA4, Tra-1-60, and Tra-1-80 (Wu *et al*., 2009). Another group reported their iPSCs were positive for SSEA1 and shared mouse ESCs morphology (Ezashi *et al*., 2009). But, the reprogrammed cells still expressed exogenous gene and relied on them, which indicated that these cells were not fully reprogrammed even if they had some pluripotent characterizations. Another possibility is that the culture conditions are not sufficient for the reprogrammed cells, and unknown supplement factors may be needed. Later on, one group reported they generated chimeric offspring with their porcine iPSCs (West *et al*; 2010). They transduced porcine MSCs with human OCT4, SOX2, KLF4, cMYC, NANOG, and LIN28 delivered by lentiviral vectors and finally got mESC like iPSCs positive for SSEA1.

1.7 Cell synchronization and gene targeting

1.7.1 Cell synchronization

Cell synchronization is a process to halt cells at a single stage in the cell cycle. There are several methods to synchronize cells. Based on their different mechanisms, they can be classified as physical fractionation and chemical blockade.

Serum starvation is a commonly used method to arrest the cells at G0 Phase. Cells need mitogen to pass the G1 phase, and later on, the mitogen is not necessary for the cell cycle after the cells enter into the S-phase. When the cells suffer from serum starvation, the lack of mitogen forces the cells to stay in G0 phase. Once the cells are released from the serum starvation, they can complete the cell cycle synchronously.

There are also chemical inhibitors which can block the cell cycle at different stages, such as thymidine, hydroxyurea, nocadozole, and colcimid (Davis *et al*., 2001).

1.7.2 Synchronization and targeting

Due to the finite lifespan of somatic cells, the advancements in gene targeting are slow. The rate of homologous recombination is determined by DNA repair mechanism and the balance between homologous recombination and non-homologous end joining (Hanson and Sedivy, 1995).

The homologous recombination prefers to occur in late S/G2 phase. Enhancement of gene targeting during S-phase is consistent with this phenomenon (Takata *et al*., 1998). A potential explanation for this enhancement was that targeting construct without nuclear localization signal cannot enter the nucleus and must wait when the nuclear membrane breaks down (Mir and Piedrahita, 2004). To maximize the efficiency of homologous recombination in targeting, it is necessary to synchronize the cells to get most of the cells in late S/G2-phase during the transfection. Cell synchrony by thymidine incorporation increased the ratio of homologous recombination to non-homologous end joining 5-fold by reducing the overall rate of non-homologous end joining (Zaunbrecher *et al*., 2008). Targeting efficiency increased 7-fold by using cell synchronization and nuclear localization signals (Mir and Piedrahita, 2004). These indicated that this approach might be useful to facilitate targeting in somatic cells by reducing the numbers of colonies that need to be analyzed before a targeting event could be identified.

1.8 Aim

The aim of this thesis was to isolate or derive porcine cell types which could be used for the generation of gene targeted animals. As the derivation of porcine ESCs has so far been unsuccessful, porcine iPSCs were hoped to provide a feasible alternative for cell mediated transgenesis and gene targeting. Different methods for generating porcine iPSCs should be assessed and their pluripotency as well as their ability to produce gene targeted animals should be evaluated. At the same time an alternative approach, gene targeting in somatic cells and derivation of cloned animals, should be carried out. In particular it should be assessed if synchronization of somatic cells improves gene targeting efficiency at the APC and TP53 loci.

2 Material and methods

2.1 Material

2.1.1 Chemicals

Bovine Serum Albumin (BSA)	PAA, Pasching, Austria
Chloroform	Sigma, Steinheim, Germany
Dimethylsulfoxid (DMSO)	Sigma, Steinheim, Germany
DNA remover	Minerva biolabs, Berlin, Germany
Ethanol absolute	Riedel-de-Haen, Seelze, Germany
Ethidiumbromid solution	Sigma, Steinheim, Germany
Ethylene diamine tetraacetic acid (EDTA)	Sigma, Steinheim, Germany
Formalin	Sigma, Steinheim, Germany
Formamide	Sigma, Steinheim, Germany
GenAgarose L.E.	Genaxxon Bioscience GmbH, Biberach, Germany
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol (2-Propanol)	Carl Roth GmbH, Karlsruhe, Germany
Methanol	Sigma, Steinheim, Germany
Phenol:Chloroform:IsomylAlcohol 25:24:1	Sigma, Steinheim, Germany
poly-DL-ornithine	Sigma, Steinheim, Germany
Rnase away	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride	Sigma, Steinheim, Germany
Sodium dodecyl sulfate (SDS)	Sigma, Steinheim, Germany
Tris hydrochloride (Tris HCl)	Sigma, Steinheim, Germany

Triton X-100	Sigma, Steinheim, Germany
Trizol	Invitrogen GmbH, Darmstadt, Germany
Tween 20	Sigma, Steinheim, Germany
Valproic acid sodium salt	Sigma, Steinheim, Germany
β-mecarptoethanol	Sigma, Steinheim, Germany

2.1.2 Plastic wares and consumables

14ml polypropylene round bottom tube	Becton Dickinson Company , Franklin Lakes, USA
40 µm cell strainer	BD biosciences, Heidelberg, Germany
Cell culture flasks (25, 75 and 150 cm2)	Corning Inc., New York, USA
Cell culture plates (6-, 12-, 24-, 48-, 96- well)	Corning Inc., New York, USA
Centrifugation tubes (15 and 50 ml)	Corning Inc., New York, USA
Cryopreservation tube	Corning Inc., New York, USA
Electroporation Cuvettes (2 and 4 mm)	Peqlab Biotechnologie GmbH, Erlangen, Germany
Filter Stericup and Steritop (0.22 µm)	Merck KGaA, Darmstadt, Germany
Glassware (bottles, flasks)	Marienfeld GmbH, Lauda-Königshofen Germany
Hybond-N+ nylon transfer membrane	GE Healthcare Ltd., Little Chalfont, United Kingdom
Petri dish (10 cm)	Brand GmbH, Wertheim, Germany
Photometer Cuvette	Eppendorf AG, Hamburg, Germany
Pipette tips with filter (20, 200 and	Mettler Toledo GmbH, Germany

1000 µl)	
Pipette tips without filter (20, 200 and 1000 µl)	Brand GmbH, Wertheim, Germany
Rainin pipette tips (20, 200 and 1000 µl)	Carl Roth GmbH, Karlsruhe, Germany
Reaction tubes (1.5 and 2.0 ml)	Brand GmbH, Wertheim, Germany
Sterile Filter (0.22 µm)	Sartorius AG, Göttingen, Germany
Sterile plastic pipettes (1, 2, 5, 10, 25 ml)	Corning Inc., New York, USA

2.1.3 Cell culture medium

Advanced Dulbecco's Modified Eagle's Medium (Advanced DMEM)	Invitrogen GmbH, Darmstadt, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	PAA, Pasching, Austria
Neurobasal Medium	Invitrogen GmbH, Darmstadt, Germany
Dulbecco's Modified Eagle's Medium F12 (DMEM/F12)	Invitrogen GmbH, Darmstadt, Germany
Kockout DMEM	Invitrogen GmbH, Darmstadt, Germany

2.1.4 Cell culture enzymes and supplements

Accutase	PAA, Pasching, Austria
ALK-5 inhibitor (A 83-01)	Biotrend GmbH, Cologne, Germany
B27 supplement minus Vitamin A	Invitrogen GmbH, Darmstadt, Germany
Cell Culture Water, EP-grade	PAA, Pasching, Austria
Dulbecco's PBS, w/o Ca & Mg	PAA, Pasching, Austria

Fetal calf serum (FCS)	PAA, Pasching, Austria
GlutaMAX	Invitrogen GmbH, Darmstadt, Germany
GSK inhibitor (CHIR99021)	AXON medchem, Groningen, Holland
Hank's Buffered Salt Solution	PAA, Pasching, Austria
Human Fibroblast Growth Factor (bFGF)	Genaxxon, Biberach, Germany
Human insulin solution	Sigma, Steinheim, Germany
Hypoosmolar Buffer	Eppendorf AG, Hamburg, Germany
Laminin	Carl Roth GmbH, Karlsruhe, Germany
Leukemia inhibitory factor (LIF)	Self-made
Lipofectamine 2000	Invitrogen GmbH, Darmstadt, Germany
MEK inhibitor (PD0325901)	AXON medchem, Groningen, Holland
N2 supplement	Invitrogen GmbH, Darmstadt, Germany
Non-essential amino acids (NEAA)	PAA, Pasching, Austria
Opti-MEM Reduced Serum	Invitrogen GmbH, Darmstadt, Germany
Progesterone	Sigma, Steinheim, Germany
Putrescine	Sigma, Steinheim, Germany
Retinoic acid	Sigma, Steinheim, Germany
Sodium pyruvate	PAA, Pasching, Austria
TGF-β1	PromoCell GmbH, Heidelberg, Germany
Trypsin powder	Sigma, Steinheim, Germany
Trypsin-EDTA	PAA, Pasching, Austria

2.1.5 Antibiotics

Blasticidin	InvivoGen, San Diego, USA
G418	PAA, Pasching, Austria

Penicillin/Streptomycin	PAA, Pasching, Austria

2.1.6 Softwares

Adobe reader	Adobe, USA
AxioVision 3.1	Zeiss AG, Oberkochen, Germany
Basic local alignment search tool (BLAST)	NCBI, Bethesda, USA
Finch TV	Geospiza Inc., Seattle, USA
Microsoft office	Microsoft, Seattle, USA
Primer 3	Whitehead Institute, Cambridge, USA
VectorNTI	Invitrogen GmbH, Darmstadt, Germany

2.1.7 Bacteria medium

Difco Luria Bartani B Agar, Miller	Becton Dickinson Company , Franklin Lakes, USA
Difco Luria Broth Base	Becton Dickinson Company , Franklin Lakes, USA
S. O.C. medium	Invitrogen GmbH, Darmstadt, Germany

2.1.8 Equipments

+4°C fridge	Beko Technologies GmbH, Dresden, Germany
-20°C freezer	Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany
-80°C ultra-low temperature freezer	Thermo Electron GmbH, Karlsruhe, Germany

AxioCAM MRC camera	Zeiss AG, Oberkochen, Germany
Axiovert 25 microscope	Zeiss AG, Oberkochen, Germany
Bio Imaging System Gene Genius	Synoptics Ltd, Cambridge, UK
BioPhotometer	Eppendorf AG, Hamburg, Germany
Digital graphic printer UP-D895MD	Synoptics Ltd, Cambrisge, UK
Electrophoresis chamber Perfect Blue mini	Peqlab GmbH, Erlangen, Germany
Electrophoresis chamber HE 33 Mini	GE Medical Systems GmbH, München, Germany
Electrophoresis chamber CSSU1214	Thermo Electron GmbH, Karlsruhe, Germany
Electrophoresis chamber CSSU78	Thermo Electron GmbH, Karlsruhe, Germany
Electrophoresis Power Supply EPS 301	GE Medical Systems GmbH, München, Germany
Handy Step multi pipette	Brand GmbH, Wertheim, Germany
Ice maker	Brice Italia S.r.l., Villa Cortese, Italy
Incubator	Binder GmbH, Germany
Laboratory centrifuge 1-15	Sigma, Steinheim, Germany
Laboratory centrifuge 3-16	Sigma, Steinheim, Germany
Laboratory centrifuge 4K15C	Sigma, Steinheim, Germany
Laboratory Centrifuge 5810	Eppendorf AG, Hamburg, Germany
Laminar Flow Hood HERAsafe Type HSP	Heraeus Instruments, Germany
Microwave MDA MW12M706	Haushaltswaren GmbH, Germany
Microwave NN-E202W	Panasonic, Kadoma, Japan
Minispin centrifuge	Eppendorf AG, Hamburg, Germany

Multiporator	Eppendorf AG, Hamburg, Germany
Nucleofactor II	Lonza Amaxa, Cologne, Germany
PCR Unit	MJ Research Inc., Waltham, USA
Pipette BioHit (10, 100, 1000µl)	Biohit Group, Helsinki, Finland
Pipettes (20, 200 and 1000 µl)	Gilson Inc., Middleton, USA
Rainin Pipet-Lite (2, 20, 200 and 1000 µl)	Mettler-Toledo GmbH, Giessen, Germany
Scale 440-33N	Kern & Sohn GmbH, Balingen-Frommen, Germany
Scale APX-1502	Denver Instruments GmbH, Göttingen, Germany
Shaker, Forma orbital shaker	Thermo Electron GmbH, Germany
Steri-Cycle CO2 incubator	Thermo Electron GmbH, Germany
Stink cupboard	Wesemann, Germany
Vortex Mixer	VELP Scientifica, Italy
Waterbath Haake C10	Thermo Electron GmbH, Germany

2.1.9 Molecular cloning materials

2.1.9.1 Miscellaneous

dNTPs	Biomers.net GmbH, Ulm, Germany
100bp Ladder	New England Biolabs, Frankfurt, Germany
1kb Ladder	New England Biolabs, Frankfurt, Germany
DNA remover	Minerva biolabs, Berlin, Germany
TURBO DNA-free	Applied Biosystems, Foster City, USA

2.1.9.2 Enzymes

Calf Intestinal Phosphatase (CIP)	New England Biolabs, Frankfurt, Germany
DNA Polymerase I, (Klenow)	New England Biolabs, Frankfurt, Germany
Antarctic Phosphatase (AP)	New England Biolabs, Frankfurt, Germany
Proteinase K	Sigma, Steinheim, Germany
T4 DNA Ligase	New England Biolabs, Frankfurt, Germany
Rnase A solution	Sigma, Steinheim, Germany

2.1.9.3 Reverse transcriptase and polymerases

5 Prime PCR Extender System	5 Prime GmbH, Hamburg, Germany
BigDye Teminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, USA
GoTaq Hot Start Polymerase	Promega, Mannheim, Germany
Phusion High-Fidelity DNA	New England Biolabs, Frankfurt, Germany
PlatinumTaq DNA Polymerase	Invitrogen GmbH, Darmstadt, Germany
SuperScript III Reverse Transcriptase	Invitrogen GmbH, Darmstadt, Germany
SuperScript One-Step RT-PCR with Platinum Taq	Invitrogen GmbH, Darmstadt, Germany

2.1.9.4 DNA isolation

GenElute Mammalian Genomic DNA Miniprep Kit	Sigma, Steinheim, Germany
High Pure RNA Isolation Kit	Roche Diagnostics, Mannheim, Germany
Nucleo Bond, Xtra Maxi	Macherey-Nagel GmbH & Co. KG, Düren, Germany

Nucleo Bond, Xtra Midi	Macherey-Nagel GmbH & Co. KG, Düren, Germany
NucleoSpin Plasmid Quick Pure	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Wizard SV Gel and PCR Clean-Up System	Promega, Mannheim, Germany

2.1.9.5 Plasmids

name	discription	source
pGEM-T-easy	cloning vector	Promega
pJet1.2/Blunt	cloning vector	Fermentas
pSL1180	Superpolylinker cloning plasmid	Amersham
pcDNA3-CMV-p53	expression vetor	Dr.Claudia Merkl
pCAGGS-Cherry-neo	expression vetor	M.Sc. Tobias Richter
pEBV-reproVI	expression vetor	Dr. Ralph Kühn
pEBV-reproVII	expression vetor	Dr. Ralph Kühn
pEBV-reproII	expression vetor	Dr. Ralph Kühn
pbs-salI	cloning vector	Dr. Ralph Kühn
pbs-pacI	cloning vector	Dr. Ralph Kühn
pCAG	cloning vector	Dr. Ralph Kühn
pSIN-hOCT4	expression vetor	Addgene
pLOVE-N-Myc	expression vector	Addgene

2.1.10 Oligonucleotides

2.1.10.1 Oligonucleotides for reprogramming vector construction

Oligonucleotide	Sequence 5' – 3'
pNANOGNheIKozF1	gctagcgccaccatgagtgtggatccagcttgt
pNANOGBsrGIR1	tgtacatcacatatcttcaggctgtatgttc
hOCT4EcoRVKozF	gatatcgccaccatggcgggacacctggct
hOCT4EcoRVR	gatatctcagtttgaatgcatgggaga
hOCT4FseIKozF	ggccggccaccatggcgggacacctggct
pKLF4 FseI F	ggccggccatggctgtcagcgacgcact
pKLF4 BamHI R	ggatccaaagtgcctcttcatgtgta
pSOX2 XbaI F	tctagaatgtacaacatgatgg
pSOX2 FseI R	ggccggcctcacatgtgagagagaggca
pCMYC FseI F	ggccggccatgcccctcaacgtcagctt
pCMYC FseI R	ggccggccttatgggcaagagttccgta

2.1.10.2 Oligonucleotides for microRNA

Oligonucleotide	Sequence 5' – 3'
miR_PvuI_F	gcgccgatcgaccccgatgacatggactc
miR_PvuI_R	cgcgcgatcgacaccccatcaccattgcta
miR exp F1	ccagtgtgctggaattcact
BGH pA R1	gcgatgcaatttcctcattt

2.1.10.3 Oligonucleotides for gene expression

Oligonucleotide	Sequence 5' – 3'	Fragment size
EGFP 11 F	ggccacaagttcagcgtgtc	633bp
EGFP 12 R	gtccatgccgagagtgatcc	
pAAT(en)(J)F	gaccatttctggaacctatgatc	284bp
pAAT(en)(J)R	ccatgaagaggacagctttgg	
pAFP(en)(J)F	cgcgtttctggttgcttacac	483bp
pAFP(en)(J)R	acttcttgctcttggccttgg	
pCDX2(trf)F	agaacccccaggtctctgtctt	101bp
pCDX2(trf)R	cagtccgaaacactccctcaca	
pDES meso F	ccaagcaggagatgatggag	244bp
pDES meso R	agggccatcttgacattgag	
pEnolase(me)F	tctgtgactgaatctatccagg	252bp
pEnolase(me)R	ctttgggttacggaacttgcg	
pigGAPDH F	actcactcttctacctttgatgct	100bp
pigGAPDH R	tgttactgtagccaaattca	
pigNanog_1F	ttccttcctccatggatctg	467bp
pigNanog_1R	aggtctggttgctccatgat	
pigNanog_F	ccagaaccagcgaatgaaat	199bp
pigNanog_R	aggtctggttgctccatgat	
pigOct4 3UTR	caaactgaggtgcctgcccttc	190bp
pigOct4 3UTR	attgaacttcaccttccctccaacc	
pNANOG globin F	gaaactgctggggaaaatca	260bp
pNANOG globin R	ttttggcagagggaaaaaga	

pNeuD(ect)(J)F	gacttgcgttcaggcaaaagc	207bp
pNeuD(ect)(J)R	gggcgactggtaagagtagg	
pPTI(trf)F	gctgccttccaaatgggttgag	252bp
pPTI(trf)R	gatagaaggccagaggttgaagcc	
pREX1 f2	gcatttttgattggggacag	249bp
pREX1 R2	tgcgatattaagtcccatatcc	
pSOX2 3UTR F	gttccatgggctcagtggtcaag	347bp
pSOX2 3UTR R	aagcgtaccgggtttttctccatac	

2.1.10.4 Oligonucleotides for gene targeting screening

Oligonucleotide	**Sequence 5' – 3'**	**Fragment size**
BSf	gagcaacggctacaatca	331bp
BSr	ggcagcaattcacgaatc	
APC TVR1	tccgaactcctggaatgtga	3747bp
BSr	ggcagcaattcacgaatc	
P53 endo F2	ccagggagtccatctaaaagtg	3161bp
P53 Int1_5 R	ttccaccagtgaatccacaa	
P53 endo F2	ccagggagtccatctaaaagtg	3308bp
P53 targ SA R	gaaagaccgcgaagagtttg	

2.1.10.5 Oligonucleotides for amplification of the probe for southern blot

Oligonucleotide	5'-3' sequence	Frangment size
IRES-BS-probe-for	ttactggccgaagccgcttg	915bp
IRES-BS-probe-rev	atggacagccgacggcagtt	

APC_S_XhoI_BglII_f	aagtcaggcggctaccactt	1206bp
APC_S_XhoI_BglII_r	ggacagtcctcgattctcac	
P53 Ex2 1F	gcaatggaggagtcgcagt	517bp
P53 Ex3 R	ctgccagggtaggtcttctg	

2.1.11 Cells

Cell	discription	Source
pBMMSCs	mesenchymal stem cells isolated from porcine bone marrow	lab store
pADMSCs	mesenchymal stem cells isolated from porcine adipose tissure	lab store
MEFs	mouse embryonic fibroblast cells	lab store
MEFs neo mito	mouse embryonic fibroblast cells neo resistant mito-C treated	lab store
rEFs	embryonic fibroblast cells isolated from rat	lab store
rADMSCs	mesenchymal stem cells isolated from rat adipose tissure	lab store
pKFCs	kidney fibroblast cells isolated from porcine kidney	lab store
Oct4-GFP pFFCs	genetically modified GFP positive fetal fibroblast cells isolated from porcine	Prof. Dr. H. Niemann
CMV-GFP pBMMSCs	genetically modified GFP positive mesenchymal stem cells isolated from porcine bone marrow	Dr. S. Karne-Scheiber

2.1.12 Antibodies and dilutions

Antibody	Antigen	Raised in	Company	Dilution
Primary antibodies				
Anti-Oct4	Mouse Oct4	Goat	Santa Cruz	1:200
Anti-SSEA1	Mouse SSEA1	Mouse	Santa Cruz	1:200
Anti-SSEA4	Mouse SSEA4	Mouse	Santa Cruz	1:100
Anti-Tra-1-60	Mouse Tra-1-60	Mouse	Santa Cruz	1:100
Anti-Tra-1-80	Mouse Tra-1-80	Mouse	Santa Cruz	1:100
Anti-Tubulin III	Mouse Tubulin III	Mouse	Sigma Aldrich	1:5000
Anti-α-actinin	Mouseα-actinin	Mouse	Sigma Aldrich	1:5000
Secondary antibodies				
Anti-Goat	Goat IgG	Chicken	Santa Cruz	1:1000
Anti-Mouse	Mouse IgG	Rabbit	Santa Cruz	1:1000

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Bacteria culture

For picking colonies and small amount DNA isolation, the *Escherichia coli* (*E. coli*) were cultivated at 37 °C overnight either on agar plates or with 5 ml Luria Broth medium (LB medium) in 14 ml culture tubes. For large amount DNA Isolation, 100-300 ml of LB medium was used to cultivate the bacteria in 500 ml or 1000 ml conical beaker. All of the media were supplemented with ampicillin at a final concentration of 100 μg/ml.

2.2.1.2 Storage of *E. coli*:

For short-term storage (less than one month), the plates and media containing the bacteria were stored at 4 °C. For long-term storage, 0.5-1 ml overnight culture mixture from a single colony was carefully mixed with sterile 99% glycerol at a volume ratio of 1 to 1 or 2 to 1 and the mixture finally was stored at -80 °C.

2.2.1.3 Transformation of *E. coli*:

1 ng of plasmid or 2 μl ligation reaction was mixed with 50 μl electro-competent cells, and then the mixture was gently (without bubbles) added into pre-cooled electroporation cuvette. The cells were electroporated by a pulse of 2500 V for 5 ms. Immediately 600 μl pre-warmed LB medium was added, and the mixture was incubated at 37 °C and 230 rpm in a shaker for 45 to 50 min. Then 10 to 200 μl of the bacteria mixture was plated on agar plates supplemented with appropriate antibiotic and incubated at 37 °C overnight.

2.2.2 Molecular biological methods

2.2.2.1 DNA Isolation

Plasmid DNA isolation from *E. coli*: For small amount plasmid preparation (Miniprep), alkaline lysis method was used routinely. 5 ml LB medium supplemented with appropriate antibiotics was seeded with pure single clone or stored bacteria and

incubated over night at 37 °C in an orbital shaker at 230 rpm. 2 ml bacteria culture was collected into a 2 ml centrifugal tube and centrifuged at 10000 rpm for 2 min. The pellet was resuspended in 100 μl resuspension solution by vortexing, and then 150 μl lysis buffer was added to the mixture. After 2 to 5 min incubated at room temperature (RT), 200 μl neutralization solution was added, and the mixture was incubated on ice for about 30 min and centrifuged at 13000 rpm for 15 min. The supernatant was transferred into a new 1.5 ml centrifuge tube and the pellet was discarded. 1 ml 99.8 % ethanol was mixed with the supernatant by vortexing and the mixture was centrifuged at 13000 rpm for 15 min. The pellet was washed with 0.5 ml of 75% ethanol and centrifuged at 13000 rpm for 5 min. The DNA pellet was then air dried at RT and dissolved in 50 µl water containing 20 µg/ml RNase A to remove RNA.

For Large amount plasmid preparation (Midi- and Maxiprep), 100 to 300 ml LB medium supplemented with appropriate antibiotic was seeded with pre-cultured mixture or stored bacteria. The mixture was incubated overnight at 37 °C in an orbital shaker at 230 rpm. Then the bacteria was collected and centrifuged at 5000 rpm for 10 min at 4 °C. The pellet was used to isolate plasmid DNA with the NucleoBond Xtra Midi/Maxi kit according to the manual.

Genomic DNA isolation from mammalian cells: For small amount genomic DNA isolation, the detached cells were centrifuged at 12000 rpm for 1 min after detaching from the culture plate. The supernatant was removed carefully and the cell pellet was resuspended in 50 µl Igepal buffer (50 mM Tris-HCl, 50 mM KCl, 3.15 mM $MgCl_2$, 0.25% (v/v) Igepal, 0.5% (v/v) Tween 20, pH 8.0) containing 100 µg/ml proteinase K Then the mixture was incubated at 65 °C for 60 min and followed by an incubation at 95 °C for 15 min. Then, the mixture was centrifuged at 16000×g for 15 min, and 5 µl of the supernatant was used for one screening PCR.

For high quality genomic DNA isolation, GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) was used according to the manual.

For large amount genomic DNA isolation, cells were expanded till confluent on T-75 flask. The cells were detached with accutase and centrifuged at 324g for 5 min. The pellet could be washed with PBS once if necessary. The pellet was resuspended in

500 µl cell lysis buffer (0.1 M Tris, 5mM EDTA, 0.2% M NaCl, 100 µg/ml Proteinase K) and incubated at 37 °C overnight. Next day, the mixture was mixed well with phenol-chloroform-isoamylalkohol at volume ratio 1 to 1 and incubated for 10 min at RT followed with centrifuge at 14000 rpm for 10 min. The upper phase was then mixed well with chloroform at volume ratio 1 to 1 in a new tube. The mixture was centrifuged at 14000 rpm for 10 min. The upper phase was mixed with 0.7 volume of Isopropanol and centrifuged at 14000 rpm for 10 min. The DNA pellet was washed with 70% ethanol and dried at final. 50-100 µl water or buffer was added to dissolve the DNA and stored at 4 °C.

2.2.2.2 DNA manipulation

Polymerase Chain Reaction (PCR): For PCR, depending on purpose, different polymerases were used. For standard DNA fragments amplification, GoTaq polymerase was used. When high accuracy DNA fragments amplification was concerned, proofreading polymerase (5 Prime or Phusion) was used. The PCR program depended on the polymerase kits and the annealing temperature of the oligonucleotides. The programs of the mainly used polymerase kits are:

1. Go-Tag Polymerase kit (Promega):

Component	Final concentration
5×Green buffer	1×
dNTPs	0.2mM each dNTP
Upstream primer	200-500 nM
Downstream primer	200-500 nM
Template DNA	Up to 500 ng
GoTaq polymerase	1.25U/50µl
ddH_2O	Up to 50 µl

Pre-denaturation	95°C	5 min	
Denaturation	95°C	30 s	30-40 cycles
Annealing	X °C	30 s	
Extension	72°C	1kb/min	
Final extention	72°C	5 min	
Store at	8°C	forever	

2. 5Prime PCR extender system (5Prime):

Component	Final concentration
10×High Fidelity buffer	1×
dNTPs	0.2mM each dNTP
Upstream primer	200-400 nM
Downstream primer	200-400 nM
Template DNA	Genomic DNA10-100 ng Plasmid 0.1-1 ng
Polymerase Mix	0.2U/50µl
ddH_2O	Up to 50 µl

Pre-denaturation	94°C	2 min	
Denaturation	95°C	30 s	30-40 cycles
Annealing	X °C	30 s	
Extension	72°C	1kb/min	
Final extention	72°C	5 min	
Store at	8°C	forever	

3. Phusion® High-Fidelity PCR Kit (New England Biolabs):

Component	Final concentration
5×High Fidelity buffer	1×
dNTPs	0.2mM each dNTP
Upstream primer	500 nM
Downstream primer	500 nM
Template DNA	Up to 250 ng
Phusion polymerase	1U/50µl
ddH_2O	Up to 50 µl

Pre-denaturation	98°C	30min	
Denaturation	98°C	10 s	30 cycles
Annealing	X °C	10-30s	
Extension	72°C	1kb/30s	
Final extention	72°C	5 min	
Store at	8°C	forever	

Restriction enzyme digestion of DNA: For plasmid DNA digestion, 3U of restriction enzyme per µg of plasmid DNA was used and incubated at proper temperature for 1 to 2 h. In some cases the incubation time was extended.

Blunting of overhanging DNA ends by Klenow: Klenow, also known as DNA Polymerase I, was used to blunt the overhanging DNA ends to blunt ends. The procedure followed the manual.

Dephosphorylation of plasmid DNA: Antarctic phosphatase (AP) and calf intestine phosphatase (CIP) were used to prevent DNA fragment from self-ligation by catalyzing the 5' phosphate from DNA. The reactions were performed according to the instruction.

Agarose gel electrophoresis: Agarose gel electrophoresis was used to separate DNA fragments according to their sizes. The agarose gel was supplemented with 0.1 µg/ml ethidium bromide to visualize the nucleotides under the UV light at 254 nm excitation wavelength in the Gene Genius Bioimaging System. DNA samples were mixed with 5 ×gel loading buffer and loaded on the gel. The gel was run at 80-120 V for 0.5-5 h depending on DNA fragment size. The DNA fragment sizes and concentration could be estimated by comparing the sizes and brightness levels of the bands with the standard DNA marker. 0.8-1.0 % TAE/TBE agarose gels were used for fragments bigger than 500bp. For fragments smaller than 500bp, 1.2%-1.5% gels were used.

Extraction of DNA fragments from agarose gels: Wizard SV Gel and PCR Clean-Up System were used to extract DNA from the TAE agarose gel according to the manual.

Precipitation of DNA with sodium chloride and ethanol: Precipitation was used to purify the DNA and concentrate or sterilize the DNA solution. After mixed with 0.1 volume of 3 M NaCl and 2 volumes of ice-cold 99.8% ethanol by vortexing, the DNA solution was centrifuged at 13000 rpm for 15 min. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged at 13000 rpm for 5 min. The DNA was dried under the hood and resuspended in sterilized or unsterilized ddH_2O.

Determination of DNA or RNA concentration: DNA or RNA concentration was measured by using Eppendorf BioPhotometer according to the instruction.

Ligation of DNA fragments: The ligation of DNA frangments with pGEM-T-easy vector was performed with promega pGEM T easy kit according to the manual.

The ligations of DNA fragments with other vectors were performed in a 20 µl reaction system containing 50 ng backbone fragments, proper amount of insert fragments. The molar ratio of vector to insert was in the range of 1:3 to1: 10. The ligation was incubated at RT for 1-2 h or 4°C overnight.

2.2.2.3 DNA sequencing

DNA sequencing was performed with the BigDye Terminator v1.1 Cycle Sequencing

Kit. 100 to 200 ng plasmid DNA were mixed with 2 µl of BigDye Reaction mix, 2 µl BigDye Sequencing Buffer, and 2.5 pmol sequencing primer and filled up to 10 µl with nuclease free water.

Further processing of the sequencing samples was performed by Dr. K. Flisikowski.

Besides, some of the sequencings were performed by Eurofins MWG Company.

2.2.2.4 RNA isolation

Generally, Trizol method was used for RNA isolation from mammalian cells and tissues. First, 1 ml of Trizol reagent per 1 well of 6-well plate was added to the cells or tissues and mixed well. The lysed mixture was frozen in liquid nitrogen or -80 °C if the RNA isolation was not performed immediately. The mixture was incubated at RT for 5 min, and then the chloroform was added to the mixture at a ratio of 1:5. After mixed well, the mixture was incubated at RT for 2 min followed by a centrifugation for 15 min at 12000×g. The upper water phase was transferred to a new centrifuge tube and mixed with pre-cooled isopropanol at a ratio of 1:1. The well-mixed mixture was incubated at RT for 20-30 min, and then centrifuged at 7500 g for 10 min. The supernatant was discarded and the pellet was washed with 500 µl of pre-cooled 75% ethanol. Finally, the RNA was air-dried on ice and dissolved in RNase-free water. To remove the DNA contamination, the DNase Kit was used according to the manual.

For high quality RNA isolation, RNA isolation kit (Roche) was used according to the instruction.

2.2.2.5 Assessing RNA integrity on agarose gels

To assess the integrity of total RNA, a denaturing agarose gel was run. Good quality RNA was expected to run as two clear bands: 28S and 18S rRNA.

2.2.2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse tanscriptase polymerase chain reaction was used to amplify DNA fragments from RNA. Two kinds of RT-PCR were used: one-step and two-step RT-PCR.

Two-step RT-PCR includes first strand reaction and PCR. The following PCR is the same as the normal PCR, 2 µl cDNA product was used for one PCR reaction.

Two-step RT-PCR:

First-strand cDNA synthesis:

Component	Final concentration
Oligo $(dT)_{20}$	1 µM
total RNA	10 pg-5 µg
dNTPs	200 µM
ddH_2O	13 µl
Heated at 65 °C for 5min, incubated on ice for 1 min	
5 ×First-strand buffer	1×
DTT	5 mM
Revers transcriptase	20U/µl
Incubated at 55 °C for 45 min, and 70°C for 15 min	

For one-step, a SuperScript™ One-Step RT-PCR kit from Invitrogen was used accoding to the manual. For two-step RT-PCR, 100-500 ng RNA was reverse transcribed to cDNA firstly. Then 2µl or more of the cDNA product was used as template for the following PCR.

One-step RT-PCR:

Component	Final concentration
2×Reaction Mix	1×
Upstream primer	200 nM
Downstream primer	200 nM
Template RNA	10 pg-1µg
RT/ Platimum Taq mix	1U/50µl
ddH_2O	Up to 50 µl

cDNA synthesis	55°C	30 min	
Pre-denaturation	94°C	2 min	
Denaturation	94°C	15 s	35-40 cycles
Annealing	X °C	30 s	
Extension	68-72°C	1kb/min	
Final extention	72°C	5 min	
Store at	8°C	forever	

2.2.2.7 Southern blot analysis

Southern blot hybridization is a method for detection of a specific DNA sequence in DNA samples by hybridizing specific labeled probe with genomic DNA. It was used to identify correctly targeted clones.

At the beginning, gene specific probe was labeled with DIG. Genomic DNA isolated from APC targeted colonies were digested with BglII and genomic DNA isolated from TP53 targeted colonies was digested with ScaI. The digested DNA ran a 1% TAE gel to separate the fragments according to their sizes. The DIG-labeled molecular weight marker VII (Roche) and 1kb NEB ladder were loaded on the gel for size indication. All samples and markers were pre-mixed with 5× loading buffer. To minimize the blot area, the gel was cut into a smaller piece according to the ladder after run at 40V for 16h and 80V for 12h. Afterward, the gel was depurinated with HCl and washed with water. Then, the gel was denatured by incubating in denaturation solution for 15 min twice and rinsed with water. Followed with neutralization for 15 min, to be equilibrated, the gel was transferred into 20×SSC solution for 10 min. According to the manual, the DNA fragments were transferred onto the membrane. After baked for 30 min at 120 °C, for pre-hybridization, the membrane was incubated with DIG easy Hyb at RT for 1 h in a 50 ml rotating falcon tube. In parallel, 2.5 µl of APC/TP53 gene specific probes were diluted in 50 µl water and denatured at 95 °C for 5 min. Afterward, the probe was mixed with pre-warmed DIG Easy Hyb immediately and

added to replace the pre-hybridization solution. The membrane was incubated at 37 °C overnight. And then the membrane was gentle shake in low stringency buffer for 15 min. Pre-heated at 54 °C high stringency buffer, was added to the membrane twice. After washed with washing buffer for 2 min, the membrane was blocked by blocking solution for 1 h with gentle shaking. The membrane reacted with the antibody solution at RT for 30 min with shaking. Following this step, the membrane was washed twice with washing buffer for 15 min and equilibrated in detection buffer for 3 min. After sealing into plastic wrap, the membrane was exposed to chemiluminescent substrate which was diluted at a ratio of 1:100. Five minutes later, the liquid was carefully removed from the membrane. Finally, the membrane was exposed to X-ray film for 5 min to 15 min.

2.2.3 Mammalian cell culture

2.2.3.1 Isolation and cultivation of porcine mesenchymal stem cells (pMSCs)

Porcine bone marrow mesenchymal stem cells were isolated from 6 month old German Landrace (male) or German Landrace × Pietrain (male) pigs. The muscle and other tissues were removed from the bones. Then the bones were cut by the saws at the ends of two sides in a low contamination area to expose the bone marrow. The HBSS (Hanks Balanced Salt Solution) –heparin solution was used to flush the bone marrow into clean culture dishes. The harvested bone marrow solution was added to 25 ml of lymphocyte separation medium and centrifuged at 1200 × g for 20 min. By centrifugation, the MSCs were separated from red blood cells and plasma. The interphase containing the MSCs was washed with 20 ml HBSS and centrifuged 5 min at 324 × g. The pellet was resuspended in MSC medium containing antibiotics (100 U/ml of Penicillin, 100 µg/ml of Streptomycin, 2.5 µg/ml of Amphotericin B) and plated in T150 culture flask. After 2 or 3 days, the medium was changed and the antibiotics were removed from the medium. The cells were split and/or frozen in liquid nitrogen when confluent.

2.2.3.2 General cell culture

In general, cells were cultivated in cell culture flasks with ventilation lids at 37°C in a humidified environment containing 5% CO_2. The growth medium was replaced every 1 to 2 days with fresh pre-warmed to 37°C medium. If necessary, selection reagents

(Blasticidin or G418) or antibiotics were added. Cells were passaged when they were confluent in the plate. First, the medium was removed and the cells were washed once or twice with PBS. Then the cells were detached with accutase by incubation at 37°C in 5 min and the accutase was inactivated by medium supplemented with serum. The cell suspension were centrifuged at 324g for 5 min, resuspended in growth medium and plated in new plates or used for the following experiments.

Cell Counting: After detached, 10 µl of cell suspention was added carefully onto a Neubauer counting chamber. After counting four large squares, the cell number per ml was calculated according to the following formula:

Number of cells per ml = number of cells in 4 large squares / 4 × dilution factor × 10^4

Mediums for different cells:

DMEM+ medium	**MSC medium**
DMEM (high glucose)	Advanced DMEM
2 mM GlutaMAX	2 mM GlutaMAX
1× Non essential amino acids	1× Non essential amino acids
1 mM Sodium pyruvate	5 ng/ml bFGF
10% (v/v) FCS	10% (v/v) FCS

KFC medium	**Mouse ESC medium**
DMEM (high glucose)	DMEM (high glucose)
2 mM GlutaMAX	2 mM GlutaMAX
1 × Non essential amino acids	1 × Non essential amino acids
1 mM Sodium pyruvate	1 mM Sodium pyruvate
15% (v/v) FCS	0.1 mM beta-Mecaptoethanol
	1 × LIF
	15% (v/v) FCS

Porcine iPSC medium	Rat ESC medium
1/2 Neurobasal Medium	1/2 Neurobasal Medium
1/2 DMEM/F12 medium	1/2 DMEM/F12 medium
0.1 mM beta-Mecaptoethanol	0.1 mM beta-Mecaptoethanol
2 mM GlutaMAX	2 mM GlutaMAX
1 × B27 supplement	1 × B27 supplement
1 × N2 supplement	1 × N2 supplement
1 × LIF	1 × LIF
0.375 μM GSK inhibitor	3 μM GSK inhibitor
0.8 μM MEK inhibitor	0.5 μM MEK inhibitor
10% (v/v) FCS	0.5 μM ALK5 inhibitor
	10% (v/v) FCS or 20% (v/v) KOSR

2.2.3.3 Transfection of mammalian cells (electroporation, nucleofection)

The cells were transfected with different vectors (depending on purpose) by electroporation or nucleofection.

For electroporation, 1×10^6 cells were mixed with 800 μl buffer and maximum of 16 μg of pure circular or linear plasmid DNA, and then were pulsed at 1200 V for 5 ms. After incubate at RT for 5 to 10 min, the cells were plated in a T-75 flask or 10 cm dish.

For nucleofection, 5×10^5 cells were mixed with 100 μl buffer and 0-6 μg of DNA, and pulsed with the AMAXA nucleofector. The cells were immediately mixed with 500 μl medium and plated on a T-75 flask or 10 cm dish.

2.2.3.4 Colony picking and cultivation of putative piPSCs

When the putative piPSC colonies were dense and big enough, plastic rings were used to isolate single cell clones with the use of accutase. The cell clusters were sucked and incubate at 37°C with accutase in less than 5 min. Then the mixture was transferred onto feeders with proper medium. To get more single colonies, they were picked under the microscope with the lid open. The cell clusters were sucked out by thin glass tube and transferred into pre-warmed accutase. The mixture was added onto feeders with proper medium supplemented with penicillin and streptomycin (Pen/Strep).

The cells were passaged on new feeders or gel matrix when they were almost confluent or had a tendency to differentiate. The cells were seeded on 12 well, 6 well plates or 10 cm dishes at a ration from 1:1 to 1:12. The culture medium was changed every day and the cells were washed with PBS if necessary.

2.2.3.5 Freezing and thawing of mammalian cells

When the cells were not in culture, they were stored in -80 °C or liquid nitrogen (about -160 °C to -130 °C).

Firstly, the cells were detached with either accutase or trypsin-EDTA and the reactionwas inactivated by medium containing serum. After centrifugation, the cells were resuspended in the freezing medium which normally contained 60% medium, 30% FCS and 10% DMSO. The aliquoted cells were added into the freezing vials and placed in a freezing device (Mr. Frosty) at -80 °C immediately.

After at least 6h, the cells were taken out from the Mr. Frosty. For short-term storage, the cells were stored at -80 °C; for long-term storage, to put the cells into liquid nitrogen was necessary.

When the cells were needed in cell culture again, they would be thawed from the storage. Firstly, the freezing tubes were transferred from the storage to the a 37 °C water bath as soon as possible, and then the cells were mixed with warm medium and centrifuged after defrosting. The cell pellet was resuspended in the medium and plated into culture flasks or plates.

2.2.3.6 Alkaline phosphatase (AP) staining

Firstly, to prepare the AP solution, 1 Sigma fast BCIP/NBT tablet was dissolved in 10 ml cell culture grade water. The cells were washed twice with PBS and fixed with fixation solution (90% methanol, 10% formalin) for 15 min at RT. Then the cells were washed with Rinse buffer. After removing the Rinse buffer, the cells were stained by the AP solution for 10 min. Pictures were taken after the staining.

2.2.3.7 Immunostaining of iPSCs

The purpose of the protocol is to detect the surface makers or intracellular factors in iPSCs by immunochemistry.

The cells were seeded on 12-well or 6-well plate and cultivate to a 30% density. When the cells were confluent, they were washed with PBS twice. The cells were fixed with fixative for 15 min at RT and washed twice with Rinse buffer.

For intracellular antigens, the cells were permeabilized with 0.1% TritonX-100 at RT for 20 min. After washed twice with Rinse buffer, the cells were blocked with blocking solution for 1 h at RT. Then the blocking solution was replaced by first antibody diluted in blocking solution and the cells were stored at 4°C overnight.

After the first antibody was removed, the cells were washed with Rinse buffer three times and the diluted secondary antibody was added. Then the cells were incubated at RT in dark for 1 h. The results were observed under fluorencence microscope after the cells were washed three times with Rinse buffer.

The secondary antibody control was performed by skipping the first antibody. The feeder cells were used as a negative control.

2.2.3.8 Separation of cells with microbeads

Microbeads are polymer particals which can be used to separate the molecules or cells by bio-reactions. Anti-SSEA-1 microbeads and columns from Meltenyi Biotech were used to separate the SSEA1 positive cells from the negative ones. All the procedures were performed accoding to the manual. After washed with three or four times with PBS, 2 ml trypsin was added into 10 cm dish or 6-well plate and incubated

at 37 °C for less than 5 min. The putative piPSC clusters were digested into single cells and the enzymatic reaction was inactivated by adding 8 ml medium containing serum. The single-cell suspension was dissociated by pipetting up and down. The cells were counted and the population for labeling was controlled up to 10^7. The centrifuged pellet was resuspended in 80 μl buffer. All the work should be done fast and on ice to keep the cells cold. 20 μl Anti-SSEA1 microbeads was mixed with the cell suspension and incubated at 4 °C for 15 min. Then the cells were washed by adding 1-2 ml buffer, and centrifuged at 300×g for 10 min. The pellet was resuspended in 500 μl buffer and the cell suspention was applied onto the prepared magnet column. The flow-through containing unlabeled cells was collected. Then the column was washed with 500 μl buffer for 3 times. The column was removed from separator and placed on a suitable collection tube. 1 ml buffer was applied on the column, and the labeled cells were flushed out by firmly pushing the plunger into the column.

2.2.3.9 Differentiation of putative porcine iPSCs

Embryoid bodies (EBs) formation: There are several methods for EBs formation. The traditional method is suspension method, hanging drops method, and the newly developed AggreWell (STEMCELL Technologies) method. For the suspension method, the detached cell was separated to single cells by pipeting up and down, and the single cells were resuspended in piPSC medium at a density of 4×10^5 per ml. Then 10 ml of cell suspension was cultivated in a 10 cm Petri dish at 37 °C in cell incubator. The medium was changed gradiently into DMEM+ medium. One day post the suspension, the medium was changed to differentiation medium I (75% piPSC medium and 25% DMEM+ medium). Two days post the suspension, the medium was changed to differentiation medium II (50% piPSC medium and 50% DMEM+ medium). Three days post the suspension, the medium was graduall changed to differentiation medium III (25% piPSC medium and 75% DMEM+ medium). Finally, the cells were cultivated in DMEM+ medium. Eight days post the suspensions, the EBs were plated onto gelatin-coated plates.

Another method is hanging drop method. The detached cells were separated to single cells by pipeting up and down and resuspended in either DMEM+ or piPSC medium. The cells were counted and diluted to 3000 to 5000 cells per 40 μl of

medium. Hanging drops were dripped on the lid of 10 cm culture dishes at a density of 20 to 30 drops per plate. The lid was turned over quickly and PBS was added into the plate to form a humidity atmosphere. The drops were cultured for 2 days in the incubator to form EBs.

AggreWell plate is a kind specific plate designed for EBs formation. Single cells were added into plate at a density of 3000 to 5000 per well. By centrifuging the plate, the cells aggregated and formed EBs.

Neural differentiation: EBs were generated with suspension method. During the first three days, retinoic acid was not added in the differentiation medium. From day 4, the EBs were cultured with 5μM retinoic acid for 4 days. On day 8, the EBs were separated into single cells by trypsin and the cells were separated with the 40 μm cell strainer to avoid the cell clusters. Then the cells were plated on laminin pre-coated plates at a density of 2×10^5 / cm^2 in DMEM/F12 medium with N2 supplement and 25 μg/ml of insulin. The medium was changed at 2h and 4h post-plating. 48h post-plating, the medium was changed to Neurobasal medium with B27 supplement. The medium was changed on day 4, day 8, and day 12 post-plating. RNA isolation or immunostaining was performed after day 12.

2.2.3.10 Cell synchronization

One day before starvation, $0.7\text{-}1.0\times10^6$ cells were plated on T-75 flask. After washed twice with PBS, the cells started to be starved in medium containing 0.5% serum. To release the cells, the starvation medium was changed to medium containing 20% serum.

2.2.3.11 Selection, picking and screening of targeted colonies

After transfection with the vector, the cells were passaged into the selection medium after at least 24 h. For each cell types, a killing curve was performed to optimize conditions for selection with selectable markers (BS or G418). The cells were passaged into 150 mm dishes or 48 well plates at a ratio of 1:5.

About 10 to 15 days later, the selected colonies were visible under a microscope. To pick the colonies from the 150 mm dishes, the plates were washed with PBS and the

colonies were picked either with sterilized filter wafers soaked in a warm accutase or sticky rings.

The sticky rings were used to cover the colonies, and the accutase was added inside the ring. The cells were transferred into 6 well plates (each colony separate).

PCR from cell culture: To detect correctly targeted colonies, targeting PCR across the short arm was performed. The cells were detached with accutase and the reaction was stoped with serum supplemented medium. After centrifuged, the cell pellet was lysised by adding Igepal lysis buffer and heated at 95 °C for 1 h. Then the mixture was centrifuged at 12000×g for 5min and the supernatant was used for PCR.

2.2.3.12 Preparation of cells for SCNT

The positive colonies were seeded on 6 well plates in MSC medium. Two days before nuclear transfer, MSC medium was replaced with serum starvation medium (MSC medium containing 0.5% FCS).

3 Results

To get higher targeting efficiency and generate transgenetic animals, pluripotent stem cells were considered as the best choice, because of their long lifespan, high frequency of homologous recombination and germline transmission capability. In view of the lack of such cells for livestock species, we tried to identify or derive the most appropriate cell type for gene targeting.

Pig, as a common large animal model for disease studies, plays a crucial role in many areas. Generating porcine pluripotent stem cells and investigating their *in vitro* characteristics could provide a much needed tool for genetic manipulation and the derivation of genetically defined porcine models. So far neither stable porcine ESC line has been established, nor have culture medium or surface markers been defined.

Since the success in isolating mouse and human iPSCs, several groups used similar methods to reprogram porcine somatic cells to induced pluripotent stem cells but without satisfying results. In the work presented here, several methods were tested to generate porcine induced pluripotent cells from pMSCs, and the pluripotency was evaluated by different methods.

As an alternative option, cell synchronization was also assessed to improve gene targeting of porcine bone marrow mesenchymal stem cell (pBMMSCs) and to produce cloned animals.

3.1 Reprogramming of porcine somatic cells with different methods

In this part, several constructs containing mouse, human or porcine reprogramming factors were used for porcine iPSCs (piPSCs) generation. The newly found pluripotent-related microRNAs were also tested for reprogramming. Porcine kidney fibroblasts (pKFCs), Oct-GFP transgenic fetal fibroblasts, BMMSCs containing a CMV-GFP transgene, normal BMMSCs and adipose derived mesenchymal stem cells (ADMSCs) were used as starting cells. Finally, a putative piPSC line was established and tested for developmental potential.

3.1.1 Constructs used for reprogramming

3.1.1.1 Episomal vectors with mouse factors

Two episomal vectors (pEBV-reproVI and pEBV-reproVII), which were shown to generate mouse iPSCs successfully, were provided by Dr. Ralf Kühn. PEBV-reproVI contains mouse Oct4 (mOct4), mcMyc, mSox2, and mKlf4. MOct4 and mcMyc are on one side of the promoter, and mSox2 and mKlf4 are on the other side. PEBV-reproVI has similar structure with pEBV-reproVII. But instead of mKlf4 and mcMyc, mOct4 and mSox2 are one side of the promoter while mNanog and hLin28 are on the other side. Besides, there is a red fluorescent protein (RFP) as a reporter to indicate the expression level (Figure 8). To ensure protein translation T2A and P2A were used to link the DNA sequences of the factors.

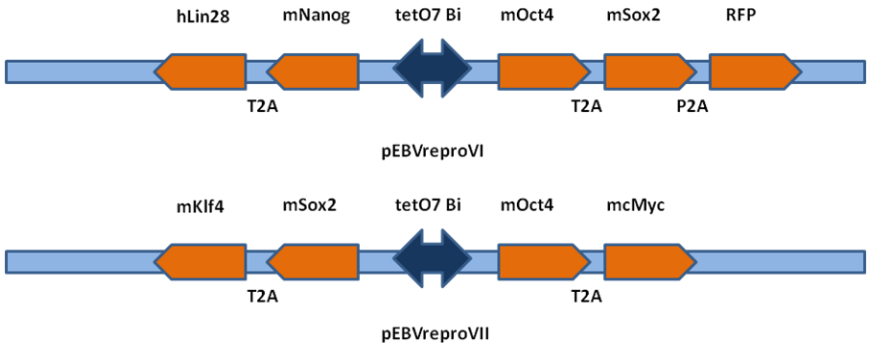

Figure 8: Episomal vectors pEBV-reproVI and pEBV-repro VII.
The pEBV-reproVI contains mouse Oct4, Sox2, Nanog, human LIN28 factors and a red fluorescent protein gene. The pEBV-reproVII contains mouse Oct4, Sox2, Klf4 and cMyc.

These two vectors contain a tet-on system: tetracycline controlled transactivator (tTA) expression cassette and the tTA responsive promoter directing the transcription of interested gene. The tet-on system controls the expression of factors. The promoter was turned on or off precisely according to the presence of doxycycline (Figure 9).

The vectors also contain the Epstein–Barr virus (EBV) episomal origin of replication (oriP) and the Epstein-Barr nuclear antigen 1 (EBNA1) gene. The protein coded by EBNA1 gene controls the oriP, which controls replication and keeps it as an episomal vector and thus avoiding integration into the host genome.

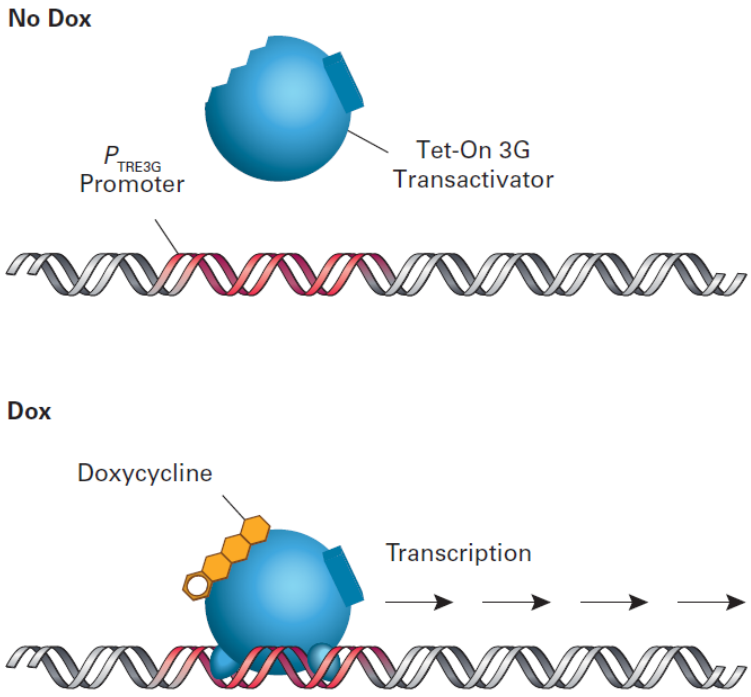

Figure 9: Doxycycline dependent tet-on system.
In the absence of doxycycline, the transactivator is free from the promoter region and the genes were inactive. When the doxycycline is added, it binds to the transactivator and makes them activate the expression.

3.1.1.2 Plasmid vectors with porcine factors

Although the reprogramming factors are much conserved, there are still differences between species. According to sequence comparison of porcine, human and mouse factors, the DNA sequences of the porcine factors are closer to human than mouse. Therefore it may be advantages to try the porcine and human factors in the reprogramming.

In order to use porcine factors, pKLF4, pSOX2, and pCMYC were cloned by RT-PCR from the porcine mRNA. In the early phase of the research, the porcine Oct4 coding sequence was failed to be cloned by RT-PCR. As an alternative choice, human Oct4 (hOCT4) was subcloned from pSin-EF2-Oct4-Pur vector (Addgene) by PCR. Flanking restriction enzyme sites were added to both ends of the amplified genes. All four DNA fragments were sequenced, and the mutations were repaired. The hOCT4 was cloned into a series of intermediate vectors to add the promoter and polyA. PKLF4 and pSOX2 were joined together by using the same enzyme and then cloned

into one vector. PCMYC was cloned using the similar protocol. The doxycycline dependent bidirectional promoter was used to drive all the factors. AttB site was cloned into the vectors containing hOCT4, pKLF4 and pSOX2 to allow directed integration, but there is no attB site in pCMYC vector to avoid integration. The final 3 vectors were designated as: pSL-attB-hOCT4, pSL-attB-pKS, pBS-pCMYC (Figure 10).

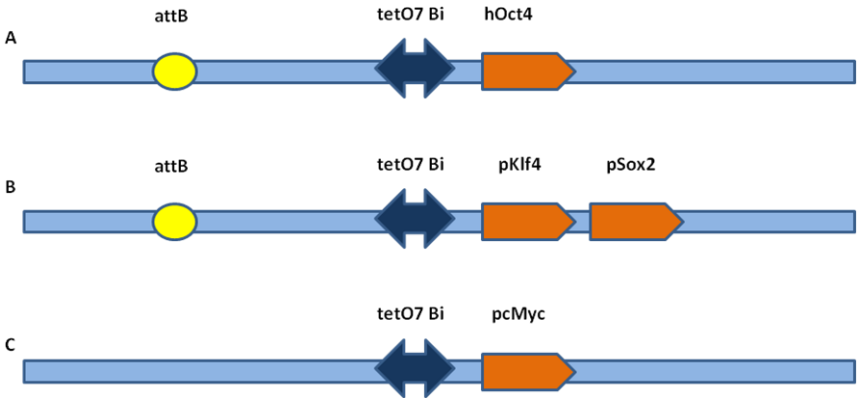

Figure 10: Constructs of reprogramming vectors.
A, pSL-attB-hOCT4; B, pSL-attB-pKS; C, pBS-pCMYC.

3.1.1.3 MicroRNA vector

MicroRNA 302-367 cluster is specific for pluripotent stem cells and reported to be able to reprogram mouse somatic cells into pluripotent stem cells with the help of histone deacetylase inhibitor valproic acid (VPA). Specific oligonucleotides (miR_PvuI_F and miR_PvuI_R) with flanking enzyme sites were designed to bind on both ends of the porcine miR-302-367 DNA sequence. The porcine microRNA 302-367 region was amplified from genomic DNA by PCR. Then it was cloned into an expression vector containing the CMV promoter, called pSL-attB-miR (Figure 11).

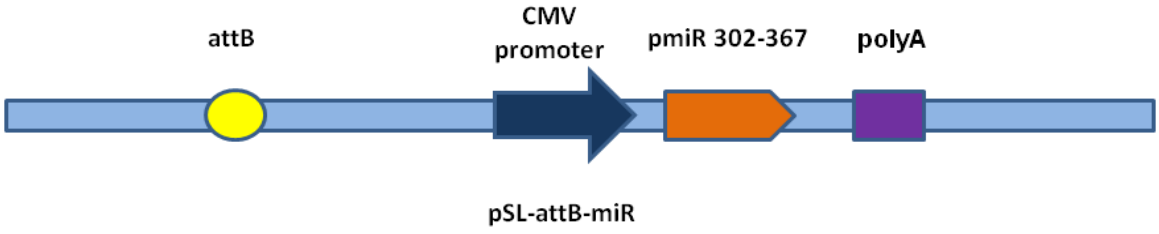

Figure 11: MicroRNAs vector for reprogramming.
Porcine miR-302-267 was driven by a CMV promoter.

3.1.1.4 Other vectors used for the reprogramming

PLOVE-N-Myc vector is a retroviral vector from Addgene. It contains an N-Myc expression cassette driven by CMV promoter (Figure 12).

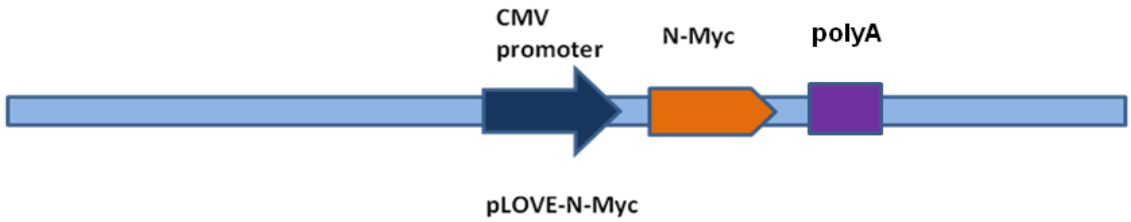

Figure 12: pLOVE-N-Myc vector.
The avian N-Myc gene was driven by a CMV promoter on the retroviral vector.

Porcine Nanog was cloned from the porcine ADMSCs by RT-PCR. After sequencing, the pNANOG was cloned into an expression vector containing the CAGGS (CAG promoter, chicken beta actin promoter/enhancer coupled with the cytomegalovirus immediate-early enhancer). On the back bone, there is a neo resistance cassette driven by SV40 early promoter which can be used for the selection (Figure 13). Pint-ØC31 vector was used to express the integrase which catalyzes the integration based on attB site.

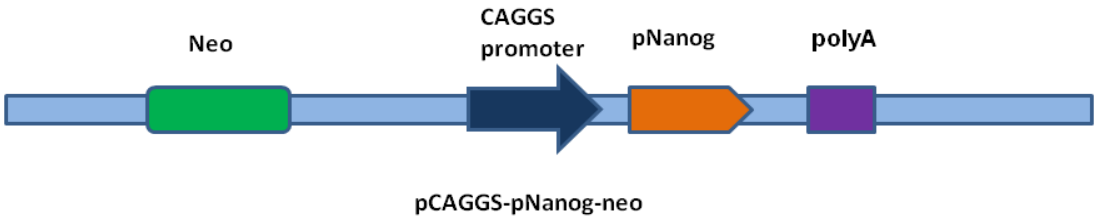

Figure 13: pCAGGS-pNANOG-neo vector.
The porcine NANOG gene was driven by a CAGGS.

3.1.2 Reprogramming of porcine somatic cells and identification of putative pluripotent stem cell

3.1.2.1 Reprogramming of porcine Nanog-KFCs with factors

Nanog has proven to be an important pluripotency marker in human and mouse pluripotent cells. Over expression of Nanog may help the somatic cells to reprogram into pluripotent state. 10 µg of sterile pCAGGS-pNANOG-neo was transfected into 1×10^6 porcine kidney fibroblasts by electroporation and the cells were plated on a T-

75 flask afterward. One day post transfection, the cells were passaged onto a new T-75 at a ratio of 1:1. The medium was changed to the medium containing 300 µg/ml of G418 to start selection. During the following two weeks, the cells were passaged for 3 passages at a ratio of 1:1 under selection. The morphology of the cells didn't change during the selection. Subsequently, to confirm that exogenous pNANOG was expressed. RT-PCR was carried out amplifying a 260bp fragment. The selected cell clones (pNANOG-KFCs) were used for reprogramming in the next step.

The three porcine reprogramming factors, Oct4, Sox2, and Klf4 were used to generate the iPSCs with the help of avian derived N-myc. N-myc is a member of the Myc family. It also has the potential of tumorigenicity, but lower than cMyc. N-myc is abundant in kidney cells (Malynn *et al*., 2000). The pLOVE-N-Myc vector is a lentiviral vector, but in this experiment, it was used as plasmid for transfection. Two transfections were performed. On day 0, the pSL-attB-hOCT4, pSL-attB-pKS, pLOVE-N-Myc were nucleofected into 5×10^5 of pNANOG positive cells at a ratio of 2:1:1. To get the vector integrated into the genomic DNA, the vector expressing the PhiC31 integrase was cotransfected with the reprogramming vectors. The amount of the integrase vector was equivalent to the sum of pSL-attB-hOCT4 and pSL-attB-pKS. The total transfected DNA amount was limited to 3 µg. The cells were plated on collagen pre-coated T-75 flask in the porcine KFC medium. In the following two days, the medium was changed into fresh porcine iPSC medium every day. The medium was supplemented with doxycycline at a final concentration of 1.5µg/ml. The expressions of hOCT4, pKLF4 and pSOX2 were controlled by the doxycycline dependent tet-on system, while N-Myc was expressed all the time. 3 days after the first transfection, the cells were detached and counted. The same transfection was repeated with the same vectors. This time, the vectors were nucleofected into all the cells from a T-75 flask. The cells were re-plated on a collagen pre-coated T-75 flask. During the following two days, the medium was changed. On day 6, the cells were passaged onto two feeder coated 10 cm dishes (Figure 14), and cultivated in the iPSCs medium for more than 4 weeks with doxycycline. The medium was changed every other day and the cells were passaged on new feeders every 4-5 days. The pNANOG-KFCs changed their morphology to long fibre shape and formed netlike structure with their neighbour cells. The mesh became bigger and lots of cells died in the first week. Several colonies were detected after 2 weeks. They were detached

with accutase and transferred onto feeders on 12-well plates. But no colonies grew out. The experiment was repeated but no colony showed up later.

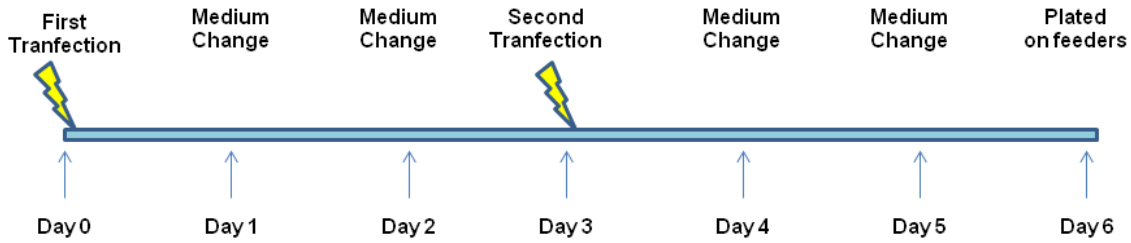

Figure 14: Double-transfection procedure for reprogramming.
The cells were transfected twice on day 0 and day3. The medium was changed to porcine iPSC medium after transfection. On day 6, the cells were plated on feeders.

3.1.2.2 Reprogramming of porcine ADMSCs with Oct4, Klf4, Sox2, and N-Myc

5×10^5 porcine ADMSCs were twice cotransfected with pint-ØC31 and a 2:1:1 mix of pSL-attB-hOCT4, pSL-attB-pKS and pLOVE-N-Myc. The total amount of DNA was 2µg. One day post the first transfection, the cells were plated on mitomycin-C treated mouse embryonic fibroblasts (MEFs) in porcine iPSC medium supplemented with 1.5 µg/ml doxycyline. The second transfection was carried out on day 3 (see timeline Figure 14). Cells were passaged every 4-5 days for 4-5 weeks by which time the experiment was terminated as no cell clones with iPSC morphology were obtained.

3.1.2.3 Oct4-GFP as a visible marker for reprogramming

Oct4-GFP transgenic porcine fetal fibroblasts (Oct4-GFP pFFs) were kind gift from Prof. H. Niemann (FLI, Mariensee) who had produced transgenic pigs carrying 18kb GFP expression vector under the control of the mouse Oct4 promoter. GFP expression in these animals was restricted to germ cells and cells of the early embryo while somatic cells of the fetus and the fetal fibroblast were GFP negative (Nowak-Imialek *et al*., 2010). Reprogramming of Oct4-GFP pFFs to a pluripotent state should reactivate the endogenous OCT4 gene as well as the Oct4-GFP transgene.

In this experiment, 1×10^6 porcine Oct4-GFP pFFs were transfected with 15 µg of the episomal pEBV-reproVII vector, which was successfully employed for the derivation of mouse iPSCs. The cells were plated in 10 cm dish and cultured in DMEM+

medium. One day post-transfection, the cells were passaged onto feeders and cultured in porcine iPSCs medium plus 50 ng/ml of bFGF. About 7 days post-transfection, many colonies appeared on the plates. The morphology of the colonies was similar to mouse ESCs colonies, but the shape was not round enough and the edge was not clear enough and no GFP expression was detected (Figure 15).

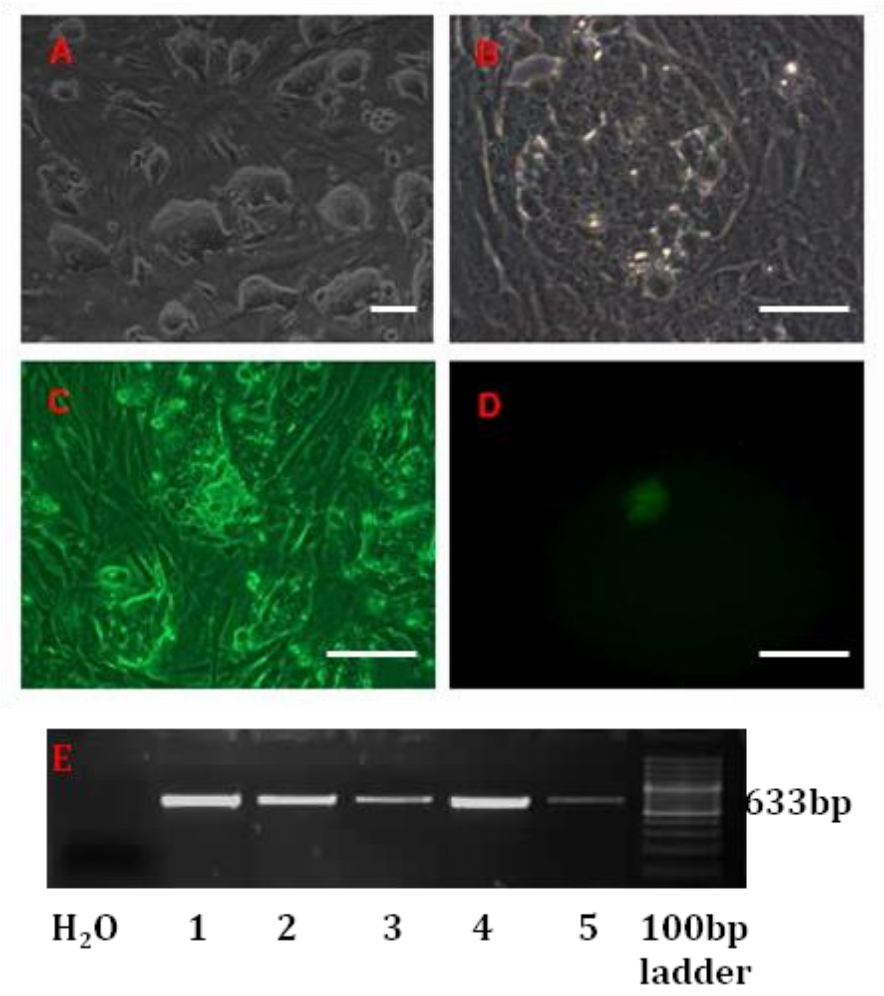

Figure 15: Reprogrammed cells from porcine Oct4-GFP fetal fibroblasts.
A and B, colonies of reprogrammed Oct4-GFP fetal fibroblasts; C, contrast under normal light; D, UV-light, E, RT-PCR of GFP, 1-5, 5 different cell clones of reprogrammed Oct4-GFP cells. Scale bars= 50 µm.

Nevertheless, some of the colonies were picked onto feeder coated 6-well plates. Cell duplication time was similar to mouse ESCs. They required passaging every 2 or 3 days at a ratio of 1 to 6. At passage 9 RNA was isolated from several cell clones and expressions of GFP was detected by RT-PCR. Figure 15 shows a clearly visible

amplification product, although only few fluorescent signals were observed (could be from dead cells or unspecific background).

Although the morphology of the cells was similar to iPSCs, and the cells were GFP positive according to the RT-PCR result, Oct4-GFP expression was not sufficient for visualisation. This may be an indication that reprogramming neither resulted in full reactivation of transgene, nor of the endogenous OCT4 gene.

3.1.2.4 CMV-GFP as a visible marker for reprogramming

Porcine CMV-GFP expressing BMMSCs were derived from 6 months old transgenic animal in which the GFP gene is under the control of the CMV (cytomegalovirus) promoter. Viral promoter such as the CMV promoter is generally silenced in pluripotent stem cells but is active in somatic cells (Liew *et al*., 2007; Wang *et al*., 2008c). If the cells are reprogrammed to iPSCs, the CMV promoter should be silenced and the green fluorescence should be turned off. In this study, 1×10^6 porcine CMV-GFP BMMSCs were electroporated with 15 µg of pEBV-reproVII and passaged into a new 10 cm dish one day post transfection. About one week later, the colonies appeared. They were picked and cultured in porcine iPSC medium plus 50 ng/ml of bFGF on feeders. After several passages, all of the cells were still green fluorescened. Compared to the untransfected CMV-GFP BMMSCs, the fluorescent intensity of some cells was however reduced, which may indicate that the activity of CMV promoter in these cells were down regulated by the reprogramming factors. But the cells were not fully reprogrammed so the CMV couldn't be silenced.

3.1.2.5 Generation of piPSCs with microRNAs

The microRNA miR-302-367 construct was used to transfect porcine ADMSCs, rat BMMSCs, rat ear fibroblasts and MEFs. The rat cells and mouse cells were designed as controls for testing the function of the construct.

The double-transfection method was used. At first, 5×10^5 porcine ADMSCs or rat BMMSCs were nucleofected with 1 µg of pSL-attB-miR and 1 µg of pint-ØC31 with the nucleofection program U-23. Rat ear fibroblasts were nucleofected with 3 µg of pSL-attB-miR and 3 µg of pint-ØC31. One day post transfection, the porcine ADMSCs, rat BMMSCs and rat ear fibroblasts were cultured in rat ESC medium

supplemented with 2 mM valproic acid (VPA). The cells were passaged to new 10 cm dishes at a ratio of 1 to 1 two days post nucleofection. 3 days post transfection, the second nucleofection was performed for each sample. On day 6, the cells were passaged onto feeder coated 10 cm dishes at a ratio of 1 to 2. The medium was changed every other day. No iPSC colonies could be detected after about one month by which time many cells had died.

To prove that miR-302-367 was expressed RNA was isolated from two independent transfections of pADMSCs and analysed by RT-PCR using primers miRexpF1 and BGHpAR1. A weak but clearly visible amplification product could be detected (Figure 16).

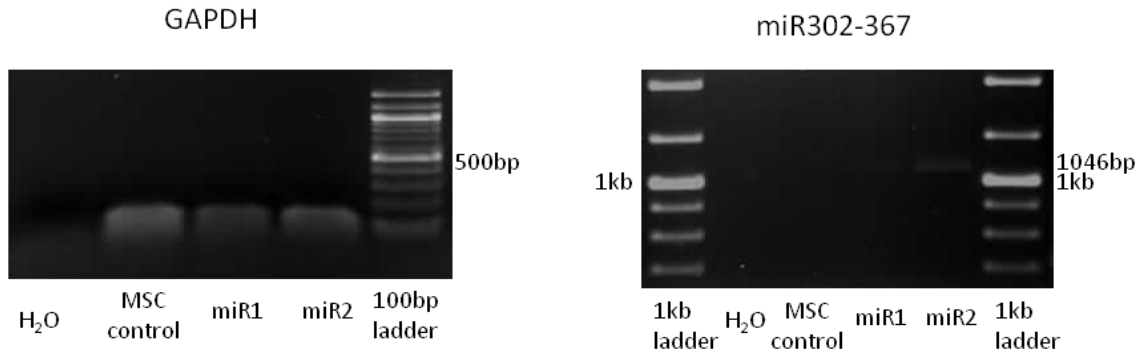

Figure 16: RT-PCR test of the expression of miR-302-367.

GAPDH RT-PCR showed positive in MSC, miR1 and miR2 samples, which indicated the presence of RNA. The RT-PCR products of MSC and water control were negative and the weak bands were observed for miR1 and miR2, which means the microRNA was transcribed in the transfected cells.

Since successful generation of iPSCs with the use of miR-302-367 was published for mouse (Anokye-Danso *et al*., 2011) and all attempts to reprogram porcine and rat cells failed, it was decided to use MEFs as a control for miR-mediated reprogramming. For this purpose, MEFs were plated on 10 cm dish at a 60% density and cultured in DMEM+ medium. The mouse embryonic fibroblasts starter kit (Lonza) was used to determine the optimal condition for nucleofection. The MEFs were nucleofected with 0.5 µg of pSL-attB-miR, 0.5 µg of pint-ØC31 and 1 µg of pmaxGFP (visual marker) and the program A-23 and T-20 were used respectively. The transfection efficiency was evaluated by assessing the expression level of GFP. 48h

post nucleofection, around 20% of cells were brightly green in both program. One day after transfection, the medium was changed to mouse ESC medium supplemented with 2 mM VPA. 3 days post the first nucleofection, the MEFs were nucleofected with 1 µg of pSL-attB-miR, 1 µg of pint-ØC31. Again no colonies were detected after one month in culture, which indicates that the pSL-attB-miR was also not able to reprogram mouse cells to pluripotent state.

3.1.2.6 Reprogramming of porcine ADMSCs with human and porcine factors

To assess if reprogramming of porcine cells might be more efficient when using porcine or human pluripotency factors instead of mouse factors, pADMSCs were transfected with pSL-attB-hOCT4, pSL-attB-pKS, pBS-pCMYC and pint-ØC31 at a ratio of 1:1:1:1 or 4:2:2:1. Both the single and double-transfection methods were also used.

Both single-transfection and double-transfection gave rise to colonies, which appeared 6 days post transfection. Their morphology was similar to the reprogrammed cells generated with mouse factors (pEBV-reproVII). About 50-100 colonies appeared per 10 cm dish. About 20 colonies were picked from one plate and passaged onto feeder coated 12-well or 6-well plates. After 5-6 passages, doxycycline was removed from the medium. All of the colonies differentiated and died within 3 to 4 days or during passaging. This showed that the fast proliferation of these colonies depended on the doxycycline, in other words, the exogenous factors.

From the morphology, no difference was observed. Compared with colonies published by Yamanaka's group (Nakagawa *et al*., 2007; Takahashi *et al*., 2007), the initial colonies from ours were similar to the non-ESC like colonies which were considered as a result caused by cMyc. To verify if they were background colonies, experiments were performed with or without cMyc.

3.1.2.7 Reprogramming of porcine ADMSCs with or without porcine CMYC

The porcine ADMSCs were nucleofected with the constructs containing human and porcine reprogramming factors in three parallel groups. The first group was transfected with pSL-attB-hOCT4, pSL-attB-pKS, pBS-pCMYC and pint-ØC31at a ratio of 2:1:1:3. The second group was transfected with pSL-attB-hOCT4, pSL-attB-pKS and pint-ØC31at a ratio of 2:1:3. The third group was transfected with only pBS-

pCMYC. Either single-transfections or double transfections were used for this experiment. Colonies were obtained only from the cells transfected with all four factors. No colonies were derived from the second group which expressed 3 factors hOCT4, pKLF4 and pSOX2 or the third group which expressed only pCMYC. The generation of colonies required all four factors.

3.1.2.8 Reprogramming of porcine BMMSCs with episomal vector

Compared with ADMSCs, the BMMSCs formed fewer colonies with same amount starting cells, which made the picking easier. It made sense to transfect cells with the two episomal vectors which generated mouse iPSCs successfully. As free episomes, they were integration-free and maintained stable numbers during passages.

1×10^6 porcine BMMSCs were electroporated with 15 µg of either pEBV-reproVI or pEBV-reproVII at one time. One day post-electroporation, the cells were cultured with 1.5 µg/ml doxycycline in either MSC medium or piPSC medium. No colonies appeared on pEBV-reproVI plates after the cells were cultured for more than 2 weeks and no fluorescent marker gene expression was detected. This vector was not functional for porcine cells to form colony. However, for cells transfected with pEBV-reproVII, colonies appeared as early as seven days post transfection, with about 20 colonies at day 10 post-transfection. Some of these colonies were detached and separated into single cells or smaller clusters by accutase and passaged on 6-well plates pre-coated with feeders. AP staining was performed with the rest of the colonies. Results showed that most of the colonies were AP positive (Figure 17). As a control 1×10^6 untransfected BMMSCs were cultured in MSC medium with or without doxycycline for 7 days. At this point, the cells were over confluent, but didn't show any colony formation (see Figure 17 C).

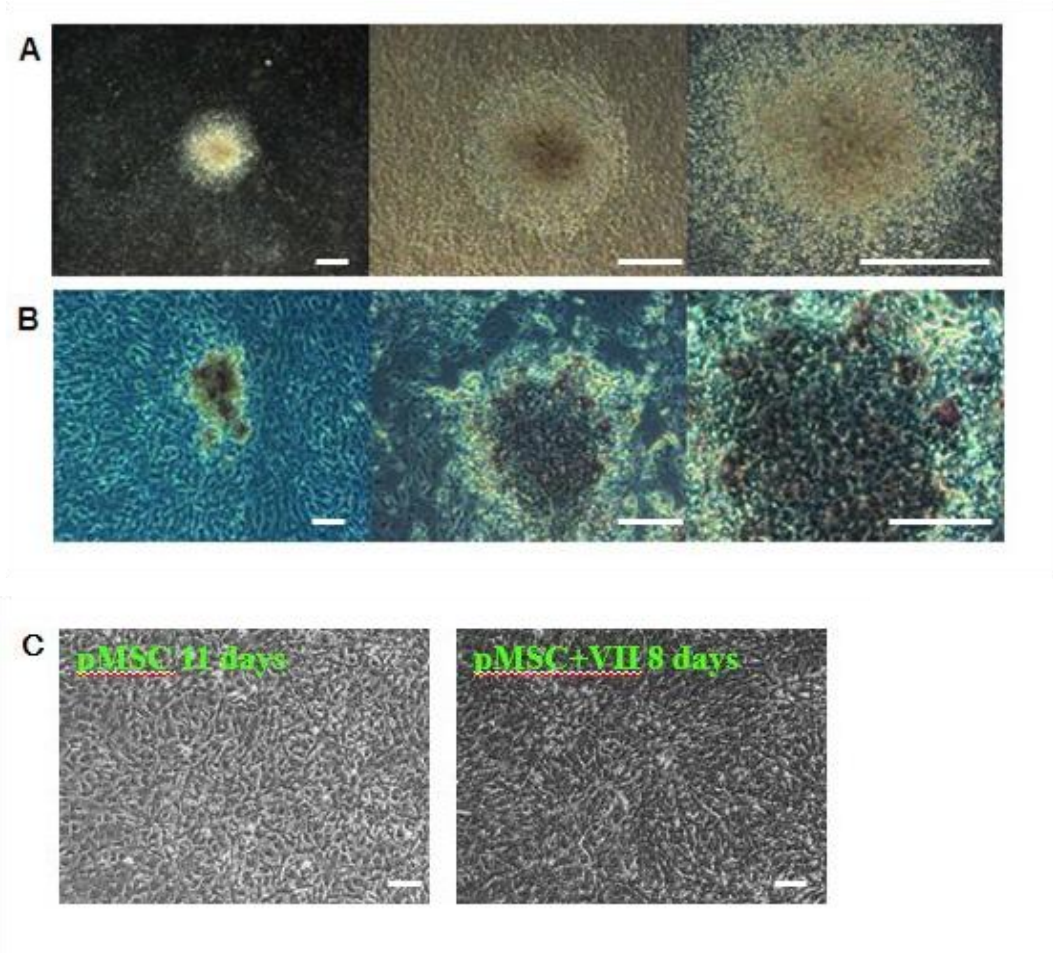

Figure 17: Putative piPSC colony (7 days post transfection) and pBMMSCs.
A, outgrown colony on the plate over confluent with transfected pBMMSCs. Scale bars=100 µm. B, AP staining of putative piPSC colony. Scale bars= 50 µm. C, left: untransfected pBMMSCs cultured for 11 days as a control; right: transfected pBMMSCs cultured for 8 days without doxycycline as a control. Scale bars= 50 µm.

3.2 Assessment of pluripotency of reprogrammed cells

To test the pluripotency of the putative iPSCs, several aspects were considered, including doxycycline dependency, expression of endogenous pluripotency factors, and marker genes, karyotype and differentiation abilities of cells.

3.2.1 Doxycycline dependency

To identify if the cells have reactivated the endogenous pluripotency factors and disengaged from the exogenous factors, doxycycline was removed from the culture medium at different passages.

At earlier passages (passage 8-15), removal of doxycycline resulted in changes of the cell morphology within 24 hrs. The cells became flatter compared to their parental cells. Many cells died before or after the next passage.

At later passages (passage 30-40), ESC-like morphology was retained for several days after doxycycline removal, but again was ultimately lost and cell death occurred.

As cells could not survive without doxycycline induction of the exogenous factors, the minimal amount of doxycycline required was then determined, ranging from 0.1 µg/ml to 1.5 µg/ml. Even at the lowest amount cells proliferated fast and kept ESC-like morphology, but did not survive after withdraw of the doxycycline.

3.2.2 Detection of endogenous gene expressions by RT-PCR

As the above experiment showed the ES-like morphology required continuous expression of the exogenous transcription factors, this could indicate that the endogenous pluripotency factors (porcine OCT4, SOX2, and NANOG, telomerase reverse transcriptase (TERT), CMYC, and KLF4) have not been reactivated or their expression levels were too low. Therefore RT-PCR analysis was carried out. As the mouse and porcine reprogramming factors share high sequence similarity, primers were designed to bind to the untranslated regions of the porcine genes. This enabled us to distinguish the porcine endogenous expressions from mouse exogenous gene expression. As a positive control RT-PCR analysis for pGAPDH was performed, RNA samples from mouse feeder cells and porcine BMMSCs were also included in the analysis as control. To compare the expressions with differentiated cells and microRNA transfected pMSCs, two samples of EBs and one sample of miR-302-367 transfected pMSCs were analyzed by RT-PCR.

The putative piPSCs were divided into two groups: SSEA1 positive population and SSEA1 negative population (for explanation see 3.2.5). RT-PCR products from piPSC samples were purified and sequenced to verify the amplified fragments from porcine RNAs.

The results showed that the water control was negative for all genes. The feeder control was only positive for pGAPDH. The pMSC was positive for all genes. SSEA1 positive cell was positive for all genes except pCMYC. SSEA1 negative cell was positive for pOCT4, pSOX2, pTERT and pGAPDH. EB1 was positive for all genes

except pCMYC and EB2 was positive for all genes. The microRNA transfected pMSC (miR pMSC) was positive for pOCT4, pSOX2 and pGAPDH (Figure 18).

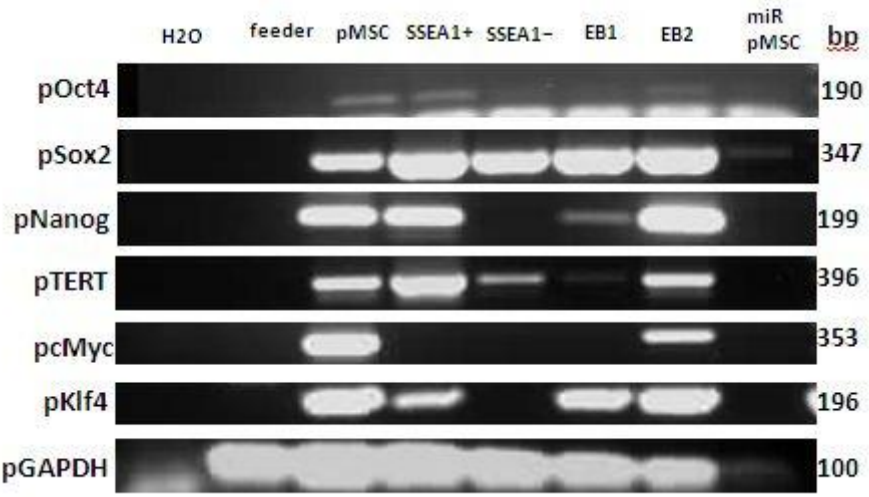

Figure 18: Detection of endogenous expression by RT-PCR.

Water control was negative for all genes and feeder control was only positive for pGAPDH. PBMMSC was positive for all the genes.

3.2.3 Karyotype

When the putative piPSCs were passaged for more than 20 passages, the karyotype of cells was checked (Figure 19). The putative piPSCs were passed on Gel-Matrix to remove the feeder cells before performing the metaphase spread. At passage 22 a total of 57 chromosome spreads were counted. The results are summarised in Table 3.

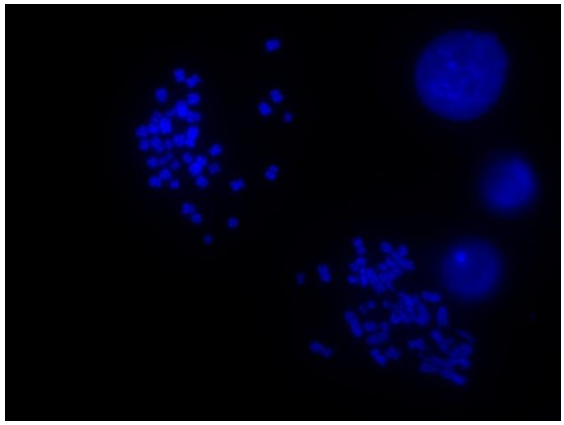

Figure 19: Metaphase spread of chromosomes from putative piPSCs at passage 22.

Table 3: Karyotype of putative piPSCs at passage 22.

Chromosome number	27	32	33	34	36	37	38	39	40	41
Number of metaphase spreads counted	1	1	1	2	9	8	27	6	1	1

In the 57 metaphase spreads, 27 had 38 chromosomes, so 47.4% of cells showed a normal diploid karyotype.

3.2.4 Immunostaining for pluripotency markers

To identify if the reprogrammed porcine cells expressed pluripotency markers, which are associated with pluripotency for human or mouse ESCs and iPSCs, immunostaining was performed to detect the Oct4, SSEA1, SSEA4, Tra-1-60, and Tra-1-80 (Figure 20).

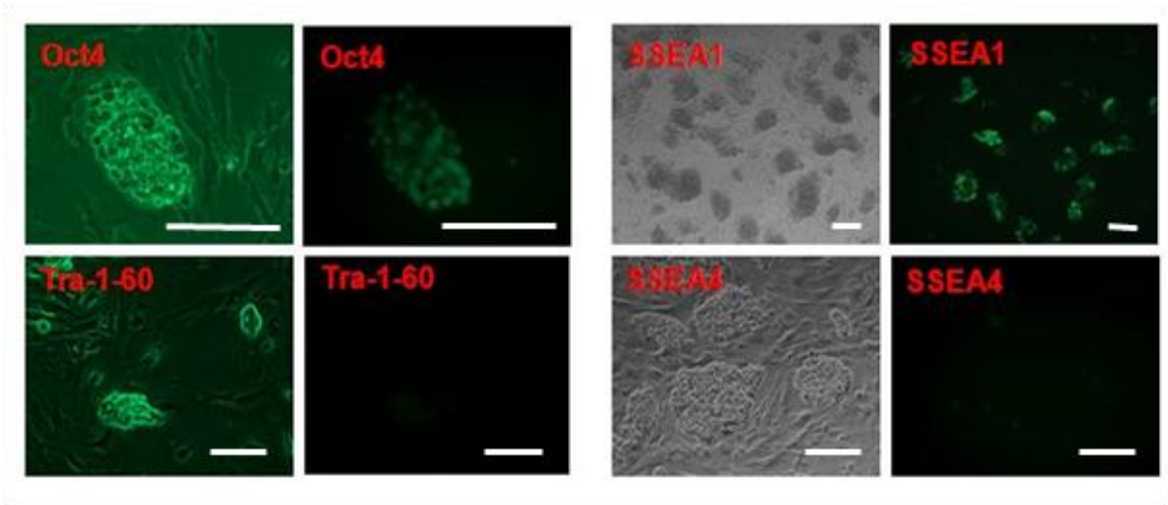

Figure 20: Pluripotency marker immunostaining of putative piPSCs.

Anti-mouse Oct4, SSEA1, SSEA4, Tra-1-60 and Tra-1-80 antibodies were used. Putative piPSCs were stained at passage 17. For Oct4, cells were treated with 0.1% Trixon-X100 to expose the inside factors. Scale bars=100 µm.

Except Oct4, all other markers are surface markers. All colonies were strongly positive for Oct4, and most of the colonies were positive for SSEA1 or partially

positive for SSEA1. Staining for SSEA4, Tra-1-60, and Tra-1-80 was negative. Feeder cells were negative for all the markers tested.

Mouse ESCs expressed SSEA1 but not the rest surface markers. Human ESCs were positive for SSEA3, SSEA4, Tra-1-60 and Tra-1-80 and negative for SSEA1. The result of immunostaining of Oct4 couldn't identify weather the Oct4 was from the endogenous pOCT4 or not, since exogenous mOct4 existed

3.2.5 Separation of the SSEA1+ and SSEA1- cells

Human ESCs were SSEA1 negative and mouse ESCs were SSEA1 positive. From the immunostaining results of putative piPSCs, it was obvious that some colonies contained mixed cell populations of SSEA1 positive and SSEA1 negative cells. This indicated that there were two populations of the putative piPSCs which differed in their reprogramming state. They tended to be a mixture of different subgroups rather than pure ones. For further investigating, the cells were separated using SSEA1 antibody coated microbeads. The separated cells were plated on new feeders. After several passages the SSEA1+ cells and SSEA1- cells were immunostained again with the SSEA1 antibody to check the efficiency of separating.

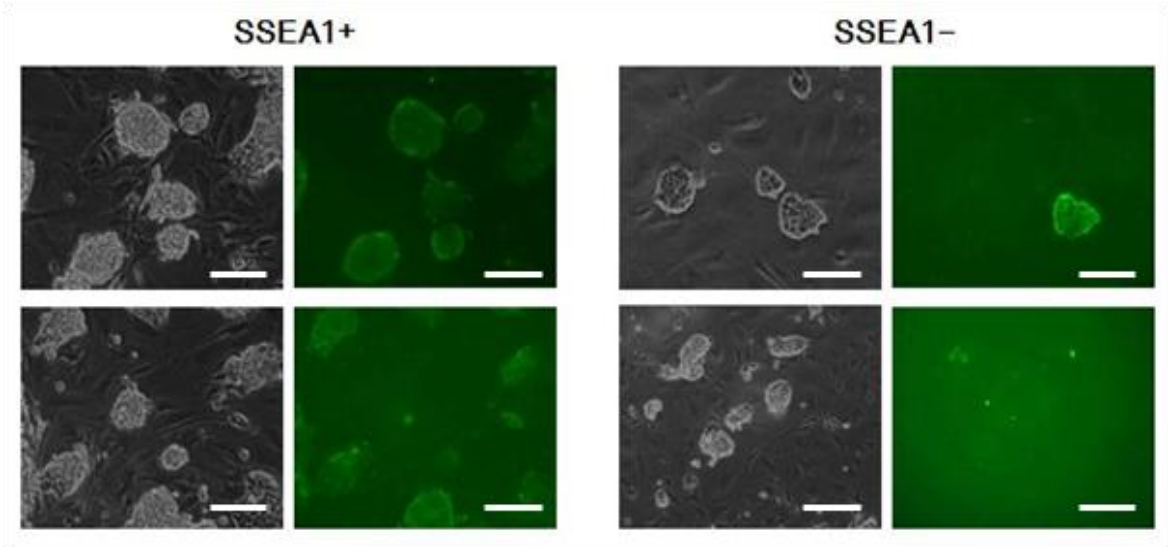

Figure 21: Immunostaining of SSEA1 for SSEA1+ and SSEA1- cells.
At passage 38, almost all the cells in SSEA1+ population were still SSEA1 positive. But a few negative colonies or cells were observed. The cells in SSEA1- population were mostly negative whereas a few colonies were positive for SSEA1. Scale bars=100 µm.

The results showed that more than 80 % cells in the SSEA1+ plates were still SSEA1 positive. Most of the cells in SSEA1- plates were negative but a few positive cells could be found (Figure 21).

To analyze if there is a difference in expression of pluripotency factors between SSEA1+ and SSEA1- cells, RT-PCR was performed. The results showed that there were differences between them (Figure 18). Both of them were negative for pCMYC. SSEA1+ cells were positive for pNANOG and pKLF4, but SSEA1- cells were negative for these two genes.

3.2.6 Differentiation of putative piPSCs

To test the differentiation ability of putative piPSCs, SSEA1+ and SSEA1- piPSCs were induced to differentiate. EB formation is often used as a method for initiating spontaneous differentiation toward the three germ lineages. The SSEA1- cells failed to form EBs, only few EB-like structures were observed compared with SSEA1+ cells which could form EBs more efficiently. The SSEA1+ piPSCs were used for the formation of EBs (Figure 22).

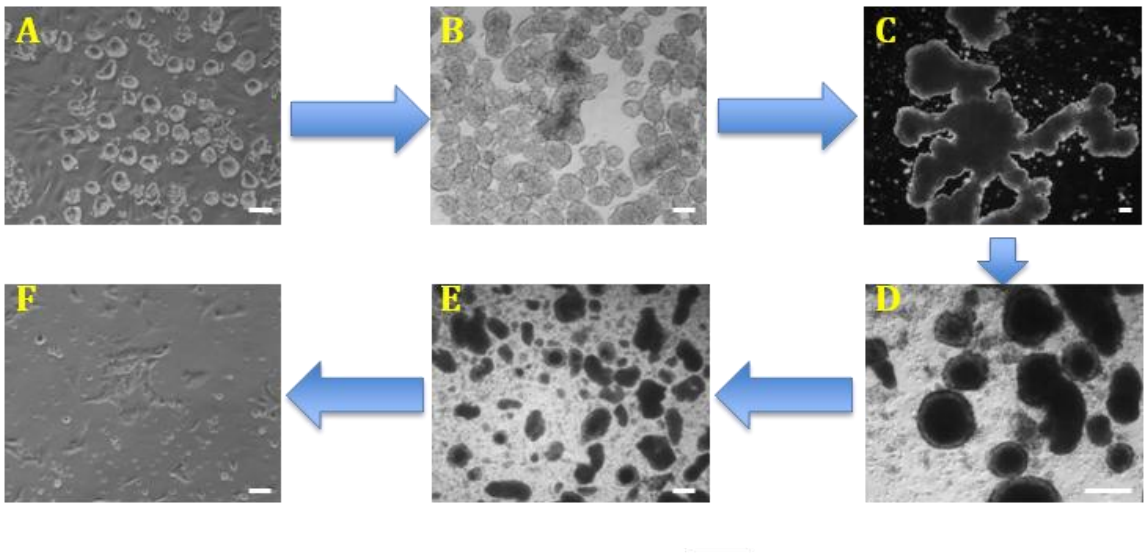

Figure 22: Formation of embryoid bodies.

The EBs were generated with suspension method. The EBs became darker in the middle and formed a clear boarder gradiently. A, The SSEA1+ iPSCs were cultured in rat ESC medium supplement with 1.5 µg/ml doxycycline; B, 2 days in suspension; C, 4 days in suspension; D: 6 days in suspension; E, 8 days in suspension; F, Plated on gelatine. Scale bars=100 µm.

The SSEA1+ piPSCs were passaged on feeders at passage 40 and cultured in rat ESC medium supplemented with 1.5 µg/ml of doxycycline. The cells were cultured till 60% confluence, and then were detached and separated into single cells. Subsequently, the single cells were resuspended in rat ESC medium with 1.5 µg/ml of doxycycline at a concentration of 4×10^6 cells per 10 ml medium. Then 10 ml of cell suspension was transferred to a 10 cm bacterial Petri dish and the rat ESC medium (supplemented with 1.5 µg/ml of doxycycline) was stepwise exchanged for DMEM+ medium (percentage DMEM+ medium was changed from 0% to 100%). On day 4, EBs were cultured in the DMEM+ only medium. On day 9 the EBs were trypsinized to separate the aggregates to single cells and plated on a gelatin coated 12-well plate. When the cells were confluent RNA was isolated.

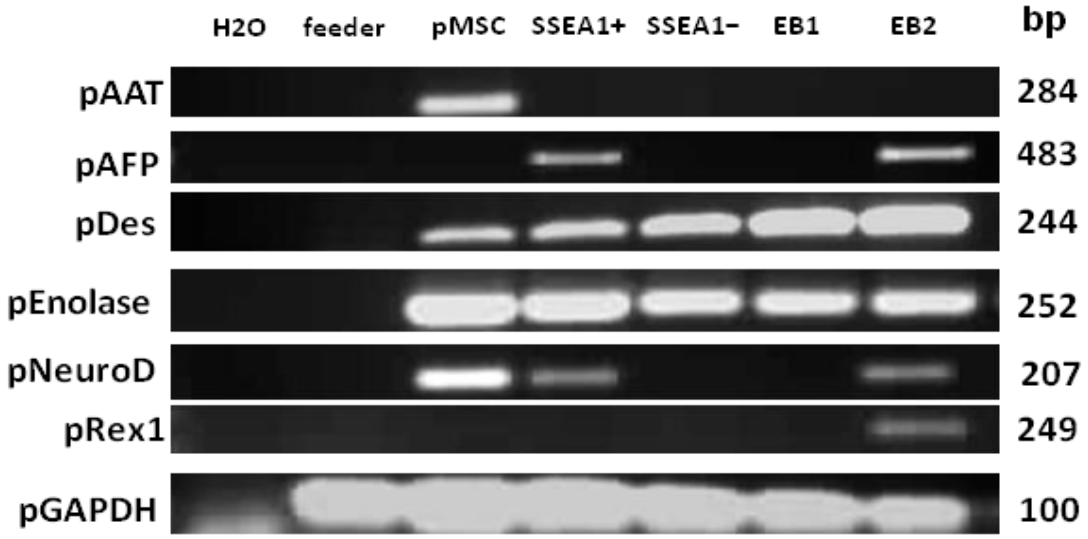

Figure 23: Gene expression of three germ layers.

The GAPDH was performed as control. As negative control, feeder was negative for all the genes but pGAPDH. PREX1 was also performed and only EB2 was positive for it. The EB2 was almost positive for all the genes except pAAT.

The expressions of marker genes for the three germ layers were tested by RT-PCR. RNA from two different EB experiments (EB1 and EB2) were analysed and RNA from pBMMSCs, SSEA1+ piPSCs, and SSEA1- piPSCs was also included as controls. Porcine Alpha-1-antitrypsin (pAAT, ectoderm), porcine desmin (pDES, mesoderm), porcine enolase (mesoderm), porcine Alpha-fetoprotein (pAFP, endoderm), and porcine neurogenic differentiation (pNEUROD, ectoderm) were tested by RT-PCR

amplification (Figure 23). Results showed that the EB1 RNA was positive for pDES and pEnolase. The EB2 sample was positive for expression of all genes tested except pAAT. In comparison, pBMMSCs were positive not only for pDES and pEnolase (typical for mesoderm), but also for pAAT and pNEUROD. Both of SSEA1+ and SSEA1- cells were positive for pDES, pEnolase and pGAPDH. The SSEA1+ cells were positive for pAFP and pNEUROD. This may indicate the putative piPSCs were not fully reprogrammed or there were differentiated cells in the putative piPSCs population. Rex1 used to be a marker of undifferentiated ESCs, but now it's considered not essential for pluripotency in mouse ES cells (Masui *et al*., 2008). It was reported Rex1 positive cells primarily differentiate into primitive ectoderm (Toyooka *et al*., 2008). Only EB2 was positive for pREX1.

Next directed differentiation towards neuron was attempted. For this, the culture medium of EBs was supplemented with 5μM of retinoic acid from day 4 onwards. On day 8, the EBs were separated into single cells by trypsin, and the mixture went through the 40 μm cell strainer to avoid the cell clusters. Then the cells were plated on laminin pre-coated plates at a density of 2×10^5 per cm^2 in DMEM/F12 medium with N2 supplement and 25 μg/ml of Insulin. The medium was changed at 2h, 4h post plating. 48h post-plating, the medium was changed to Neurobasal medium supplemented with B27. And the medium was changed on day 4, day 8, and day 12 post plating. Immunostaining was performed after day 12.

The results showed that there were a few neuron-like cells in the differentiated population. They had long and thin axons with small bright dots. Their long and branched axons crossed with the close neuron-like cells occasionally. Most of the rest cells shared a smooth muscle-like morphology which was flat, stentering and full of filaments.

Anti-beta-Tubulin III (mouse) antibody was used for identification of neurons and Anti-α-actinin (mouse) antibody for the smooth muscle cell type. Almost all the cells were positive for both Tubulin III andα-actinin (Figure 24).

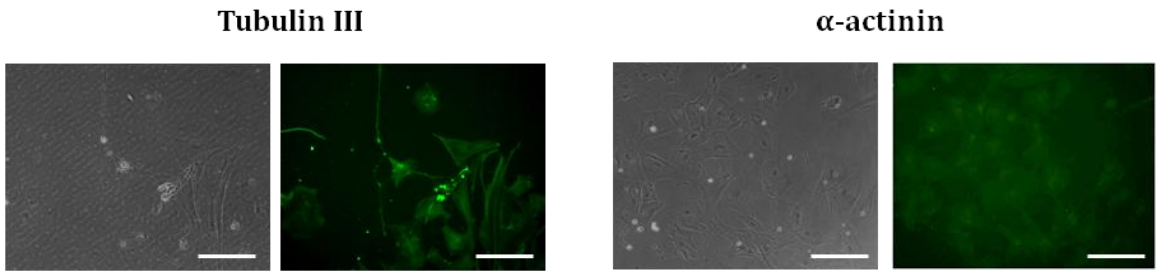

Figure 24: Immunostaining of differentiated cells with antibodies of specific markers.
All of the cells were positive for Tubulin III. The neuron-like cells showed long and thin axons with bright dots. Most of cells were positive for α-actinin. The smooth muscle-like cells contained lots of thin filaments. Scale bars= 50 µm.

Porcine cell with some characteristics of iPSCs could be isolated. Two distinct populations could be identified (SSEA1+/-). One of these expressed the endogenous pluripotency markers, although the levels were not sufficient as requirement for minimal expression of exogenous factors. These cells formed EBs and differentiatied into cells of all 3 germlayers could be observed. However the parental MSCs also expressed several of these markers, making interpretation of results difficult.

3.3 Cell synchronization and gene targeting

As the overall goal of the project was to develope methods for improved genetic manipulation of the pig genome and as it was not guaranteed that this could be achieved by iPSC technology, an alternative approach was also attempted: improving gene targeting in somatic cells, which compared to ESCs or iPSCs is generally very low. As a result of low efficiency, few successful gene targeting experiments have been reported for livestock.

As gene targeting by homologous recombinant is presumed to occur during the S-phase of the cell cycle synchronization of cells might result in improved efficiency. To test this, two tumor suppressor genes (APC and TP53) were chosen for targeting and both targeting vectors were already available.

3.3.1 Gene targeting of APC and TP53 in synchronized pBMMSCs

A protocol to synchronize pBMMSCs was developed by M. Sc. Denise Nguyen, but had never been employed for gene targeting experiments.

PBMMSCs were plated at a density of 60-70% confluence on T-75 flask and cultured in normal MSC medium containing 10% FCS. 24h later, the medium was changed into serum starvation medium containing 0.5% FCS. 14-16h post-starvation, the cells were released from the serum starvation by changing the medium into MSC medium containing 20% FCS.

24h post releasing, the cells were detached and collected for electroporation. 1×10^6 pBMMSCs were electroporated with either 13 µg of linearized APC1061-BS targeting construct or 10 µg of linearized p53-BS targeting construct. The cells were plated on 10cm dish and cultured in MSC medium (containing 20% FSC and 3 µg/ml blasticidine). The transfection and selection of the unsynchronized groups were performed with the same method.

3.3.2 Screening of the targeted colonies by PCR

After 10-12 days in selection, cell colonies become visible under the microscope. Cell colonies were counted and subsequently single cell clones were picked and cultured on 24-well plates and later expanded on 12-well plates. Once confluent, half of the

cells were then collected for DNA isolation and PCR screening to determine if the targeting vectors had integrated by homologous recombination. The rest of the cells were frozen or expanded for additional analysis.

In the APC1061 PCR screening, the oligonucleotides (BSf and BSr) binding to BS resistant cassette, were used to amplify a fragment of the BS gene as a positive control. The primer pair BSf and TVR1 (Figure 25), which bind to the endogenous APC gene and BS resistant gene, were used to screen for the targeting event. The amplified fragments were 3.7 kb for the targeted cells.

A pair of TP53 endogenous primers (p53 endo F2 and p53 Int1_5R) was used to amplify a 3.1 kb fragment as endogenous control. Another pair of primers (p53 endo F2 and p53 targ R) binding to the TP53 gene and a sequence on the targeting construct was used to screen for a TP53 targeting event (amplify a fragment of 3.3 kb). The targeting efficiencies as determined by the PCR screening are shown in Table 4.

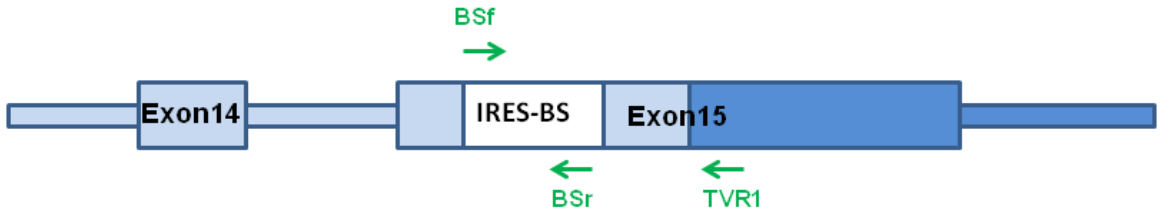

Figure 25: Oligonucleotides binding sites of APC.

BSf and BSr bind in the IRES-BS region. TVR1 binds in APC gene.

Table 4: Targeting efficiencies tested by the PCR screening.

	APC1061			TP53		
	Selection survived	PCR positive	%	Selection survived	PCR positive	%
Synchronized	29	17	58.6%	64	1	1.6%
Unsynchronized	19	16	84.2%	76	5	6.6%

Based on the PCR results, the efficiency of unsynchronized cells was higher than synchronized cells for both genes. For APC targeting, more colonies from synchronized cells survived after selection, but the positive colony number was only one more than the unsynchronized cells. For TP53 targeting, more colonies appeared in the unsynchronized group, and also more positive colonies in this group.

3.3.3 Validation of targeted colonies by southern blot hybridization

To confirm the results obtained by PCR, southern blot analysis was carried out. Where possible colonies were expanded on T-75 flask and cultured until confluent, the cells were collected, and the genomic DNA was isolated.

For APC1061, 10 µg of DNA was digested by BglII to distinguish the wild type and targeted alleles. If the targeting vector recombined correctly, one of the BglII will cut in the vector and the other one will cut in APC gene resulting in a fragment size of 5.2kb for the wild type and 6.0kb for the targeted allele (Figure 26). Two probes were used: BS probe only detects the targeting construct while the APC probe can bind to both wild types APC and targeted allele detecting two bands. Cell clones 2, 4, and 6 were targeted as they showed the two correct bands: 5.2 kb and 6.0 kb (Figure 27). Cell clones 5 and 8 were not targeted, since they only showed a single band of 5.2 kb, the same size as the wild type allele.

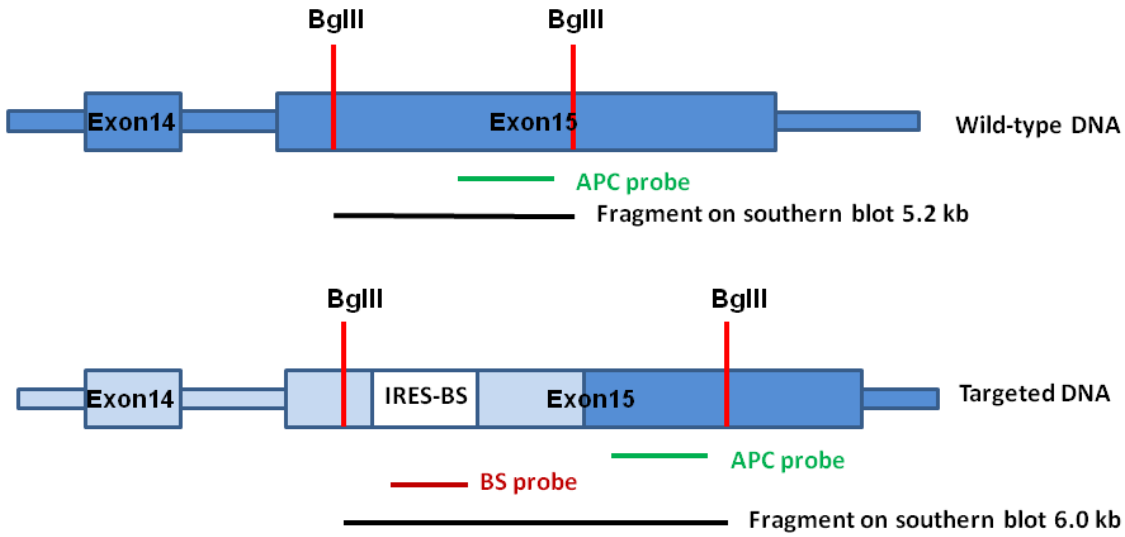

Figure 26: Schematic picture of the binding sites of APC probe and BS probe.
The APC probe (green) binds flanking regions 3' of the homology sequence. The BS probe (red) detects the IRES-BS-polyA-cassette (white).

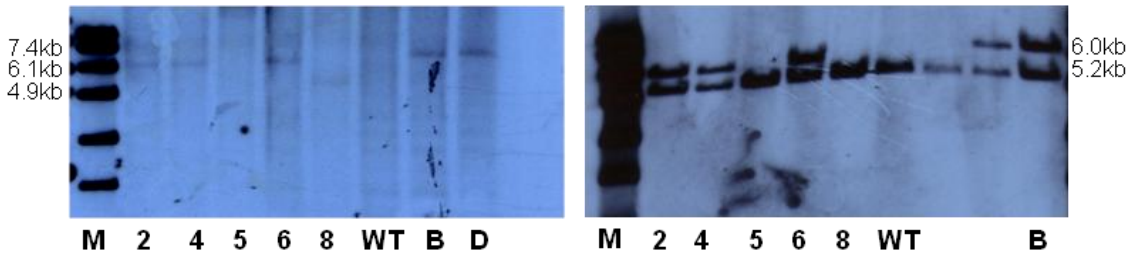

Figure 27: Southern blot of porcine APC1061-BS gene targeting cells.

5 of APC1061-BS targeted colonies were analyzed. Left: southern blot with BS probe; Right: southern blot with APC probe. Cell clones 2, 4 and 6 showed the 6.0 kb band in both southern blots. B and D were samples isolated from APC targeted pigs. Their results in southern blot were the same as cell clones 2, 4 and 6.

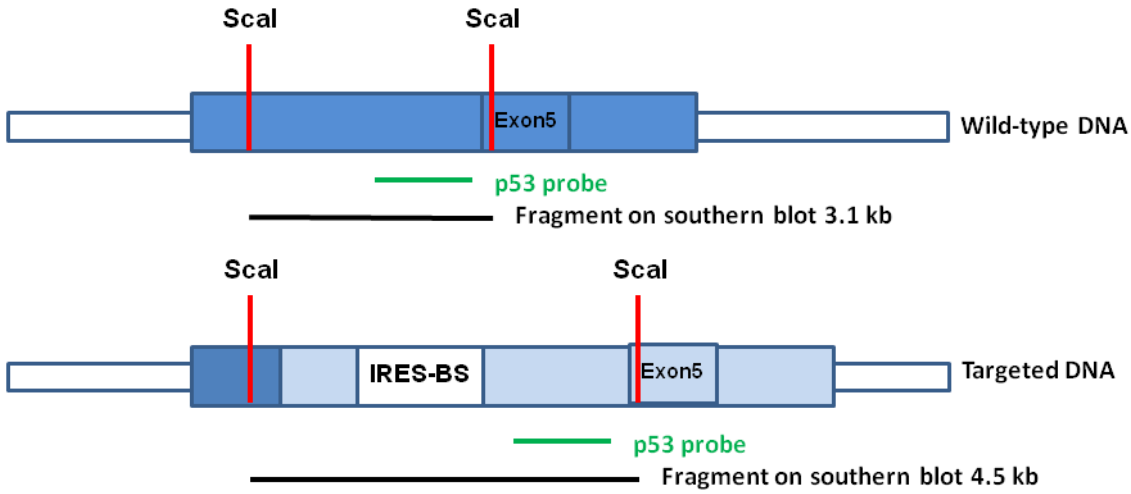

Figure 28: Schematic picture of the binding site of p53 southern blot probe.

The p53 probe binds flanking regions 3' of the homology sequence from targeting vector.

For TP53 targeted cells 10 µg DNA was digested with ScaI to distinguish the wild type and targeted alleles. Five ScaI sites locate within the TP53 gene. If the targeting vector recombines correctly, one of the ScaI will cut in the vector and the other one will cut in the TP53 gene resulting in a fragment size difference between the wild type (3.1kb) and the targeted (4.5kb) locus (Figure 28). The p53 probe used can bind to both wild type TP53 allele and the targeted allele. Cell clone 29 was targeted according to the southern blot results (Figure 29). It showed two bands: 3.1 kb and 4.5 kb. Cell clones 1 and 16 were not targeted, and they showed only a single band of 3.1 kb, the same as the wild type. Cell clones 7 and 22 showed two bands at 3.1

kb and 4.9kb. This indicated that cell clones 7 and 22 were not targeted. The 4.9 kb bands could be from the random integration.

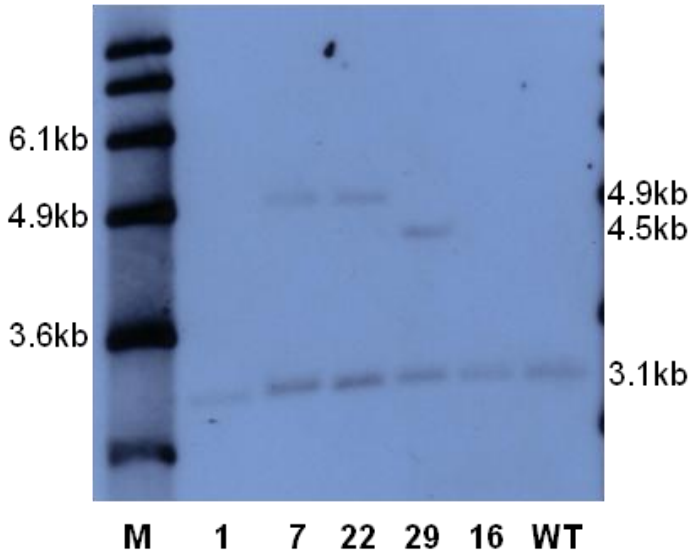

Figure 29: Southern blot of porcine p53-BS gene targeting cells.

Cell clone 1, 16 and wild type showed one band at 3.1 kb. Cell clone 29 showed two bands at 3.1 kb and 4.5 kb. Cell clone 7 and 22 showed two bands at 3.1 kb and 4.9 kb.

3.3.4 Somatic cell nuclear transfer with gene-targeted clones

Somatic cell nuclear transfer was carried out with three APC1061 targeted clones. Three piglets were born. PCR and southern blot analysis (performed by Dr. Claudia Merkl) confirmed that these pigs were derived from the targeted cells clones. The results of APC1061 targeting were published in Gastroenterology (Flisikowska *et al*., 2012).

4 Discussion

For biotechnology and medical research, animal models are still irreplaceable, and human disease models are widely used for basic research and the development of new treatment strategies and for gene therapies.

Small animals, like zebra fish and mouse, have been studied for a long time and offer good animal models. But due to their differences to human in their genetic affinity and body size, their usefulness is limited. Now, genetic modification of livestock species, such as pig, cow and sheep become possible and open the possibility to use these as models for human diseases. In particular, the pig has been suggested as the animal of choice as it has similar organ size and physiology to human, which may make results obtained more predictable for the transfer into the clinic (Telugu *et al*., 2010). Pigs are also deemed as the preferred donor animal for cell and organ xenotransplantation (Rogers *et al*., 2008).

Gene targeting is a common research tool to genetically modified animals. It has been used very successfully in the derivation of mouse models, which shows that it is a powerful and promising tool in transgenic animal research. The optimal choice of cells for gene targeting in the mouse is the ESCs (Capecchi, 2005). Though the mouse ESCs and human ESCs have been established for a long time, there still remains a large gap in our understanding how to isolate and cultivate genuine ESCs from large animals. Up to now, all efforts to derive porcine or bovine ES cells have been unsuccessful. Somatic cells in combination with nuclear transfer were used for gene targeting to make up for the lack of ESCs and successfully generated transgenic and targeted large animals (Laible and Alonso-González, 2009; Samiec and Skrzyszowska, 2011) However, with a lower efficiency compared to mouse ESCs. The somatic cells do not grow well as ESCs and often their lifespan is not long

enough for expansion and analysis. Beside, somatic cells necessitate the use of nuclear transfer to generate the transgenic animal, which again is inefficient.

The use of ESCs for genetic manipulation can save time and enhances the efficiency dramatically. It is assumed that the frequency of homologous recombination is higher in ESCs than somatic cells. Therefore gene targeting in the pluripotent stem cells is easier, and targeted cells can be used directly to generate chimeric animals with blastocyst injection. It has been shown that in mouse ESCs the targeting efficiency was 10-fold higher than in somatic cells (Arbonés *et al*., 1994). However, a comparison of targeting efficiencies between ESCs and somatic cells from livestock species has so far not been possible, due to the lack of pluripotent stem cells.

Another question which has not been solved is the donor cells for blastocyst injection in large animals. Induced pluripotent stem cells (iPSCs) could be the answer. After the mouse iPSCs and human iPSCs were created, they were used successfully for gene targeting (Zou *et al*., 2009).

Some notable results regarding porcine iPSCs have been achieved (Esteban *et al*., 2009; Ezashi *et al*., 2009; Wu *et al*., 2009; West *et al*., 2010). Putative iPSCs could be isolated and showed partial pluripotency, though all isolates differed from each other. Even chimeric pigs were born as reported by one group, but there are still queries if the cells are definitive iPSCs. Even if these putative iPSCs are not fully reprogrammed, they have some properties of the ESCs and may be more efficient in gene targeting than the somatic cells.

The purpose of this research was to search better cell types for transgenic animal generation in particular large animals with precise genetic modifications. Porcine somatic cells were reprogrammed to generate piPSCs. As none of the published attempts to generate iPSCs were completely successful, other methods for their

isolation were attempted in this work and results indicate that partial reprogramming of somatic cells has been achieved. It provides a basis for further experiments.

As an alternative to iPSCs, in the second part of this thesis, cell synchronization method to improve gene targeting efficiency in somatic cells was assessed. Two different targeting constructs were used to modify different alleles in multipotent MSCs. The synchronized and unsynchronized cells were compared in the efficiency of genetic manipulation. The results showed that though targeting was performed successfully, the synchronization did not result in higher efficiency. Targeted cells were then used for somatic cell nuclear transfer and live piglets obtained. These animals provide a model for the human FAP, since these patients have a strong predisposition for colorectal cancer (Flisikowska *et al*. 2012).

4.1 Methods for generation of iPSCs

Following the first iPSCs report (Takahashi and Yamanaka, 2006), many groups tried to isolate iPSCs from different species with different methods. By delivering the reprogramming factors with lentiviral vectors (Yu *et al*., 2007), retroviral vectors (Takahashi *et al*., 2007), adenoviral vectors (Stadtfeld *et al*., 2008b), episomal vectors (Yu *et al*., 2009), transponsons (Kaji *et al*., 2009), and plasmid (Okita *et al*., 2008), mouse or human iPSCs could be derived. It was investigated whether the 4 or 6 classic factors could be replaced (Nakagawa *et al*., 2007). Some groups generated iPSCs with Oct4 and Sox2 or even only Oct4 (Kim *et al*., 2008; Kim *et al*., 2009a; Giorgetti *et al*., 2010). To eliminate the potential mutation caused by integration of the exogenous expression vectors, factors were delivered as mRNA or protein. However the efficiency was generally lower and the methods were more complex (Warren *et al*., 2010; Kim *et al*., 2009b). At the beginning of this project the generation of iPSCs by delivering mRNA or protein into the cells had not been reported. For this reason and because the use of expression vectors was reported to give the most consistent results, this strategy was employed for the derivation of porcine iPSCs.

Reprogramming was attempted using vectors containing either mouse, human or porcine genes. Instead of cMyc also N-Myc was tested and cells which expressed fluorescent marker genes indicating either pluripotency (Oct4-GFP) or a differentiated state (CMV-GFP) were employed.

During the study of this project, two groups reported the successful generation of iPSCs by expressing microRNAs (Anokye-Danso *et al*., 2011; Miyoshi *et al*., 2011). One group achieved highly efficient reprogramming by lentiviral expression of miR-302-367 with the help of valproic acid (Anokye-Danso *et al*., 2011). The other group generated iPSCs by transient transfection of mature miRNA, miR-200c, miR-302s, and miR-369s (Miyoshi *et al*., 2011). I therefore also tried to express the miR-302-367 cluster to reprogram porcine somatic cells but with little success.

Best results were obtained when using the reprogramming vector pEBV-reproVII. This vector was successfully tested to generate mouse iPSCs. By using this vector, putative porcine iPSCs with morphology reminiscent of mouse ES cells could be obtained.

4.2 Construction of reprogramming vectors

4.2.1 Reprogramming factors

In previous studies with mouse and human iPSCs different reprogramming factors were assessed. Several of them were proven to be the key factors for the pluripotency. In the first iPSCs publication (Takahashi and Yamanaka, 2006), 24 candidate transcriptional factors were selected to generate the iPSCs, finally 4 factors were considered as the pivotal factors in generation of iPSCs from mouse embryonic fibroblasts. Since then, Oct4, Sox2, Klf4, and cMyc have been deemed to be the key reprogramming factors. They also tried to generate iPSCs with only 2- or 3-factor combinations. But no colonies were obtained with the 2-factor combination.

With the 3-factor combination there were some colonies but none of them were considered to be real iPSCs. However when other cell types were employed such as neural stem cells, a single factor Oct4 could reprogram the cells although with low efficiency (Kim *et al*., 2009b).

Unlike for human or mouse no porcine ESCs have yet been derived, in view of the difficulties it was decided to begin with the classic 4-factor method, the "OKSM method" for reprogramming porcine primary cells. Nanog and Lin28 were also considered for the generation of piPSCs based on their importance in ESCs and their functions in reprogramming human somatic cells.

Klf4 and cMyc are tumour related genes. CMyc was considered to accelerate the proliferation and transformation of the cells, which may be important in the early time of the iPSC colonies emerging.

Considering that porcine pluripotent stem cells may have different signalling pathway compared to mouse or human, other factors could be involved in their reprogramming. For example Oct4 may not be the core factor for porcine cells, as one group generated putative piPSCs with 3 factors omitting Oct4. The analysis of the Oct4 expression of porcine early embryo also indicated that Oct4 might not be the key factor in porcine embryogenesis (Kuijk *et al*., 2008). Other combinations of reprogramming factors could be attempted in future research.

The established naïve iPSCs and ESCs in human showed a dependence of extra Oct4 and Klf4 or Klf4 and Klf2 (Hall *et al*., 2009; Hanna *et al*., 2010). The similar experiment could be done with putative porcine iPSCs to test if extra Oct4 and Klf4 or Klf4 and Klf2 combinations can maintain the undifferentiated state in the absence of doxycycline.

It is know that there is a strict expression threshold for the reprogramming factors in particular Oct4. Higher or lower levels of the factors may trigger the differentiation. Only cells which express all four factors in the right level may become iPSCs. It may be an explanation why there are so few cells transformed into pluripotent stem cells.

4.2.2 The functions of cMyc and N-Myc in reprogramming

As a proto-oncogene, cMyc greatly enhances reprogramming efficiency, although it is dispensable for reprogramming (Nakagawa *et al*. 2007; Sridharan *et al*. 2009). The negative role of cMyc in the self-renewal of hESCs was reported (Sumi *et al*., 2007), and forced expression of cMyc induced differentiation and apoptosis of human ESCs. This contrasts significantly to the positive role of cMyc in mouse ESCs (Cartwright *et al*., 2005). During iPSC generation, transgenes delivered by retroviruses were silenced when the transduced fibroblasts acquire ESC-like state. The role of cMyc in establishing iPSCs could be as a booster of reprogramming rather than a controller for the maintenance of pluripotency.

CMyc and N-myc are essential for completion of murine embryonic development. But their roles in regulating pluripotency remain unclear. Previously, cMyc null mESCs were found to retain the potential for self-renewal with slight changes in differentiation capacity (Baudino *et al*., 2002), and N-myc can functionally replace cMyc in murine development, cellular growth, and differentiation (Malynn *et al*., 2000). Investigation of other Myc family members showed that both cMyc and N-Myc independently were sufficient to maintain the pluripotency (Smith *et al*., 2010). L-myc seems to be dispensable for embryonic development (Varlakhanova *et al*., 2010).

CMyc is an oncogene contributing to many human cancers (Beroukhim *et al*., 2010). Defects in the Wnt-APC pathway found in human colon carcinoma result in enhanced Tcf transcriptional activation of Myc (He *et al*., 1998). Retroviral insertional mutagenesis further identified cMyc as a major murine oncogene (Akagi *et al*., 2004).

These transgenic mouse studies provided the evidence that deregulated expression of Myc is sufficient to drive tumorigenesis (Adams *et al*., 1985; Leder *et al*., 1986; Chesi *et al*., 2008).

N-Myc showed lower tumorigenesis and could replace cMyc in an iPSCs cocktail (Blelloch *et al*., 2007; Nakagawa *et al*., 2007). In this study we therefore tried to use N-Myc to replace cMyc but no colony were obtained.

A previous study in human showed that cells formed colonies and proliferated fast when only Oct4 and cMyc were transfected. But these colonies were not really reprogrammed to pluripotent state. These colonies were considered to be a result of cMyc expression (Lowry *et al*., 2008). We therefore wanted to test if this could be true for our porcine cell clones. Transfections were carried out with or without cMyc. If only Oct4, Sox2 and Klf4 were transfected no colonies appeared. If cMyc was added to the cocktail ES like colonies did develop. To exclude that the change in cell morphology was due to cMyc, cells were transfected with pCMYC only, no colonies could be detected. This was a strong indication that only the combination of factors affected cell morphology and growth behaviour.

When comparing reprogramming factor combination of Oct4, Sox2, Klf4, and cMyc with combination of Oct4, Sox2, Nanog and Lin28 using episomal vector, the first combination resulted in large number of piPSC colonies and the latter in none. In combination with the above results it seems that cMyc is crucial in the reprogramming process.

4.2.3 Delivery system

Viral transduction usually leads to higher efficiency than non-viral methods (McMahon *et al*., 2006). Initial methods used to derive human iPSCs employed viral vectors, in which both the vector backbone and transgenes were permanently

integrated into the genome (Yu *et al*., 2007; Takahashi *et al*., 2007). Such vectors can produce insertional mutations that interfere with the normal function of iPSC derivatives, and residual transgene expression can influence differentiation into specific lineages (Yu *et al*., 2007), or even result in tumorigenesis (Okita *et al*., 2007).

Integration free iPSCs were also generated successfully with adenoviral vectors or repeated plasmid transfections (Stadtfeld *et al*., 2008b; Okita *et al*., 2008). But the efficiency is quite low and not suitable if the cells need long exposure to the vectors. Cre/LoxP was also used for removing the reprogramming factors, but the vector backbone could still stay in the genome (Kaji *et al*., 2009; Soldner *et al*., 2009). Transposon vectors are another option allowing removal of the integrated fragments from the genomic DNA without leaving a trace (Woltjen *et al*., 2009).

Episomal vector was used widely because of their unique properties. Theoretically, they don't integrate into the genome and remain independent of the genomic DNA. They only duplicate when the cells start to split and separate into the daughter cells equally.

In the research, presented here two different types of vectors were used: episomal vectors and vectors which could integrate into pseudo attP sites (requires addition of integrase) and could later be excised via the Cre/Lox system. They were delivered to the cells by single or repeated plasmid transfections. However there is no guarantee that the episomal vector could be excluded from genome even by using the oriP/EBNA1 system integration.

If Oct4, Sox2, Klf4, and cMyc were used, the efficiency of colony generation was same either episomal vector or attB-LoxP system. The difference was that the episomal vector always contained all four factors, while different attB-LoxP vectors were constructed containing between one to four dedifferentiation factors, which could be transfected at different ratios, for example, increasing the amount of Oct4

DNA or minimising the amount of cMyc DNA. After successful reprogramming, the possible but unwanted integration of the episomal vector may have to be assessed. For the attB-LoxP system the excision of the vector by Cre transduction still need to be tested. In most experiments, however these vectors should be transfected without the addition of Phi integrase, in the hope that only transient expression occurs and no vector integration. This still needs to be verified.

4.2.4 The promoter and doxycycline dependent tet-on system

Promoter that drives expression of the reprogramming factors is also of importance. First of all, the promoter should allow for abundant expression of all factors. Second, the promoter should be silenced or suppressed after the reprogramming process. Comparison of several promoters used during ES cell differentiation showed that CMV promoter drove the transgene expression only during the late stage. Similarly, the CAGGS and phosphoglycerate kinase (PGK) promoter drove transgene expression at a significant level only during late stages. Human elongation factor 1 alpha (EF1α) promoter directed robust transgene expression at every stage of mouse ES cell differentiation (Hong et al., 2007) So far, the beta-actin-based promoter is the only ubiquitous promoter that was reported to have little loss of gene expression during human ES cell propagation and in vitro differentiation. The CAGGS is comprised of the beta-actin promoter and may affect the behaviours of the reprogrammed cells. In this work, a bidirectional promoter was used for expression. This promoter contained two minimal CMV promoters. The tet-responsive element (TRE) was between two promoters.

The tetracycline-dependent gene system allowed a strict control over the expression of the pluripotency factors. Here a bidirectional promoter with an improved tet-on system was used. By adjusting the concentration of the doxycycline, the expression of the exogenous genes was controlled. In the absence of doxycycline the

exogenous genes couldn't be expressed and the partially reprogrammed cells changed their phenotype. This indicated that the tet-on system was very tight.

4.2.5 MicroRNA vector

Several reports demonstrated that specific miRNAs were highly expressed in ESCs and played a critical role in the control of pluripotency related genes (Houbaviy *et al*., 2003; Suh *et al*., 2004; Judson *et al*., 2009; Miyoshi *et al*., 2011).

One group transfected Nanog promoter-driven GFP mouse cells with mature miRNA mir-200c, mir-302s, and mir-369s at 48 h intervals. After 15 days, they observed 5 colonies from 5×10^4 cells and by 20 days post transfection they got 2 colonies from 1×10^5 cells (Miyoshi *et al*., 2011).

The miR-302-367 cluster contains a short DNA sequence less than 1kb. Alignment analysis showed that the miR-302-367 was highly conserved across species (Card *et al*., 2008; Rosa *et al*., 2009). In combination with valproic acid, it was a promising approach for porcine iPSC derivation and the experiment were based on those published for mouse iPSCs isolation (Anokye-Danso *et al*., 2011). The porcine miR-302-367 sequence was cloned into an expression vector driven by the CMV promoter. Although the mature porcine miR-302-367 was expressed in the cells no iPS colonies appeared.

4.3 Different cell types used for generation of iPSCs

Widely used cell types for iPSCs generation are fibroblasts and MSCs. Some terminal differentiated cells were successfully reprogrammed to iPSCs, which indicated that all of the somatic cells might be able to go back to the original undifferentiated state. But the efficiency among different cell types varied dramatically. In this study, considering the expected difficulties with the isolation of porcine iPSCs, we decided to start with multipotent MSCs.

It has been shown by several researchers that MSCs are a good choice for generation of iPSCs. They are multipotent and keep the ability to differentiate into several cell types, including chondrocytes, adipocytes, and oosteocyte. They can be passaged up to about 50 passages *in vitro*. The porcine MSCs express Sox2, Klf4, cMyc, which help the reprogramming process. In some MSC lines, the expression of Oct4 and Nanog could be detected in early passages and negative in later passages. Oct4 and Nanog expression was also found in human and rabbit MSCs (Lamoury *et al*., 2006; Roche *et al*., 2007; Riekstina *et al*., 2009).

In our experiments, Oct4 expression couldn't be detected in the first isolates of porcine MSCs. Lacking a source of Oct4 expressing cells, human Oct4 mRNA, which has a high sequence similarity to pig, was used as a replacement. Later on in newly isolated pMSCs Oct4 was detected by PCR, but the expression level might have been very low as all attempts to clone Oct4 failed again.

From mouse iPSCs research, we know that the efficiency of generation of iPSCs was low even with mesenchymal stem cells. The results from Yamanaka's group showed no evidence that the iPSCs are from the multipotent stem cells (Takahashi and Yamanaka, 2006). In this research, we focused on porcine MSCs (including BMMSCs and ADMSCs), and also tested other types of cells, like fetal fibroblasts and kidney fibroblasts. ADMSCs were used preferably since they were considered as an easily obtainable cell source and the better donor cells for iPSCs in human and mouse (Sugii *et al*., 2010). Studies showed that ADMSCs generated the iPSCs more efficiently with or without feeder. This may due to the high expression of FGF, LIF and fibronectin in ADMSCs. It was found that ADMSCs could serve as feeder cells for pluripotent stem cell lines (Sun *et al*., 2009; Sugii *et al*., 2011).

4.4 The medium and supplemental factors for iPSCs culture

Since mouse and human ESCs have long been established, their culture conditions have also been standardized. They have different signalling pathway for self-renewal. Basically, the mouse ESC medium is based on DMEM and supplemented with LIF, 10-15% serum or 15-20% serum replacement. Conditioned medium collected from medium used by MEFs was used for mouse ESCs in the early time. Human medium is based on knock out DMEM and supplemented with 4-10 ng/ml bFGF, 10-15% serum or 15-20% serum replacement. But different group may modify their medium by adding inhibitors and changing the concentration of the factors and serum or serum replacement. Since the components of serum varied by their source, the serum replacement was considered as a more reliable supplement for the cells. Some of the factors in serum may lead the cells to differentiate. The serum free mediums were also widely used for the iPSCs. Normally, it is based on DMEM/F12 medium and Neurobasal medium, supplemented with B27, N2 supplement and other factors and inhibitors.

During the reprogramming process, cells should be maintained in culture conditions that support self-renewal. The most important is the culture environment, which is a key determinant for the outcome of the pluripotent state (Van Oosten *et al*., 2012).

The four factors, however, could not induce human iPSCs when fibroblasts were kept under the culture conditions for mESCs after retroviral transduction. These data suggest that the fundamental transcriptional network governing pluripotency is common in human and mice, but extrinsic factors and signals maintaining pluripotency are unique for each species (Takahashi *et al*., 2007).

In our experiments the LIF was produced by MEFs and the unit couldn't be counted. The results showed that the putative iPSCs needed a rich medium, which was based on serum-free medium and supplement with LIF, GSK3 inhibitor and MEK inhibitor.

Besides, the cells grew better with the addition of 10% serum. Withdraw of any factors or inhibitors could lead cells lose their ESC-like morphology. The cells relied on LIF and were independent on bFGF, which indicated that the putative piPSCs were closer to mESCs.

4.5 MicroRNA reprogramming

Histone deacetylase (HDAC) inhibitor VPA was used in our experiments, as it was reported to enhance the reprogramming efficiency. One group employed lentiviral vector to express the miRNA driven by CMV promoter. Their results showed that the efficiency of generating iPSCs was higher compared to the traditional 4 factors method, but they showed no colony could be generated without VPA. We tried to culture the transfected cells with VPA. Though the expression of the miRNA was detected, there were no colonies. The same results were achieved from rat MSCs and mouse MEFs. Increasing the number of repeated transfections could be tried in the future. The classic reprogramming cocktail could also be used together with microRNA.

4.6 Reprogramming of somatic cells

4.6.1 Transfection methods

Compared to nucleofection, the electroporation was less harmful for the cells and they recovered soon after transfection. But the nucleofection had higher transfection efficiency. And, in the nucleofection, the nucleic acid could be delivered directly into the nucleus. In this research, both the nucleofection and electroporation were used for cell transfection. Pre-experiments were performed to determine the optimal conditions. In some experiments, the cells needed to be nucleofected twice, and the cells weren't counted at the second time, which may lower the transfection efficiency since the nucleofection requires fixed cell numbers.

4.6.2 Colony screening, picking, passaging, and storage

Selection is not necessary for obtaining the stable iPSCs, but omission of selection increased the number of false positive colonies, such as transformed cells or cells that failed to receive all factors (Stadtfeld *et al*., 2008a). In mouse some scientists used cells which had a resistant gene inserted at the endogenous Oct4 or Nanog locus for positive selection (Wernig *et al*., 2007; Okita *et al*., 2007). Live cells imaging was also used for distinguishing bona fide human iPSCs from the partially reprogrammed cells (Chan *et al*., 2009). To screen the iPSC colonies, the easy way is to visualize the signals of pluripotency. A knock-in GFP driven by the Oct4 promoter was used to indicate the re-expression of the endogenous factors and the stable iPSCs were selected successfully by this system (Stadtfeld *et al*., 2008a).

In experiments described here, by using Oct4-GFP transgenic fetal fibroblasts, no green colonies were observed, but the GFP expression was confirmed by the RT-PCR. This indicated that the Oct4 promoter was activated but maybe not fully demethylated. In the experiment of CMV-GFP MSCs, the CMV promoter should be silenced in fully reprogrammed cells since it was suggested that CMV was inactive in ESCs. Before transfection, the MSCs were brightly green. After transfection, the putative piPSCs and the unreprogrammed cells were still green, while some cells in the colonies were only faintly green. This phenomenon didn't change after several passages. That suggested the CMV promoter was not silenced because the cells were not fully reprogrammed.

Previous studies on human iPSCs showed that there were many non-ESC-like colonies generated with OKSM (Oct4, Klf4, Sox2 and cMyc). These colonies had different morphology compared with the real iPSC colonies and the positive selection reporter didn't activate in these colonies. These colonies shared similar morphology with our piPSC colonies which had irregular bright border and darker centre. The nature of these colonies remains to be determined (Takahashi *et al*., 2007; Hotta *et*

al., 2009). It was reported that some “early colonies” were observed 14 days post infection, which were highly proliferative and changed morphology. But further characterization indicated that these colonies were only transfected with Oct4 and cMyc and they were not real iPSCs. 21 days later, new colonies emerged. These “late colonies” were reprogrammed to iPSCs (Lowry *et al*., 2008). In our study, the putative piPSC colonies appeared at about day 7. These colonies shared the morphology of those “early colonies described” above. This may indicate that they are not fully reprogrammed. But, no new colony appeared even after waiting for more than one month.

For mouse and human cells the time for generation of iPSC colonies varied among different research groups. Depending on different methods, the period varied from 9 days to 40 days (Table 1). In our research, after transfection, the putative porcine iPSC colonies appeared and were picked 7-10 days. The putative iPSCs from Tibetan miniature pig emerged by 8-10 days post-infection (Esteban *et al*., 2009). Another group which got human iPSC-like colonies, found that the colonies were visible 7 days post-infection and the colonies were large and round with clear boundaries on day 13 (Wu *et al*., 2009). However, the mouse ESC-like colonies from one group emerged 22 days post transduction (Ezashi *et al*., 2009). One group, which generated chimeric offspring from their piPSCs, reported their initial colonies appeared within 7 days.

4.7 Identification of iPSCs

In the definition of the pluripotent stem cells two characteristic are important. One is the self-renewal, which is defined as the ability of cells to keep reproducing themselves without differentiation under appropriate culture conditions. The other is pluripotency the ability to differentiate into the three germ layers. The standards of pluripotency of iPSCs are the same as established for mouse and human ESCs.

They can differentiate into all the somatic cells and produce germ line cells. The new developed porcine pluripotent stem cells were compared with them.

To evaluate the pluripotency, many aspects could be considered. The first is the morphology of the putative iPSCs. Our putative piPSC colonies showed mouse ESC-like compact morphology with three dimensional structure and clear border. These cells displayed a high nuclear-to-cytoplasmic ratio and prominent nucleoli, though some human ESC-like morphology which was flatter compared with mouse ESC was also observed (Esteban *et al*., 2009; Wu *et al*., 2009).

Beside the morphology *in vitro* proliferation, feeder dependence, surface markers, gene expression, promoter activities, telomerase activities, and embryoid formation and differentiation should be considered. *In vivo* teratoma formation, chimeric formation, and germline contribution are important for the final pluripotency evaluation.

4.7.1 Feeder dependence

The pluripotent stem cells are generally maintained on a layer of inactive MEF feeder cells. The feeder cells could affect the analysis of pluripotent stem cells, for example the gene expression analysis by RT-PCR. It also influences the differentiation bias of pluripotent stem cells in culture. In mouse ESCs the feeder layer could be replaced by adding cytokine and LIF, but this didn't work for human ESCs. Gel-Matrix was used to replace the feeder for human ESCs (Xu *et al*., 2001).

In our study, the feeder was not necessary during the iPSC derivation. With or without feeder cells, the colonies were derived with the same efficiency. Similar results were reported for human and mouse iPSCs (Sugii *et al*., 2011). But it is crucial to maintain the putative piPSCs on feeder cells for proliferation. When plated on Gel-Matrix, the cell colonies lost their dome-like morphology and became loose and flat, but they still survived.

4.7.2 Silencing event in pluripotent cells and doxycycline dependency

One of the differences between pluripotent stem cells and somatic cells is the ability to silence retroviruses (Barkis *et al*., 1986; Teich *et al*., 1977). Fully reprogrammed cells show silencing of the factors delivered by retroviral vectors (Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007), whereas partially reprogrammed cells show incomplete silencing and persistent expression of the viral factors (Takahashi and Yamanaka, 2006). Further research in mouse showed that retroviral silencing is a gradual process that is initiated early but completed late in the reprogramming process, coinciding with the activation of epigenetic regulators and pluripotent genes (Stadtfeld *et al*., 2008a).

In human ESCs, the lentiviral delivered genes were suppressed under a promoter dependent manner, whereas the suppression of transient expressions weren't observed (Xia *et al*., 2008). Their results indicated that the promoter-dependent transgene expression and suppression in human ESCs is dependent on integration of the transgenes into the host genome. In mouse, the fully reprogrammed iPSCs were largely maintained by the activity of the endogenous pluripotency factors (Wernig *et al*., 2007).

Wu *et al* (2009) described their cells depended on doxycycline to maintain the undifferentiated state. The exogenous genes were silent with the absence of doxycycline in differentiation *in vitro*. In other two groups, even though their cells could differentiate into the three germ layers, the expression of retroviral transgenes were still detected (Esteban *et al*., 2009; Ezashi *et al*., 2009). This means the reprogrammed cells still depended on exogenous factors. In our research, the exogenous factors were always expressed with the presence of doxycycline.

A major advantage of the inducible system over constitutive expression systems is that it allows for the ''self-selection'' of reprogrammed cells in the absence of drug

selection and obviates the need for ES cell expertise. After stably reprogrammed cells have been generated and doxycycline has been withdrawn, cells that survived go on to reactivate the endogenous pluripotency program, while unstable reprogramming intermediates and transformed colonies disappear, likely through differentiation or apoptosis.

Along with the theory of naïve pluripotent stem cells, the real embryonic stem cells in human were defined (Nichols and Smith, 2009; Hanna *et al*., 2009). Naïve pluripotent cells can differentiate into a primed EpiSC-like state *in vitro* by promoting the signalling of TGF-b, Activin, and bFGF, EpiSCs can epigenetically revert back to naïve pluripotency by a variety of genetic manipulations and culture conditions. The conserved hESCs were considered as hEpiSCs that were slightly different from naïve ESCs. HEpiSCs showed a flat morphology compared to the mouse ESCs (naïve state). The naïve ESCs were considered as the ground state whereas the EpiSCs were considered as the primed state. Though both of them can form teratomas, the naïve ESCs can contribute to the chimaeras while the EpiSCs can't. The naïve ESCs have no differentiation bias and the EpiSCs have variable differentiation bias. What's more, the naïve ESCs respond to LIF/STAT3 pathway while the EpiSCs respond to FGF pathway. ESCs need the pluripotency factors Oct4, Nanog, Sox2, Klf2 and Klf4 while EpiSCs only need Oct4, Nanog and Sox2 (Nichols and Smith, 2009). To investigate the naïve state of human pluripotent stem cells, the doxycycline-control system was used to generate hiPSCs. Unlike the result from mouse iPSCs, those naïve hiPSCs depended on doxycycline and differentiated upon withdrawal of doxycycline. With the expression of Oct4 and Klf4 or Klf4 and Klf2, the hiPSCs could be released from the dependency of doxycycline (Hanna *et al*., 2010). When the hESCs were transfected with Oct4 and Klf4 or Klf4 and Klf2, they changed their flattened morphology to dome-shaped colonies and packed round cells morphology, like mESCs. The generated hESC-like piPSCs also employed doxycycline-control system, similar results showed that their piPSCs changed morphology and

differentiated quickly without doxycycline (Wu *et al*., 2009). Many of our putative piPSCs showed dome-like colonies and mESCs morphology, especially the SSEA1 positive cells. They also depended on doxycycline. This indicated that these cells may also need extra factors, like additional Oct4 and Klf2, to maintain the undifferentiated state.

Contrast to previous studies, the results from our piPSCs, indicated that the iPSCs in large animals are not permissive as mouse, and they may need extra conditions to stabilize the naïve state.

4.7.3 Gene expressions of iPSCs

After several passages the putative piPSCs were analyzed for expression of several endogenous genes. The expression of pOCT4, pSOX2 and pNANOG as the most important pluripotent genes, were detected. But without qPCR, we couldn't say if they were up regulated, since our BMMSCs used for piPSCs expressed all the factors (even Oct4) before reprogramming. The GAPDH, housekeeping gene, was used to test if there was RNA or not. Though the primers were designed for porcine GAPDH, it was not specific enough as they allowed amplification of mouse GAPDH. To avoid amplification of both endogenous and exogenous fragments primers were designed to bind to the 3' or 5' UTR of the porcine sequences, so only porcine were detected. Sox2 and TERT expression was found in all samples. Two different piPSC populations were obtained: SSEA1 positive and SSEA1 negative (see 3.2.5). Interestingly both populations were negative for pCMYC expression, which might indicate that the partially reprogrammed cells down regulated or silenced pCMYC due to the presence of exogenous mcMyc. The SSEA1 negative cells were negative for pNANOG and pKLF4, which was consistent with the differences in their loose morphology and immuostaining results. As long as the doxycycline was present and the exogenous genes were expressed cells could be cultured for many passages.

4.7.4 The karyotype stability

The long-term karyotype stability is an important issue, especially for cells passaged by enzymatic digestion (Trounson, 2006). In human ESCs there was a considerable instability of some imprint genes in long-term culture (Rugg-Gunn *et al*., 2005). For the porcine iPSCs even at passage 22 half of the cells showed a normal karyotype.

4.7.5 The specific markers of pluripotent stem cells

The immunostaing results in this work showed that the putative piPSCs carried the SSEA1 surface marker. But they were negative for SSEA4, Tra-1-60 and Tra-1-80. This result supported the point that the putative piPSCs were closer to mouse iPSCs than human iPSCs. In previous studies, the putative iPSCs from different groups showed different surface markers. Two groups showed their piPSCs were SSEA1 positive (Ezashi *et al*., 2009; Telugu *et al*., 2010), while one group found their piPSCs positive for SSEA3, SSEA4, Tra-1-60 and Tra-1-80 (Wu *et al*., 2009), which is similar to human iPSCs. The piPSCs reported by one group were SSEA4 positive, but no other surface markers were tested (Esteban *et al*., 2009). The morphology also seems to have a relationship with the surface marker. The morphology of SSEA1 positive cells had more similarity with mouse iPSCs, while the SSEA3 and SSEA4 positive cells were closer to human iPSCs which are flatter (Ezashi *et al*., 2009; West *et al*., 2010).

In our study, not all of the cells were SSEA1 positive. Even in one colony, part of the colony was positive whereas the rest was weakly positive or negative. This coincided with the observation that the colonies of some putative piPSCs showed a mixed morphology. In order to get a pure population, the cells were separated with the SSEA1 marker (microbeads system). More than 80% of cells in the SSEA1 positive population were SSEA1 positive after separation and this percentage was maintained for several passages. The positive and negative populations showed different

morphology: the positive one showed “dome-like” compact colonies while the negative one seemed flat and loose. The SSEA1 positive cells also performed better in the following differentiation experiment.

4.7.6 The differentiation ability of putative piPSCs

Embryoid body-mediated differentiation was performed to test the differentiation ability of putative piPSCs. In this work, different methods were used for the EBs formation, including hanging drops, suspension and aggregate well. Even for the same cell line, not all the time the cells could form embryoid bodies. Balls and “sausages-like” EBs showed an undistinguished morphology compared to published mouse or porcine EBs. RT-PCR of these differentiated cells was performed to analyze the expression of differentiation markers. As shown in the results, the expression patterns were confusing. AFP, an endoderm marker, was positive in SSEA1 positive cells and embyoid body sample 2 (EB2). Another endoderm marker, AAT, was only found in MSCs. Desmin and Enolase were positive in all the samples. Since they are the markers for mesoderm, a possible explanation is that the cells were only partially reprogrammed and still had the memory from the MSCs, which are derived from mesoderm. NeuroD, as a marker for ectoderm, was found in MSCs, SSEA1 positive cells and EB2. Rex1, considered as a marker for epiblast cells, was found only in EB2. In all of the published works, piPSCs could differentiate into the three germ layers by embryoid body-mediated differentiation. The markers of three germ layers described above were also detected. Besides, they also checked the expression of pluripotency markers, like Oct4 and Sox2. One group showed almost silenced Oct4 and strongly down regulated Sox2 (Ezashi *et al*., 2009). The cells with hiPSC morphology showed Oct4, Sox2 and Nanog decreased in EBs differentiation (Wu *et al*., 2009). The group which generated the chimeric pigs reported that hOCT4 and pOCT4 were detected in EBs after 10 days of differentiation. This might be due to the resistance to silencing caused by lentiviral integration (West *et al*., 2010). In

this work, we found the presence of pOCT4, pSOX2, and pNANOG expression in the EBs, which means the cells weren't fully reprogrammed and couldn't get the pluripotency marker silenced during differentiation. Real time RT-PCR can be performed to analyze if the factors were down regulated after differentiation or not in the future.

By adding retinoic acid to EB culture neuronal differentiation can be induced in human and mouse. The method was used to differentiate rat iPSCs successfully into neurons and was then tested for piPSCs differentiation. Immunostaining was performed to identify the cell surface markers. There were mainly two populations in the differentiated cells: neuron-like cells and smooth muscle like cells. The mouse beta-tubulin III antibody was applied in the immunostaining. A few neuron-like cells were observed in the differentiated population. But the immunostaining result was not specific enough. The antibody was designed specific for mouse. It was possible that the antibody could bind to other things unspecifically in porcine or did not recognise porcine proteins at all. Since there were many smooth muscle-like cells, another antibody which can bind to alpha-actinin, was also used to identify smooth muscle cells. Though the antibodies were tested with different dilutions and the negative control was negative, all the cells showed a weak positive signal. Similar results were shown in previous studies: human ESC lines H7 and H9 cultured on Gel-Matrix were positive for the neuronal precursor marker beta-tubulin class III both in undifferentiated cells and EBs (Carpenter *et al*., 2001). It was also found in some cancer cells and was considered as a marker of angiogenic perivascular cell (Sève *et al*., 2007; Chen *et al*., 2011; Stapor and Morfee, 2012). Specific anti-porcine antibodies may be needed for further research.

To demonstrate multilineage differentiation, piPSCs could be injected into nude mice and generated teratomas derived from three germ layers. All the published porcine results showed that piPSCs could differentiate into three germ layers *in vivo*. We also

tried to inject nude mice with our putative piPSCs, but no teratoma was obtained successfully. Considering their doxycycline dependency, it was not surprising that they may have stopped to proliferate *in vivo* without doxycycline.

However, germ line competence wasn't reported in the first three piPSC publications, indicating that these cells do not possess the full arsenal of pluripotent properties. So far, only one group described their piPSCs could generate chimeric offspring (West *et al*., 2010). They got live chimeric fetus by injecting the piPSCs into embryos with high efficiency of 85.3%. All of the chimeric fetuses developed normally, and the young chimeric pigs developed without tumour. PCR analysis confirmed that the piPSCs contributed not only to the chimeric offsprings but also to germline. But germline transmission was very low and all offsprings derived from iPSCs only survived for 3 days. This indicates that their iPSCs were not fully competent (West *et al*., 2011).

4.8 Gene targeting and synchronization

Since the real piPSCs were failed to be generated, the alternative choice was considered for gene targeting: cell synchronization. Somatic cells were successfully applied in livestock gene targeting. Theoretically, the targeting efficiency could be improved by arresting cells in S/G2 phase in which homologous recombination occurs.

Serum starvation was used to synchronize the pMSCs at S phase in this work. By comparing the targeting efficiency between synchronized and unsynchronized cells, the result determined if synchronization could increase the rate of homologous recombination or not. Two tumour related genes, APC and TP53 were studied in this work. Porcine MSCs were successfully targeted with their targeting vectors. PCR and southern blot were used to check if the cells were targeted.

4.8.1 Synchronization method

Specific synchronization can favour the targeting efficiency by increasing the rate of homologous recombination. To eliminate the side effect of chemical inhibitors, serum starvation is a better choice to synchronize primary cells prior to gene targeting. The starvation and release from cell cycle block were controlled by changing the serum contents of the medium, which is easy to manipulate. According to the results from M. Sc. Nguyen, the serum deprivation was sufficient to arrest the pMSCs while some chemical inhibitors failed (hydroxyl urea and thymidine). So the serum starvation protocol was used in this research. The starvation could last for up to 96h (Goissis *et al*. 2007, Kues *et al*. 2000). To minimize the cellular stress, the starvation time was limited to 24h. The starter population of pMSCs was limited to a confluence of 60-70%, which is crucial for the synchronization in this protocol. The cells were arrested at G0 phase after 24h of serum starvation. Then medium with 20% serum was used to release the cells from the G0 phase. 16h post-releasing, most of the cells were stuck at the S/G2 phase, meanwhile the cells were transfected in order to get the highest efficiency, since homologous recombination occurred at S/G2 phase.

Cell populations from different isolations could have remarkable differences in cell doubling time, which may affect the efficiency of synchronization. The cells which used for establishing the serum starvation protocol may have a different growth speed from those used for gene targeting, thereby decreasing the synchronize effect.

4.8.2 Validation of the targeted colonies

A promoter-trap strategy was used to enrich for targeted cell clones. Without integration near a promoter the resistant gene couldn’t be expressed. This strategy helped to avoid the false positive clones caused by random integration. The concentration of the selection antibiotics were determined by a killing curve. The final

concentration should be just enough to kill the wild type cells but not hurt the cells with the construct integrated.

After 10 to 15 days in selection the cells formed colonies on the plates. The single colonies or minipools were expanded and screened by PCR. PCR screening was not enough to confirm that the cells are truly targeted. Further analysis, such as Southern blot analysis, were required, this should show predicted bands not only for the targeted but also the wild type alleles and exclude cells with additional random integration. In this work, the step of validation was very necessary before using the cells for nuclear transfer.

Three of APC1061 targeted colonies were used for somatic cell nuclear transfer. Piglets from these cells were born and analyzed and targeting confirmed. The result was published on Gastroenterology (Flisikowska *et al*., 2012). These transgenic pigs could provide a valuable resource for colorectal cancer research.

4.8.3 Comparison of targeting efficiencies

Even with the promoter-trap strategy, there were still false positive colonies caused by different reasons. To calculate the efficiency, it is necessary to base on the number of the colonies survived from the antibiotic selection. Different porcine BMMSC lines were pre-cultured to assess their growth speed, and the cells with fast proliferation were used for the targeting.

In order to compare targeting efficiency, all the protocols for transfection, the cells used, and the screening methods were the same between synchronized and unsynchronized cells. In previous studies in our group, high targeting efficiency of the APC locus was obtained in pBMMSCs. In this work, the efficiency was up to 84.2% based on PCR results. But not all colonies tested by southern blot were positive for targeting. Half of the tested colonies were verified to be targeted. The rest of them

were just random integrated. There were more colonies from the synchronized cells, but the targeting efficiency was lower compared to the unsynchronized cells.

In this work, for TP53, unsynchronized cells resulted in 5 targeted colonies from 76 screened (6.6%). The southern blot analysis showed that only one colony was correctly targeted (1.3%). Synchronized cells resulted in only one targeting positive colony according to the PCR screening. This colony could not be expanded to allow confirmation by southern blot analysis. In summary, the cell synchronization by serum starvation didn't increase the efficiency of targeting as was expected, on the contrary it decrease the efficiency in this study. The results indicated that synchronizing the cell cycle at the S phase increased the frequency of random integration of the targeting vector in the genome; at the same time it did not improve gene targeting. Previous study with porcine fetal fibroblasts showed that synchronizing the cell cycle at the S phase significantly increased the frequency of random integration of the targeting vector in the genome; at the same time it did not improve gene targeting (Lee, 2010).

Since the targeting efficiencies vary even in the same cell isolate, more repeat experiments need to be performed to determine the accuracy of the results. In general, the efficiency of APC1061 targeting is high according to the results obtained here and by other member of our group.

4.9 Outlook

For the pursuing of porcine iPSCs, more attempts are needed. Since the reprogramming factors are still unclear for pig, more factors may have to be involved in the reprogramming process, such as Tbx3, Klf2. Other delivery methods can be used, such as lentiviral, retroviral vectors or transposons, or a higher number of repeated transfection could be used. The medium components could be modified according to the published achievements of putative porcine ESCs culture conditions.

The porcine Oct4 and Nanog promoters could be cloned used for expression of florescent reporters or selection genes. The differentiation protocols could be improved in further experiments.

The partially reprogrammed cells can be used for gene targeting. They grow as fast as mouse ESC/iPSC and can be expanded over a prolonged period of time. Even if they cannot be injected into the early embryo to produce chimeric animals, they may be useful donor cells for nuclear transfer.

The serum starvation protocol should be modified in the future. Other synchronization methods could be tried. For example, the flow cytometry can be used for collecting the cells in same cell cycle phase.

4.10 Concluding marks

Large animal models are can support medical researchers. Pig, as an animal close to human, is much more important than any other species. However, the wildly used gene targeting method is limited in large animals because of the lack of pluripotent stem cells.

In this project a suitable cell sources or improved technology for gene targeting in pigs were developed. Several different methods were used for generating porcine iPSCs which could be considered as the optimal choice for gene targeting. Several reprogramming factors Oct4, Sox2, Klf4, cMyc, Nanog, N-Myc, and Lin28 were constructed into different vectors. Site-specific recombination vectors or episomal vectors were used to deliver the factors into the cells. ESC specific microRNAs were also tried to generate the iPSCs with the help of valproic acid. Some putative porcine iPSCs were achieved and assessed for the pluripotency. SSEA1+ and SSEA1- cells were identified and could be separated for further differentiation.

The synchronized and unsynchronized MSCs were used for gene targeting. Two different target loci APC and TP53 were studied. Results showed that the synchronization was unable to increase the targeting efficiency in both experiments. The targeted colonies were validated by southern blot. APC1061 targeted cells were used for SCNT and three piglets were born.

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6 Abbreviation

%	Percent
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometer
ADMSC	Adipose derived mesenchymal stem cell
AP	Alkaline phosphatase
APC	Adenomatous polyposis coli
bFGF	Basic fibroblast growth factor
BMMSC	Bone marrow mesenchymal stem cell
BMP	Bone morphogenetic protein
bp	Base pair
BS	Blasticidin
BSA	Bovine serum albumin
CAGGS	chicken beta actin promoter coupled with the cytomegalovirus immediate-early enhancer
cDNA	Complementary DNA
CIP	Calf Intestine Phosphatase
CK18	Cytokeratin 18
cm^2	Square centimeter
CMV	Cytomegalovirus
cMyc	cellular Myelocytomatosis oncogene
CO_2	Carbon dioxide
d	Day
ddH_2O	Double distilled water
DL	German landrace
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	1,4-Dithiothreitol

EBNA1	Epstein-Barr nuclear antigen 1
ECC	Embryonal carcinoma cell
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor 1 alpha
EGC	Embryonic germ cell
EGF	Epidermal growth factor
ERK	Extracellular signal-Regulated Kinases
ESC	Embryonic stem cell
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
g	Gram
g	Gravitational acceleration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSC	Germline stem cell
GSK3	Glycogen synthase kinase-3
h	Hour
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric acid
HPRT	Hypoxanthine phosphoribosyl transferase
HR	Homologous recombination
ICM	Inner cell mass
iPSC	induced pluripotent stem cell
IRES	Internal ribosome entry site
JAK	Janus kinase
kb	Kilobase
KO	Knockout
l	Liter
LIF	Leukemia inhibitory factor
M	Molar
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEK	Mitogen extracellular kinase

min	Minute
miRNA	MicroRNA
ml	milliliter
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NaCl	Sodium chloride
NEAA	Non-essential amino acids
ng	Nanogram
Oct4	Octamer-binding transcription factor 4
p53/TP53	Tumor suppressor protein p53
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pFF	Porcine fetal fibroblast
PGCs	Primordial germ cells
PGK	Phosphoglycerate kinase
PI3K	Phosphoinositide 3-kinase
polyA	polyadenylation site
POU5F1	POU domain, class 5, transcription factor 1
RNA	Ribonucleic acid
rpm	Rounds per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shp2	SH2 domains cytoplasmic tyrosine phosphatase
Smad	Drosophila mothers against decapentaplegic protein
Sox2	Sex determining region Y-box 2
SSEA	Stage Specific Embryonic Antigen
STAT	Signal Transducer and Activator of Transcription
SV40	Simian virus 40
Tbx3	T-box transcription factor 3
Tcf	T-cell factor

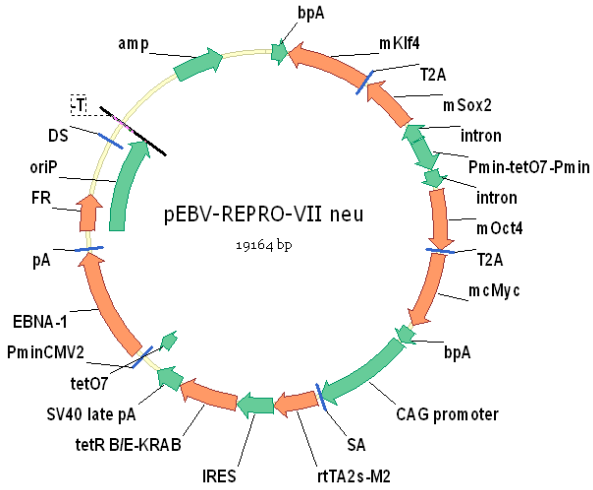
TGF-β	Transforming growth factor beta
U	Unit
UTR	Untranslated region
UV	Ultraviolet
V	Volt

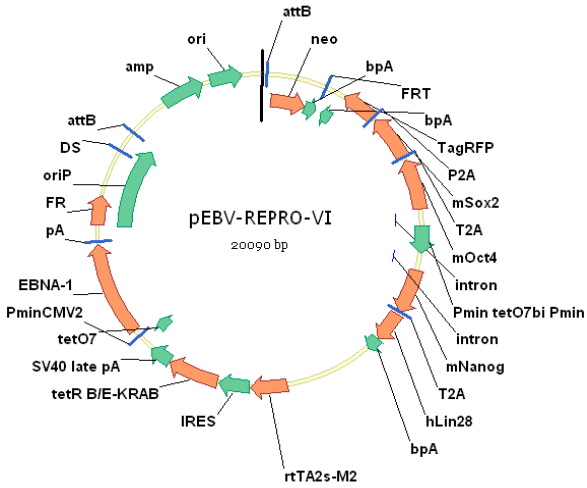
7 List of figures

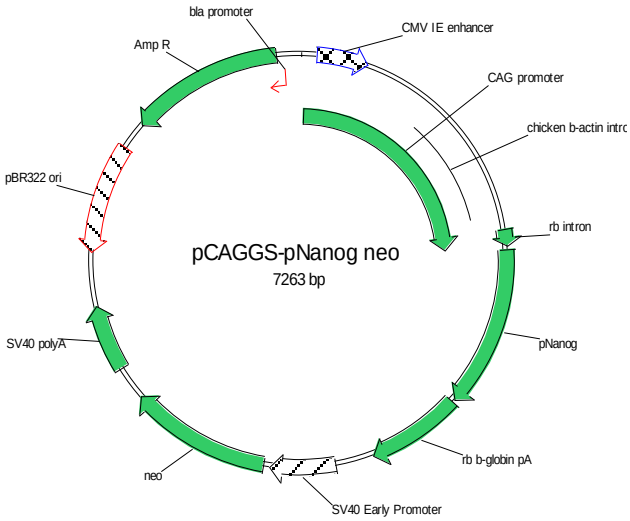
8 List of tables

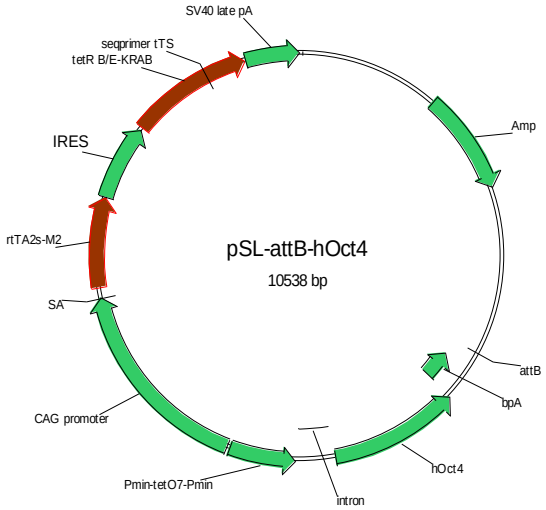
9 Appendix

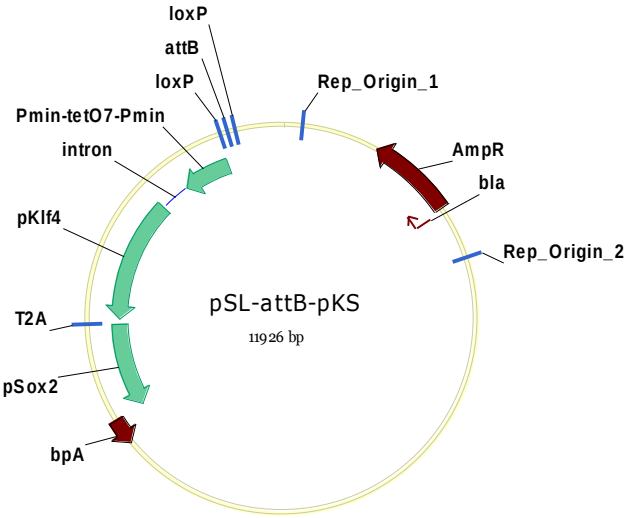
Vector maps

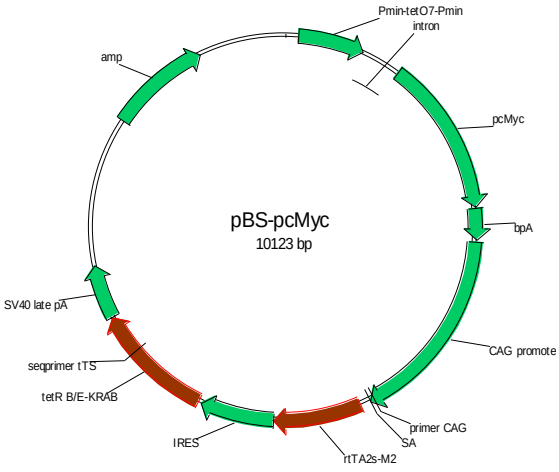


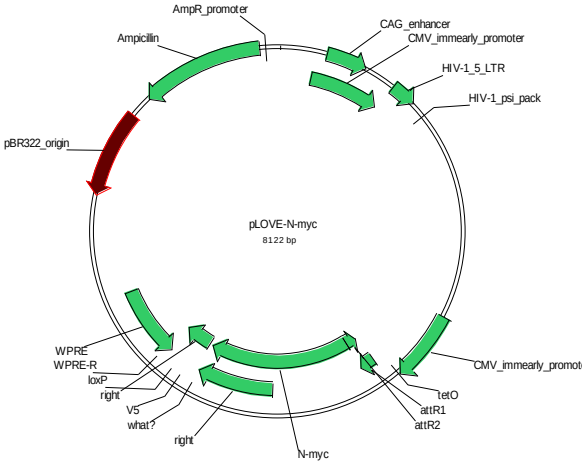








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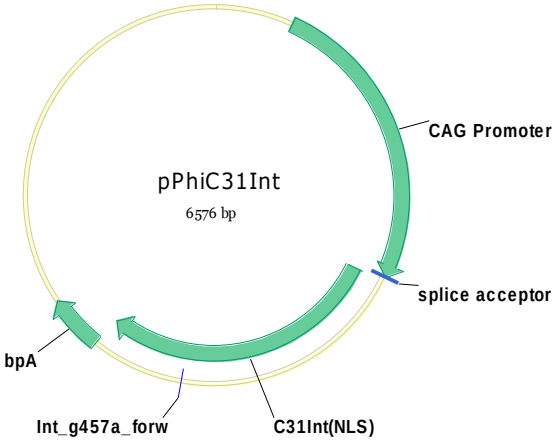
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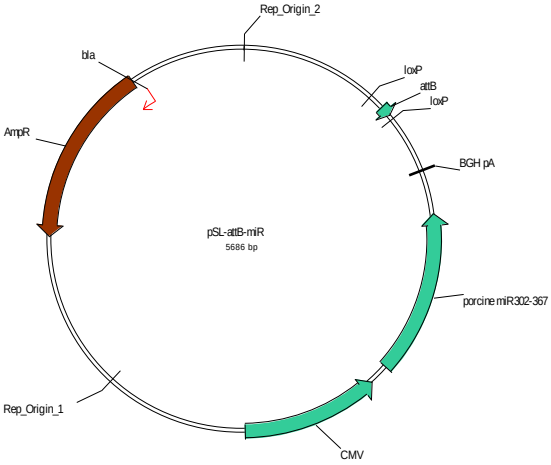
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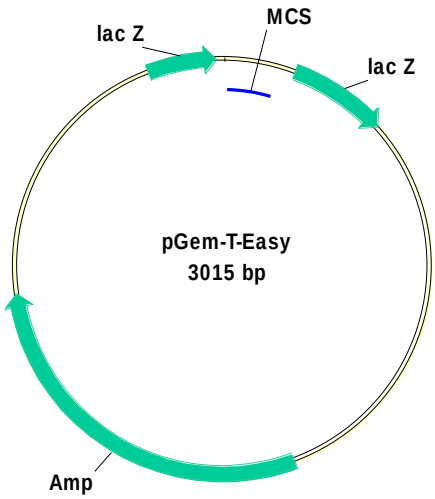
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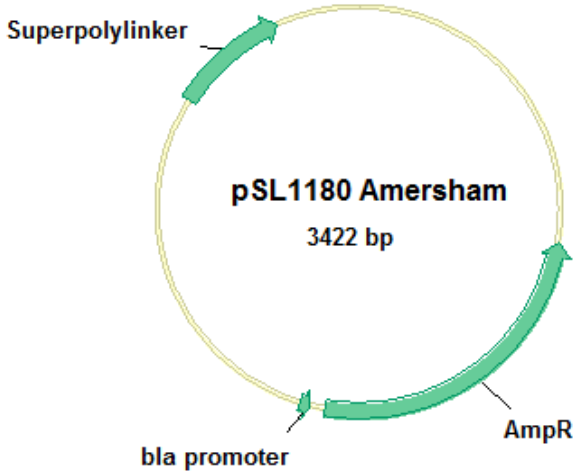
Superpolylinker
pSL1180 Amersham
3422 bp
AmpR
bla promoter

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