Analysis of *OCT4* expression in transgenic porcine embryos carrying an *OCT4-RFP* reporter construct

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TABLE OF CONTENTS

LIST OF ABBREVATIONS

BAC	bacterial artificial chromosome
bp	Base pair
BRG1	Brahma-related gene 1 protein
BSA	Bovine serum albumin
Cas9	CRISPR-associated protein 9
CDX2	Caudal type homeobox 2
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CLSM	Confocal laser scanning microscope
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Dapi	4', 6-Diamidino-2-phenylindole
DE	Distal enhancer of Pou5f1
DMEM	Dulbecco modified Eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGA	Embryonic genome activation
EP	Epiblast
EPO	Electroporation (nucleofection)
ESC	Embryonic stem cell
FCS	Fetal calf serum
FGF4	Fibroblast growth factor 4
GATA6	GATA binding protein 6
GFP	green fluorescent protein
h	Hours
HGMB1	High mobility group B1
ICM	Inner cell mass
KCl	Potassium chloride
mg	Milligram
min	Minutes
ml	Mililiter
NANOG	Tir Nan Og homeobox
OCT4	Octamer-binding transcription factor 4
PBS	Phosphate-buffered saline
PE	Proximal enhancer of *POU5F1*/*Pou5f1*

PE	Primitive endoderm
Pfa	Paraformaldehyd
PKC	Porcine kidney cell
POU	Pit-Oct-Unc
POU5F1	Pit-Oct-Unc class 5 homeobox 1
PP	Proximal promoter of *POU5F1*/*Pou5f1*
qPCR	quantitative polymerase chain reaction
RFP	Red fluorescent protein
SCNT	Somatic cell nuclear transfer
SOX2	SRY(sex determing region Y)-box 2
TALEN	Transcription Activator Like Effector Endonuclease
TE	Trophectoderm
TIE	Trypsin plus ethylenediaminetetraacetic acid
WT	Wild-type
ZFN	Zinc-Finger Nuclease
µl	Microliter
µm	Micrometer

I. INTRODUCTION

The transcription factor OCT4, also known as POU5F1 or OCT3, belonging to the Pit-Oct-Unc (POU) family, is part of the transcriptional regulation system during early development and early lineage specification in mammalian embryos (Nichols et al., 1998; Niwa et al., 2000). The murine OCT4 protein was first detected in early embryos as well as in embryonic stem cells (ESCs) and primordial germ cells (PGCs) (Scholer et al., 1989b). Nichols et al. (1998) showed that in mice OCT4 is necessary for the formation of the pluripotent inner cell mass (ICM) of the blastocyst, since *Oct4*-deficient embryos fail to form the ICM during blastocyst stage. Furthermore the *Oct4* gene is required for maintaining murine ESCs in their pluripotent state (Niwa et al., 2000) and its protein is one of the four essential transcription factors, which successfully reprogrammed somatic cells into induced pluripotent stem cells (Takahashi and Yamanaka, 2006). Differences in gene and protein detection of OCT4 and other transcription factors, altogether known to be key factors in pluripotency signaling pathways during preimplantation development, when comparing porcine embryos with murine, human and bovine embryos (Cao et al., 2014; Hambiliki et al., 2012; Kuijk et al., 2008). This could be a reason that up to now it was not possible to isolate embryonic stem cells from livestock like pig and cattle. In conclusion the *Oct4* gene emerged to be very important during embryogenesis and ES cell pluripotency.

The results of previous reports regarding *OCT4* gene expression pattern in porcine embryos during preimplantation development differ from each other (Cao et al., 2014; Kuijk et al., 2008; Magnani and Cabot, 2008). An elegant way to monitor gene expression in embryos is the usage of reporter gene constructs, since whole mount embryos can be investigated longitudinally without fixation and antibody staining. Transgenic embryos carrying the coding sequence of EGFP under the control of the murine *Oct4* promoter have been successfully generated in mice, cattle and pig (Kirchhof et al., 2000; Szabo et al., 2002; Wuensch et al., 2007).

Since murine regulatory sequences could eventually not reflect the porcine endogenous *OCT4* promoter activity adequately and the analysis of OCT4 protein localisation is not possible in the established reporter systems the aim of this

thesis was to establish pig embryos carrying an improved reporter system with the following characteristics: (i) knockin of *RFP* into the *OCT4* locus for expression of a OCT4-RFP fusion protein to avoid a lack of regulatory elements and potential phenotypes due to the reduction of the pluripotency genes to the heterozygous state and (ii) use of a RFP with short protein half-life for monitoring quick changes of gene expression.

Transgenic porcine kidney cells, generated via nucleofection using bacterial artificial chromosome (BAC) DNA, either having a knockin of *OCT4-RFP* at the *OCT4* locus or a random integration were used for somatic cell nuclear transfer (SCNT). The developing *OCT4-RFP* embryos were studied for their *OCT4* gene expression via RFP detection at specific time points and compared to OCT4-immunostained wild-type SCNT and parthenogenetic embryos.

II. AIM OF THE STUDY

The aim of this dissertation project was to investigate the endogenous *OCT4* expression and protein localisation in porcine preimplantation embryos at different time points using a reporter system and the comparison to wild-type embryos. For this, transgenic porcine embryos carrying an *OCT4-RFP* reporter system were generated, by knockin of *RFP* 3´ to the pluripotency regulator *OCT4* for OCT4-RFP fusion protein expression as outlined in Figure 1.

Experimental approach:

(A) Generation of single-cell clones with a correct targeting at the *OCT4* locus (knockin) via homologous recombination and with random integration.

Step 1: Transfection of different male and female wild-type porcine kidney cells (WT-PKC), with *OCT4-RFP* BAC-DNA construct.

Step 2: Generation of single-cell clones by antibiotic selection of the transfected cells.

Step 3: Analysis of the single-cell clones for correct integration at the *OCT4* locus by quantitative polymerase chain reaction (qPCR).

Step 4: Somatic cell nuclear transfer (SCNT) using cell clones with *OCT4-RFP* knockin or cell clones with random integration.

Step 5: Detection of RFP in blastocysts by epifluorescence microscopy and in embryos at the 8-cell stage, morula and blastocyst stage by confocal laser scanning microscopy (CLSM).

(B) Investigation of OCT4-expression in wild-type and parthenogenetic embryos.

Step 1: Generation of wild-type SCNT embryos and parthenotes

Step 2: Immunostaining against OCT4 in blastocysts

Step 3: Analysis of stained blastocysts using a CLSM.

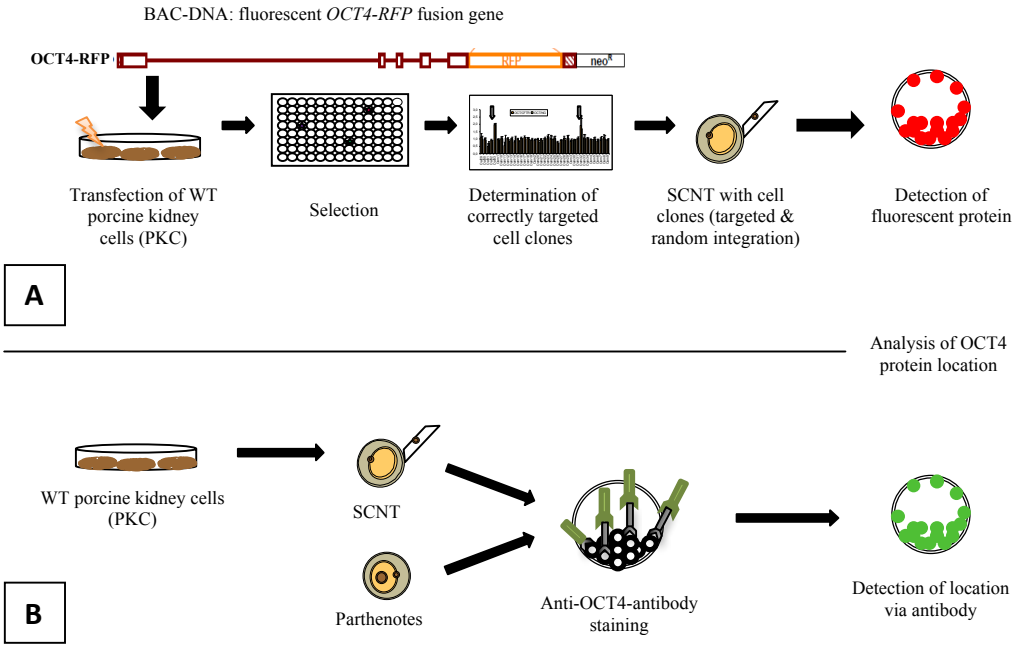


Figure 1: Aim of the study. (A) Generation and analysis of *OCT4-RFP* embryos. RFP expression should reflect the *OCT4* promoter activation and protein localisation since a fusion protein is expressed. (B) Production of wild-type blastocysts and localisation of OCT4 protein via antibody staining of OCT4.

III. REVIEW OF LITERATURE

1. The transcription factor OCT4 and its function in pluripotency signaling pathways

1.1. The transcription factor OCT4

The transcription factor OCT4 (octamer binding transcription factor 4), also known as POU5F1, OCT3, and OCT3/4, was first identified in mice as a specific transcription factor in early embryos, ESCs and the PGCs (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1989b). OCT4, encoded by the *Pou5f1* gene, is a part of the octamer binding subgroup, which belongs to the class V POU (Pit-Oct-Unc) family of transcription factors (Scholer et al., 1990b). The central POU domain, present in all POU proteins, consisting of two subdomains, the POU-specific (POU_S) and the POU homeo-domain (POU_{HD}) and a flexible linker of variable length, connecting both subdomains (Sturm and Herr, 1988). Belonging to the helix-turn-helix class for identifying DNA motifs (Sturm and Herr, 1988), both POU_S and POU_{HD} bind to a consensus octameric sequence "ATGCAAAT", with the ability to cooperate during DNA binding (Verrijzer et al., 1992; Verrijzer et al., 1990). Moreover, several POU transcription factors can form protein-protein complexes with other transcription factors, activating gene transcription (Ambrosetti et al., 1997; Nishimoto et al., 1999) and hence extending the specificity and range of POU transcription factors for identification of DNA sequences. In addition to the central POU domain, the OCT4 protein posseses two other domains: a proline rich N-terminal domain and a proline, serine and threonine rich C-terminal domain (Brehm et al., 1997). Both domains are known to function in transactivation (Imagawa et al., 1991; Vigano and Staudt, 1996), which is known to be necessary for correct function of OCT4 (Ambrosetti et al., 2000), but the C-terminal operates cell-type-specific in contrast to the N-terminal (Brehm et al., 1997). Moreover the POU_S subdomain is supposed to play an important role for OCT4 in maintaining pluripotency (Nishimoto et al., 2005), however the role of the POU_{HD} still needs further investigation to clarify its exact biological function (Nishimoto et al., 1999).

1.2. The *POU5F1*/*Pou5f1* gene – differences and homologies in human, mouse, cattle and pig

In human, the *POU5F1* gene is located on chromosome six (Krishnan et al., 1995), in mouse the *Pou5f1* gene is mapped to chromosome seventeen (Scholer et al., 1990a), in cattle this gene was determined on chromosome 23 (van Eijk et al., 1999) and in pig, the gene was found on chromosome seven (Chardon et al., 2000). The gene is closely mapped to the major histocompatability complex in all four species. Takeda et al. (1992) demonstrated that the sequence of the human *POU5F1* gene shares 87% identity to the murine gene and hence showed its importance due to the high evolutionary conservation. In all four species, the *POUF51*/*Pou5f1* gene is composed of five exons, a proximal enhancer (PE) as well as a distal enhancer (DE) and a proximal promoter (PP) (Okazawa et al., 1991; Rasmussen, 2010; Takeda et al., 1992; Yeom et al., 1996). The murine *Pou5f1* promoter was first examined by Okazawa et al. (1991) and is composed of a GC-rich region with several binding sites for transcription factors to induce transcription. However it misses a typical TATA-box sequence. The activity of the two cis-enhancers, which are located in a 5-kb region upstream from the murine promoter, is controlled in a tissue-, stage- and lineage-specific manner (Yeom et al., 1996). During murine preimplantation development as well as germ line formation, the DE activates the *Pou5f1* expression and therefore the DE is active in the corresponding cell lines, the ESCs and the PGCs (Yeom et al., 1996). On the other hand, *Pou5f1* gene expression in the epiblast and hence in epiblast derived cell lines, is driven by the PE (Yeom et al., 1996). Kirchhof et al. (2000) demonstrated that an *Pou5f1* gene reporter construct, containing the five exons of the murine *Oct4* gene and a modified 9-kb promoter 5´of the reporter EGFP, was active during preimplantation in microinjected murine, cattle and porcine embryos, although showing a a mosaic expression pattern. The EGFP fluorescence signal detection in murine, porcine and cattle embryos carrying the GOF18ΔPE-EGFP construct demonstrated that promoter sequences must be functional conserved between all these three species (Kirchhof et al., 2000; Nowak-Imialek et al., 2011; Wuensch et al., 2007). Nordhoff et al. (2001) compared the 5-kb upstream regulatory sequences of the *POU5F1*/*Pou5f1* gene between mouse, human and cattle and identified four conserved regions with a homology from 66% up to 94% among these three species. In addition Rasmussen (2010) analyzed the homology of the *OCT4* promoter sequences of pig, macaque,

human, cat, cattle, mouse and rat and concluded that the DE and the PP are highly conserved among these species. On the other hand, one part - the 1A binding side - that forms together with the 1B binding side the murine PE, is almost entirely missing in the porcine *OCT4* sequence. This heterogeneity in the sequence of the 1 A binding site has been also demonstrated for other mammals (Kobolak et al., 2009) and therefore its importance and role in controlling the *OCT4* gene still remains a mystique question.

1.3. Expression of *OCT4* in early embryogenesis

1.3.1. Early embryogenesis – from fertilization until hatching

After fertilization the totipotent zygote, a one cell embryo, which is surrounded by the zona pellucida (ZP), divides consistently into equal-sized blastomeres, which is known as the cleavage division stage (Figure 2). During cleavage, embryonic genome activation (EGA) takes place step by step, whereas the maternal mRNA is gradually degraded and the embryonic mRNA, which is required for growth and differentiation, is expressed for the first time (Galan et al., 2010). The point in time of EGA differs between species. In mouse EGA occurs at the 2-cell stage (Oestrup et al., 2009), in human between the 4-cell and 8-cell stage (Braude et al., 1988) and in pig at the 4-cell stage (Tomanek et al., 1989). The next stage is called blastulation, which includes the first differentiation of the embryo. After compaction and polarization of the blastomeres, the outer cell layer differentiates into trophectoderm (TE) cells surrounding the blastocoel, and the inner cell core forms the inner cell mass (ICM) of the blastocyst. The blastocoel is the cavity of the blastocyst, which is fluid-filled via an active fluid transfer by the TE. The ICM is known to be pluripotent and therefore has the ability of self-renewal and differentiation into every tissue of the adult organism including the germ cells, and the ICM cells are the mostly frequently used source for ESC derivation in mice and human (Ginis et al., 2004). Thereafter the second lineage derivation occurs, the exposure of the ICM cells to the fluid-filled blastocoel initiates the most ventral cells to differentiate into primitive endoderm (PE), meanwhile the residual ICM cells, that are surrounded by TE and PE, develop into the epiblast (EP). Subsequently the three different cell types develop into one fetal lineage, the EP, which will give rise to the fetus, and two extraembryonic lineages, the TE and

the PE, that will develop into the placenta and the yolk sac. The time point of the second lineage differentiation differs between species, in mouse and human it occurs prior to hatching, where the blastocysts hatches from the ZP, and in the porcine embryo the EP formation is initiated by the next stage, the hatching (Hall, 2008). The respective days post fertilization differ between mouse, human and pig, revealing the length of pregnancy and the peculiarity during preimplantation development.

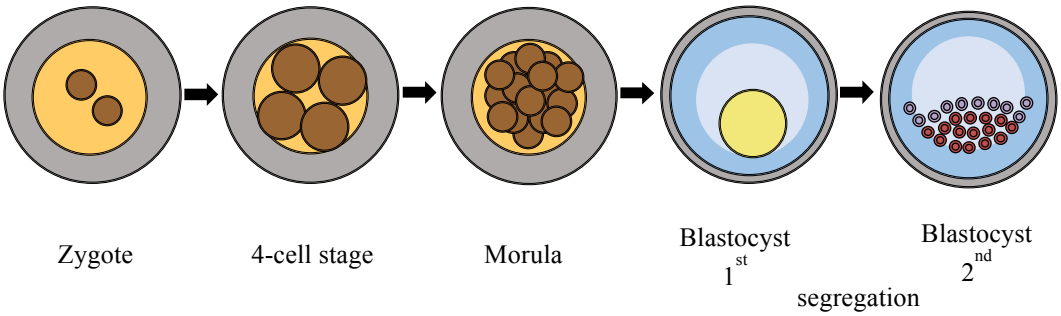


Figure 2: Early embryonic development. The zygote, which is surrounded by the ZP (gray), divides consistently into equal-sized blastomeres. In porcine embryos the EGA begins at the 4-cell stage and compaction of the embryo takes place at the morula stage. Thereafter the outer cell layer differentiates into TE (blue) and the inner cell layer form the ICM (yellow) of the blastocyst. The second lineage segregation occurs where the most ventral cells differentiate into PE (red), meanwhile the residual ICM cells develop into the EP (purple).

1.3.2. *Oct4* expression during murine preimplantation development

The *Oct4* mRNA expression and protein localisation in embryos during preimplantation were first studied and detected in murine unfertilized oocytes, zygotes, morulae and blastocysts (Palmieri et al., 1994; Rosner et al., 1990; Scholer et al., 1989b; Yeom et al., 1991). Following fertilization, the maternal *Oct4* mRNA decreases rapidly (Yeom et al., 1991), while the OCT4 protein is expressed in the nuclei throughout all cleavage stages (Palmieri et al., 1994). Embryonic *Oct4* mRNA as well as protein levels begin to increase extensively prior to the 8-cell stage, after embryonic genome activation occurred (Palmieri et al., 1994; Yeom et al., 1991). Until the morula stage, the *Oct4* mRNA and the

protein are substantially and consistently detected in all nuclei of the embryo. Following the first lineage segregation during blastulation, *Oct4* mRNA and protein are exclusively located in the ICM and not in the TE (Okamoto et al., 1990; Scholer et al., 1990a; Scholer et al., 1990b). During the second lineage segregation, the OCT4 protein is increased in the PE cells (Palmieri et al., 1994), while the *Oct4* mRNA is reduced in these cells and instead up-regulated in the cells forming the EP (Rosner et al., 1990; Scholer et al., 1990a). Guo et al. (2010) confirmed that *Oct4* mRNA is equally expressed in both the EP and the PE by single-cell gene expression analysis.

1.3.3. *OCT4* expression during human preimplantation development

The analysis of the *OCT4* gene expression in human blastocysts showed a higher expression in the ICM than in the TE (Hansis et al., 2000) as shown by Hambiliki et al. (2012), indicating a similar expression pattern as in murine blastocysts. These results were consistent with the cDNA microarray study by Galan et al. (2010) confirming the expression of *OCT4* gene being restricted to the ICM in blastocysts. Cauffman et al. (2005) demonstrated that the *OCT4* mRNA expression ranges from oocyte till blastocyst, with a decline from 6-cell stage till compaction and, in contrast to Hansis et al. (2000), an equal expression in the ICM and TE (Cauffman et al., 2005). The analysis of the OCT4 protein localisation during early human embryogenesis identified, that it is detected until compaction in the cytoplasm and thereafter in the cytoplasm and in the nuclei of the ICM and the TE in the blastocyst in almost the same intensity (Cauffman et al., 2005). The recently published study by Niakan and Eggan (2013) supports previous studies, since the OCT4 protein first appeared in the 8-cell stage, simultaneously with the EGA in human embryos (Braude et al., 1988) and is expressed in the ICM as well as in the TE of the early blastocyst. During the late-blastocyst stage the OCT4 protein was mainly limited to the nulcei of the ICM (Niakan and Eggan, 2013). Hence the localisation of the OCT4 protein is more equal between human mid- till late-blastocyst stage and murine late-blastocyst till peri-implantation stage, after the second lineage segregation had occurred (Niakan and Eggan, 2013).

1.3.4. *OCT4* expression during bovine preimplantation development

In cattle the first study investigating the *OCT4* gene expression and OCT4 protein localisation demonstrated that both, mRNA and protein could be detected from oocyte onwards until day 10 of *in vitro* produced blastocysts (van Eijk et al., 1999). The important difference compared to murine blastocysts was that the OCT4 protein was present in the ICM and TE cells in expanding blastocysts (Kirchhof et al., 2000; van Eijk et al., 1999) as well as *OCT4* transcripts (Daniels et al., 2000). One possible explanation could be that *OCT4* expression promotes TE cell fate in late implanting animals like cattle and other unuglates (Degrelle et al., 2005). In contrast with earlier studies by Kirchhof et al. (2000) and van Eijk et al. (1999), Kurosaka et al. (2004) demonstrated that the mRNA was expressed in bovine oocytes, dropped down during cleavage stage and started to increase one till two cell cycle post EGA, followed by up-regulation until blastocyst stage. In the bovine blastocyst the *OCT4* mRNA was only detected in the ICM and not in the TE cells, similar as in murine embryos, suggesting that the protein degradation is delayed (Kurosaka et al., 2004). Khan et al. (2012) showed that the *OCT4* transcript is expressed in oocytes, 4-cell and 16-cell stage at a low level, thereafter it was up-regulated in morulae, detected in the whole blastocyst until day 8 when it was restricted to the ICM. The OCT4 protein was detected from the 16-cell stage onwards and with a more intensive signal in the ICM than in the TE cells in blastocysts (Khan et al., 2012). Since the OCT4 protein is present colocalized in the ICM and the TE cells, the *OCT4* gene seems not to be the ideal marker for pluripotency in cattle.

1.3.5. *OCT4* expression during porcine preimplantation development

The first study investigating porcine *in vitro* and *in vivo* derived blastocysts was presented by Kirchhof et al. (2000) and revealed that the OCT4 protein was detectable in the cytoplasm and in the nuclei in both parts of the blastocyst, the ICM and the TE, indicating that in pig as well as in cattle, the OCT4 protein is not a marker for undifferentiated cells as in mouse (Kirchhof et al., 2000). These results were committed by Hall et al. (2009) and Kuijk et al. (2008), who additionally supposed that the cytoplasmatic OCT4 staining is likely unspecific binding of the antibody, which both authors detected. Kuijk et al. (2008) suspected that the OCT4 protein does not prevent the TE formation, like it does in

mouse, since they did not detect OCT4 in *in vivo* produced morulae. On the mRNA level *OCT4* expression had two peaks - during metaphase II - indicating its likely involvement in oocyte maturation and during blastocyst stage (Kuijk et al., 2008). In contrast, Magnani and Cabot (2008) demonstrated that *OCT4* transcripts were merely up regulated at the 2-cell stage and there was no difference in the transcript levels from the oocyte up to the blastocyst stages. Studies investigating the *OCT4* gene expression pattern in embryos derived by *in vitro fertilization (IVF)*, SCNT or serial SCNT, revealed differences between all the groups (Huang et al., 2014; Xing et al., 2009). The *OCT4* transcript from pooled embryos differed significantly between *IVF* and SCNT at the blastocyst stage (Xing et al., 2009), whereas Huang et al. (2014) identified the lowest *OCT4* mRNA expression via single cell RNA sequencing at the 2-cell stage comparing SCNT and *IVF* embryos. Both authors agreed that in recloned SCNT embryos the *OCT4* expression was much lower and that the second round of nuclear transfer (NT) did not advance the incomplete reprogramming of the donor nucleus. The recently published study by Cao et al. (2014) analyzing porcine and murine preimplantation embryos by deep sequencing showed that *OCT4* mRNA expression was detected in both species exclusively in the ICM of blastocysts, suggesting that its function for the first lineage segregation in pig is similar to that in mouse (Cao et al., 2014). In contrast, immunostaining of porcine blastocysts displayed, that both, ICM and TE were OCT4 positive (Cao et al., 2014), which is consistent with previous reports (Hall et al., 2009; Kirchhof et al., 2000; Kuijk et al., 2008). Taken together, in the porcine blastocyst, as seen in cattle, the OCT4 protein is present in both compartments, the ICM and the TE, whereas the *OCT4* mRNA seems to be restricted to the ICM. The results regarding the maternal *OCT4* mRNA expression differ significantly between different studies. The different expression pattern between the species could be one reason that it was until now not possible to establish embryonic stem cell lines from pig and cattle. Therefore the *OCT4* gene expression in porcine embryos needs further investigation to get deeper insight into the regulation of pluripotency and so probably facilitate the establishment of embryonic stem cells.

1.4. Target genes and signaling pathways of OCT4 during preimplantation development

The segregation of ICM and TE is the first lineage decision during mammalian embryogenesis and OCT4 is essential for the murine ICM cells to maintain their pluripotent state (Nichols et al., 1998). A key regulator of the trophectodermal lineage in the mouse blastocyst is caudal type homeobox 2 (CDX2) (Strumpf et al., 2005). In murine ESCs, the *Oct4* gene is repressed by a cooperation of the CDX2 with Brahma-related gene 1 protein (BRG1) (Wang et al., 2010) and in turn, *Cdx2* gene expression is directly repressed by the OCT4 protein (Niwa et al., 2005) indicating a reciprocal relationship. Hence Niwa et al. (2005) suspected that once the amounts of the OCT4 and CDX2 proteins gain imbalance, their reciprocal inhibitory relationship is stimulated, which yields in their distinct expression pattern in the ICM and the TE cells and therefore influences the segregation between these two cell lineages (Niwa et al., 2005) (Figure 3). In bovine embryos CDX2 does not prevent the *OCT4* gene expression in the TE cells (Berg et al., 2011). The newly published study from Bou et al. (2016) identified that in pig embryos CDX2 represses the OCT4 by stimulating its nuclear export, followed by proteasom degradation.

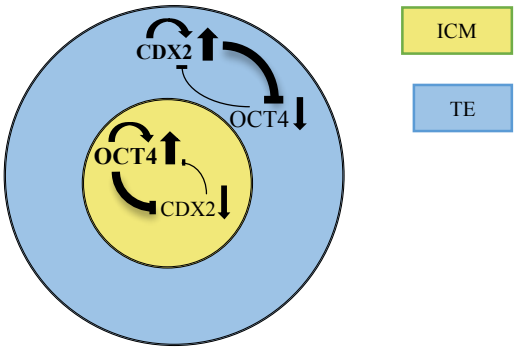


Figure 3: Model of the reciprocal relationship of OCT4 and CDX2 during first lineage segregation in mouse. Once the amounts of the OCT4 and CDX2 protein gain imbalance, their reciprocal inhibitory relationship is stimulated which increases the uneven distribution pattern. Consequently, in the TE cells (blue) the expression of the *Cdx2* gene increases, and simultaneously the *Oct4* gene expression increases in the ICM (yellow) and vice versa. (modified from Le Bin et al., 2013).

During segregation of the ICM of the mouse blastocysts into PE and EP, the three transcription factors OCT4, SRY (sex determing region Y)-box protein-2 (SOX2) and NANOG, named after a mythological Celtic land of eternal youth, Tir Nan Og, are important. These transcription factors promote the expression of PE genes in a non cell-autonomous way (Le Bin et al., 2014; Messerschmidt and Kemler, 2010; Wicklow et al., 2014), as well as the interaction with the fibroblast growth factor 4 (*Fgf4*) (Frankenberg et al., 2011; Nichols et al., 1998). The *Fgf4* mRNA is transcribed from the 1-cell stage onwards (Rappolee et al., 1994). The protein complex of OCT4 and SOX2 is able to bind to the enhancer of *Fgf4*, initiating the transcriptional activation of *Fgf4* gene during early murine embryogenesis (Yuan et al., 1995) and subsequently the increase of the FGF4 protein induces PE cell fate during embryogenesis. Frankenberg et al. (2011) suggested the following sequence of events: beginning from the 8-cell stage the FGF4 protein activates *Gata binding protein 6* (*Gata6*) gene expression. At the morula stage the *Nanog* and *Gata6* gene expression, which are both expressed in the ICM, conduct the differentiation towards PE or EP. An incidental subpopulation of cells contain an elevated level of GATA6 protein and low levels of NANOG protein. These cells are predisposed to differentiate into PE. The remaining *Nanog* expressing cells form the EP (Figure 4).

However, a newly published report (Frum et al., 2013) showed, that the OCT4 protein is able to support PE differentiation independently of the FGF4 signaling cascade, and therefore would explain why OCT4 is detected in equal amounts in the EP and PE cells (Palmieri et al., 1994) Moreover, Aksoy et al. (2013) suggested the following model: On the one side the OCT4 protein interacts with the SOX2 protein and therefore activates EP genes, on the other side the interaction between the OCT4 protein and the SOX17 protein activates PE genes (Figure 4).

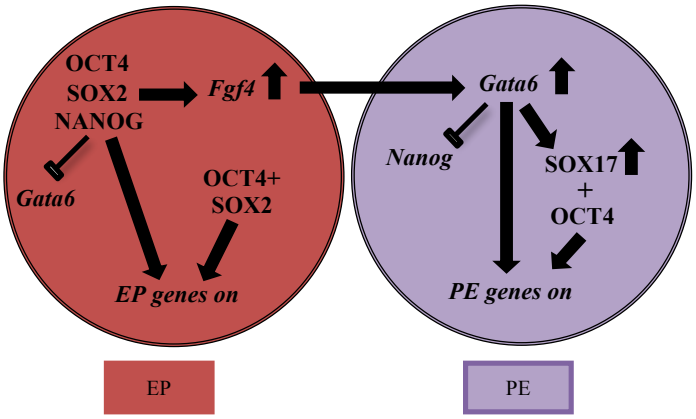


Figure 4: Model of basic transcription factor network during second lineage segregation in mouse. The proteins OCT4, SOX2 and NANOG stimulate *Fgf4* transcription in the EP followed by the activation of *Gata6* expression in the PE by FGF4. Cells with an elevated level of the GATA6 protein are predisposed to differentiate into the PE. The remaining *Nanog* expressing cells form the EP. The OCT4 protein is expressed in equal amounts in both, EP and PE and therefore its interaction with either the SOX2 or SOX17 protein promotes EP or PE gene expression (modified from Frum et al., 2015).

Regarding the signaling pathways in bovine embryos Kuijk et al. (2012) suggested that FGF4 represses *NANOG* gene expression during the second lineage segregation, however the activation of *GATA6* still remained an unsolved question. Xie et al. (2010) suggested that the OCT4 protein in bovine embryos may interact with HMG (high mobility group) B1 protein instead of SOX2 protein. This theory was supported by the fact that down-regulation of the *SOX2* gene expression had no effect on *OCT4* mRNA expression in bovine embryos (Goissis and Cibelli, 2014).

In porcine blastocysts the FGF4 signaling was supposed to contribute rather to the first linage segregation by improving the ICM cell number and supporting TE

development, than to the second one, because inhibition of FGF signaling had no effect on the proportion of NANOG and GATA6 positive cells (Li et al., 2016; Rodriguez et al., 2012).

1.5. Function of OCT4 during preimplantation development

Almost 30 years ago, the *Oct4* gene was found to be expressed in murine unfertilized oocytes, ECCs, ESCs and PGCs, but not in adult tissue (Okamoto et al., 1990; Scholer et al., 1989a; Scholer et al., 1990b). Therefore *Oct4* was suggested to play a pivotal role during early embryogenesis (Scholer et al., 1990b). The first milestone reflecting the important role of OCT4 during embryogenesis was published by Nichols et al. (1998). They generated murine *Oct4*$^{-/-}$ embryos and showed that they developed normally until the first lineage differentiation, where the embryos failed to form an ICM (Nichols et al., 1998). Moreover the *Oct4*$^{-/-}$ embryos developed into trophoblast giant cells *in vitro*, thus supporting that OCT4 is crucial for maintaining pluripotency in the early embryo by preventing differentiation of the ICM into extraembryonic TE (Nichols et al., 1998).

Since the first establishment of murine (Evans and Kaufman, 1981) and human ESCs (Thomson et al., 1998), they have been a useful tool to investigate the expression, regulation and function of pluripotency genes. In murine ESCs, Niwa et al. (2000) demonstrated that the level of *Oct4* gene expression is critically important for maintenance of pluripotency. When *Oct4* gene expression decreased below 50% normal, the ESCs differentiated into TE cells, when it increased above 150% normal, the cells underwent differentiation into PE or mesoderm (Niwa et al., 2000).

A major milestone in stem cell research was the discovery that the four transcription factors OCT4, SOX2, myelocytomatosis oncogene (c-MYC) and Krüppel-like factor 4 (KLF4) are able to reprogram murine adult fibroblasts into ESC-like cells (Takahashi and Yamanaka, 2006). This was the generation of the first induced pluripotent stem cells (iPSCs) underlining the crucial role of the *Oct4* gene in pluripotency acquisition (Takahashi and Yamanaka, 2006). Since these four transcription factors failed to activate the pluripotency gene cascade in porcine cells (Wu et al., 2009) and the *OCT4* gene is expressed in the ICM and TE

cells in porcine embryos (Kirchhof et al., 2000), the exact regulation of pluripotency in porcine embryogenesis seems to be different from murine embryos (Liu et al., 2015). Therefore new insights into the regulation of pluripotency in porcine embryos would help to establish true porcine ESCs, which researchers have failed to generate until now (Notarianni et al., 1990; Piedrahita et al., 1990).

2. Reporter molecules

2.1. General remarks

The visualization and monitoring of gene expression and detection of certain proteins in cells, embryos or whole organisms became very easy using reporter molecules (Habermann et al., 2007). Such reporter molecules could be part of gene constructs for the generation of transgenic cells, embryos or animals, which are composed in different ways depending on the scientific question: 1) the reporter can be controlled by a regulatory DNA sequence of the gene of interest to study e.g. promoter activity, and 2) a fusion protein containing the reporter protein and a protein of interest can be expressed (Habermann et al., 2007). The most commonly used reporter molecules in research are the enzymes β-galactosidase and the firefly luciferase to detect viral or cellular promoter activity in mammalian cells (An et al., 1982; Economou et al., 1989). Both reporters have been used in murine embryos, the β-galactosidase to identity the gene expression pattern of Oct4 (Yeom et al., 1996) and the luciferase to distinguish the enhancer regions of the murine Oct4 gene (Okumura-Nakanishi et al., 2005). These reporter molecules feature the disadvantage, that investigations could not be performed in living material, thus hampering longitudinal analysis (Soboleski et al., 2005). Since 1994, the first demonstration of the suitability of fluorescent reporter molecules as reporters for *in vivo* labeling, they have become popular tools. The unique formation of the chromophore of GFP acts as its own “enzyme”, that only needs molecular oxygen, no other substrates or cofactors, like reporter genes with enzymatic activity do (Habermann et al., 2007). The following three characteristics classify a fluorescent reporter as a useful reporter protein (according to Habermann et al., 2007): First the fluorophore should emit a very bright fluorescence signal and secondly a reporter requires a fast maturation rate

to monitor transcriptional dynamics and at least, a high photostability without undesirable photoconversion.

2.2. Green fluorescent proteins (GFP)

A major milestone was the discovery of the green fluorescent protein (GFP) from the Aequorea victoria jellyfish (Shimomura et al., 1962). GFP is a hollow cylinder, which consists of eleven β-strands and harbors a coaxial α-helix, with the chromophore placed in center of the helix (Ormo et al., 1996). Placing the chromophore in the center of the β-barrel protects them from degradation. The chromophore consists of the tripeptid serine, tyrosine, glycine and is a p-hydroxybenzyliden-imidazolinone, which develops fluorescence via spontaneous autoxidation (Cody et al., 1993; Prasher et al., 1992). The next important step was the identification of the sequence as well as the cloning of the GFP coding sequence (Prasher et al., 1992). Thereafter Chalfie et al. (1994) proved that GFP was suitable as a fluorescent reporter for detection of genes and proteins in eukaryotic and prokaryotic cell lines. In mutation experiments within the wild-type GFP a red-shifted GFP variant, named enhanced GFP (EGFP) with a 100-fold increased fluorescence intensity and more potent folding compared to the wild-type GFP was discovered (Cormack et al., 1996; Heim et al., 1995). Subsequently researchers have identified many variants of GFP and fluorescent proteins from other marine animals, called GFP-like proteins, offering a broad variety in colors, wavelengths and half-life time.

2.3. Red fluorescent proteins (RFP)

The first red fluorescent protein dsRED was isolated from the coral Discosoma and successfully used in mammalian cell as well as Xenopus embryo labeling experiments (Matz et al., 1999). The further analysis of the dsRED showed that it offers a longer excitation wavelength, higher chemical resistance and less photobleaching in comparison with GFP and tetramizes in living cells (Baird et al., 2000). Oligomerization of dsRED-fusion protein is problematic since this can affect the biological function and localisation of the protein of interest (Campbell et al., 2002). Hence Campbell et al. (2002) generated a monomeric RFP (mRFP) with the same brightness expressed in living cells compared to dsRED and better

discrimination from autofluorescence than dsRED. Since then many other optimized orange and red fluorescent clones of mRFP such as mTomato and mStrawberry, have been discovered (Shaner et al., 2004). TagRFP, originating from the sea anemone *Entacmaea quadricolor*, features a longer half-life time of 100 minutes and a brighter fluorescence signal compared to mCherry (Merzlyak et al., 2007). Besides serving as useful reporters in mammalian cells, eukaryotic and prokaryotic organisms, the red fluorescent proteins provide excellent toolkits to generate transgenic mice (Vintersten et al., 2004), pigs (Webster et al., 2005) and cats (Cho et al., 2010), further extending their functional range.

3. Transgenic *OCT4* fluorophore reporter animals

Fluorescent reporters have been used to investigate the *OCT4* promoter activity in divers animals. Yeom et al. (1996) demonstrated that an 18-kb genomic *Oct4* fragment (GOF18), even when the PE sequence was removed (GOF18ΔPE), was sufficient to reproduce the endogenous murine *Oct4* gene expression pattern in early murine embryogenesis. According to this, transgenic mice were generated using a gene construct, containing the coding sequence of EGFP under the control of GOF18ΔPE, to investigate the *Oct4* gene expression during embryogenesis (Szabo et al., 2002; Yoshimizu et al., 1999). The transgenic OG2 mice were widely used to study *Oct4* gene expression (Boiani et al., 2002; Boiani et al., 2004; Iqbal et al., 2007). Kirchhof et al. (2000) showed that this murine GOF18ΔPE-EGFP construct was also able to function in bovine and porcine preimplantation embryos after microinjection into zygotes. Bovine embryos generated by SCNT using stable transfected cell clones with the murine GOF18ΔPE-EGFP construct were investigated in a quantitative way to correlate quality of SCNT preimplantation embryos with *Oct4* promoter activity (Wuensch et al., 2007). Later GOF18ΔPE-EGFP transgenic pigs have been generated with a successful detection of the *Oct4* gene expression in blastocysts and the genital ridge during development (Nowak-Imialek et al., 2011). Further Oct4-FP constructs have been generated for the production of transgenic mice using mCherry, EGFP or Cerulean (Stewart et al., 2009). More recently, researchers established fluorescent *Oct4* animal models containing the species-specific promoter to monitor the actual endogenous *OCT4* gene expression such as porcine

embryos carrying *OCT4-EGFP* (Huang et al., 2011) and rabbit fetuses carrying an additive *Oct4-EGFP* (Yin et al., 2013). Berg et al. (2011) showed that a bovine *OCT4-GFP*Δ*PE* construct injected into murine zygotes resulted in a fluorescence signal in the ICM and the TE cells as seen in bovine embryos, suggesting the species-specific differences must be due to alterations in the DE of the *OCT4* gene. Hence transgenic animals containing a fluorescent protein under the control of endogenous regulatory *Oct4* sequences are a functional tool for investigation of *Oct4* expression.

After the laberatory work for this thesis was finished, Lai et al. (2016) published a study presenting porcine kockin blastocysts, fetuses and piglets carrying an *OCT4-2A-tdTomato* fusion construct. Based on this construct Lai et al. (2016) were able to monitor the *OCT4* promoter activity, but not the exact localisation of the OCT4 protein since tdTomato and OCT4 were translated independently.

4. Somatic cell nuclear transfer (SCNT)

Since the first generation of the cloned sheep "Dolly" using differentiated cells as donor cells for nuclear transfer (Wilmut et al., 1997), various other cloned animals, like mice (Wakayama et al., 1998), cattle (Cibelli et al., 1998) and pigs (Polejaeva et al., 2000) have been successfully generated using SCNT. Simultaneously the first genetically modified lambs, expressing the human factor IX, were produced via SCNT (Schnieke et al., 1997). Thus, the application of genetically modified cells for SCNT - provides a powerful tool to generate tailored animal models. Several genetically modified porcine models have been established to date using SCNT (Klymiuk et al., 2013; Renner et al., 2013; Stoltz et al., 2013). But the big disadvantage of SCNT is still the low cloning efficiency (proportion of cloned offspring out of transferred embryos), usually varying between 1 and 5% in pig (Kurome et al., 2013). Many different influencing factors have been found to affect the efficiency of SCNT. Among these factors the source of donor cell has been shown to have an important impact on the efficiency in different mammalian species including pig (Betthauser et al., 2000), cattle (Kato et al., 2000) and mice (Yamazaki et al., 2001). Furthermore the passage number of the donor cells had a major influence on blastocyst rates and varied in bovine (Liu et al., 2001) and sheep (Gupta et al., 2007) SCNT embryos. Bureau et

al. (2003) analyzed two different bovine donor cells as well as their resulting 1-4-cell stage as well as morula stage SCNT embryos for chromosomal aberrations and concluded that the increased anomalies in the embryos reflects the higher number of chromosomal anomalies observed in their corresponding donor cell line. In addition, Bureau et al. (2003) supposed that the SCNT process itself impacts correct chromosome segregation as well as their proper allocation during cell division. Incorrect spindle formation has been demonstrated in monkey (Simerly et al., 2003) and murine (Van Thuan et al., 2006) embryos generated by SCNT. Moreover the synchronization of the cell cycle of the donor cell is important, since an enucleated MII oocyte is used as the recipient cytoplast in SCNT, the donor nucleus should be in G0/G1 to maintain chromosomal diploidy of the reconstructed embryos (Campbell et al., 1996; Wilmut et al., 2002). Additionally the epigenetic status of the donor was suspected to be critical for sufficient SCNT (Enright et al., 2003). Epigenetic reprogramming implicates changing of the chromatin structure, without changing the nucleotide sequences, back to an totipotent epigenetic nature (reviewed in Surani et al., 2001). Epigenetic modifications change the expression of genes in coordination with the specialized function of the cell (reviewed in Surani et al., 2001). Reprogramming the epigenetic status of adult donor cells during SCNT is critical and concerns changes in histone modifications, DNA methylation, genomic imprinting, X chromosome inactivation as well as the telomere length (reviewed in Shi et al., 2003). Incomplete epigenetic reprogramming resulted in aberrant patterns of DNA methylation (Bourc'his et al., 2001; Dean et al., 2001; Ohgane et al., 2001), histone acetylation (Enright et al., 2003), X chromosome inactivation (Xue et al., 2002) as well as the expression of imprinted and non-imprinted genes (Daniels et al., 2001; Dean et al., 2003; Humpherys et al., 2001). Therefore inadequate reprogramming of epigenetic modifications seems to be the most important reason for the low cloning efficiency (reviewed in Shi et al., 2003).

To increase the efficiency of epigenetic porcine embryos were treated with different kinds of histone deacetylase inhibitors (Himaki et al., 2010; Jeseta et al., 2008; Zhao et al., 2010), such as Scriptaid which increased the cloning efficiency from 0.4% in the control group up to 1.6-3.7% using porcine fibroblasts from different origin as donor cells for SCNT (Zhao et al., 2010).

IV. MATERIAL AND METHODS

1. MATERIAL

1.1. Apparatuses

Apparatuses name	**Manufacturer**
AccuJet pro Pipetboy	Brand, Wertheim
AQUAline AL 12 water bath	Lauda-Brinkmann, USA
Biohit Picus multichannel pipet (300 µl)	Satorius, Göttingen
Cellavista High End System	Roche, Mannheim
Centrifuge Biofuge pico	Heraeus, Osterode
Centrifuge Labofuge M	Heraeus, Osterode
Centrifuge Rotanda 96	Hettich, Tuttlinge
Centrifuges 5810 R	Eppendorf, Hamburg
CO_2 cell incubator	MMM Group, Munich
CO_2 incubator	Heraeus, Osterode
CO_2-O_2-Incubator	Binder, Tuttlingen
Confocal laser scanning microscope LSM 510 Meta	Zeiss, Oberkochen
Confocal laser scanning microscope LSM 710 Meta	Zeiss, Oberkochen

Cool cell container	Biocision, USA
Glass pipettes	Brand, Wertheim
Microscope DM IL	Leica, Wetzlar
Neubauer counting chamber	Assistent, Sondheim
Pipettes (2 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf, Hamburg
Stemi SV 6 microscope	Carl Zeiss, Oberkochen
Steril benches Laminair® HB2448K, HB2472	Heraeus, Osterode
Zeiss Axiovert 200 M fluorescence microscope	Carl Zeiss, Oberkochen

1.2. Consumables

Consumer item	**Manufacturer**
48-well, 4-well culture dish	Nunc, Darmstadt
6-channel µ-slides VI 0.4	Ibidi, Martinsried
6-well, 96-well F-bottom culture dishes	Greiner bio-one, Frickenhausen
60 mm, 100 mm culture dishes	Sarstedt, Nümbrecht
96-well half area microplates	Corning, USA
Centrifuge tubes (15 ml, 50 ml)	Greiner bio-one, Frickenhausen

Cover glass (26 x 21 mm, 0.17 ± 0.01mm)	Karl Hecht GmbH & Co, Sondheim v. d. Rhön
Cover slip (76 x 26 mm, 0.17 ± 0.01mm)	Karl Hecht GmbH & Co, Sondheim v. d. Rhön
Cryotubes 1 ml, 2ml	TPP, Switzerland
Parafilm®M	American Can Company, USA
Perforated adhesive-backed PVC-film	Mactac, USA
Pipette tips	Eppendorf, Hamburg
Pipette tips with filter	Axygen Inc., USA
SafeGrip® Latex gloves	SLG, Munich
Steritop GP 0,22 µm Express®plus membrane	Millipore, USA
Sterivex GP 0,22 µm	Millipore, USA

1.3. Chemicals used for cell culture

Chemical	**Manufacturer**
Amaxa Nucleofector® Kit	Lonza, Cologne
CollagenR	Serva, Heidelberg
Difco™ Trypsin 250	BD, USA
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Steinheim

Dulbecco modified Eagle medium (DMEM) with stable L-Glutamine	Gibco, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe
Fetal calf serum	Gibco, Darmstadt
Geneticin (G-418)	Gibco, Darmstadt
Glacial acetic acid	Sigma-Aldrich, Steinheim
KaryoMax (Colcemid)	Gibco, Darmstadt
Methanol	Sigma-Aldrich, Steinheim
non-essential amino acids (100×)	Gibco, Darmstadt
Nucleofector™ device	Lonza, Cologne
Penicillin/Streptomycin (100×)	PAA, Austria
pmaxGFP™	Lonza, Cologne
Potassium chloride (KCl)	Sigma-Aldrich, Steinheim
Sodium pyruvate	Gibco, Darmstadt
ß-Mercaptoethanol	Sigma-Aldrich, Steinheim

1.4. Chemicals used for embryos

Chemical	**Manufacturer**
Bovine serum albumin (BSA)	Roth, Karlsruhe

Paraformaldehyde (Pfa)	Sigma-Aldrich, Steinheim
Polyvinylpyrrolidone (PVP)	Sigma-Aldrich, Steinheim
Triton X-100	Sigma-Aldrich, Steinheim
Vectashield with DAPI	Vector Laboratories, USA

1.5. Media and solutions used for cell culture

Medium/Solution	**Components**
Cell culture medium	DMEM with stable L-Glutamine 10% or 15% (v/v) FCS 1% (v/v) Non-essential amino acids (100 ×) 1% (v/v) Sodium pyruvate (100 ×) 0.1 mM β-Mercaptoethanol
Cryo medium	10% (v/v) DMSO 90% (v/v) FCS Prepared freshly before use on ice
PBS without $Ca^{2+/}Mg^{2+}$ (PBS-)	8 g NaCl 0.2 g KCl 0.2 g KH_2PO_4 2.14 g $Na_2HPO_4 \times 7H_2O$ Add 1000 ml aqua bidest pH 7.2-7.4

Selection medium	Cell culture medium 1.2 mg/ml G418
Starvation medium	DMEM with stable L-Glutamine 0.5% (v/v) FCS 1% (v/v) Sodium pyruvate (100×) 1% (v/v) Non-essential amino acids (100×) Stored at 4°C
Stop medium	DMEM with stable L-Glutamine 10% (v/v) FCS Stored at 4°C
Trypsin/EDTA	PBS without $Ca^{2+/}Mg^{2+}$ 0.5% (w/v) Trypsin 0.04% (w/v) EDTA

1.6. Media and solutions used for embryos

Medium/Solution	**Components**

0.1% PBS-PVP	0.1 g PVP 100 ml PBS
0.1% Triton-X-100-PBS	50 µl Triton-X-100 50 ml PBS
1% BSA-0.1% Triton-X-100-PBS	0.2 g BSA 20 ml 0.1% Triton-X-100-PBS
PBS	8 g NaCl 0.2 g KCl 0.2 g KH_2PO_4 1.15 g $Na_2HPO_4 \times 2H_2O$ 0.1 g $CaCl_2$ 0.1 g $MgCl_2$+$6H_2O$

1.7. Plasmids and BACs

BAC/Plasmid name	**Manufacturer**
pGEM® T-Easy Vector System	Promega, Mannheim
pL452	kindly provided by Neil Copeland
pTagRFP-laminB1	Evrogen, Russia
CH242-102G9	BACPAC Resource, Chori, USA

pJET1.2/blunt Cloning Vector	Fermentas, St. Leon-Rot

1.8. Antibodies

Primary Antibody	**Manufacturer**
Goat-anti-human Oct3/4 (sc-8628)	Santa Cruz, USA
Secondary Antibody	
Rabbit-anti-Goat–FITC (ab6737)	Abcam, United Kingdom
Blocking Peptid	
Oct-3/4 blocking peptid (sc-8628 P)	Santa Cruz, USA

1.9. Software

Software	**Manufacturer**
Adobe Photoshop CS2	Adobe, USA
AxioVision SE64Rel.4.9	Zeiss, Oberkochen
Cellavista SW Workstation Version 2.0.0.23	Roche, Mannheim
ImageJ	National institutes of health, USA
Zeiss ZEN lite 2011	Zeiss, Oberkochen
ZEN 2009 software (black edition)	Zeiss, Oberkochen

1.10. Oligonucleotides

Oligonucleotides were either designed by hand or with the primer 3 software. They were manufactured by Thermo Fisher Scientific, USA.

OCT5f	5´-caCTGAGagatatgcaaggcagagac-3`
OCT5r	5´-ttCCATGGggaaggtccagggaccTCTgtttgaatgcatg-3`
OCTpAf	5´-cgTCCGGAatgcattcaaactgaggtg-3`
OCTpAr	5´-tgGAATTCtgacctggtcaagtctatcag-3`
OCT3f	5´-acGTCGACcttgctcagtgggttaacgatctg-3`
OCT3r	5´-ttCCGCGGttaagggaagagagtgagcagac-3`

2. METHODS

2.1. Design of an *OCT4-RFP* targeting vector

The *OCT4-RFP* targeting vector was generated by Dr. Nikolai Klymiuk as described in Klymiuk et al. (2014).

For this dissertation project a BAC vector has been used for gene targeting by homologous recombination since BAC vectors are supposed to have a higher targeting efficiency due to their increased length of homologous sequences. The genomic sequence of the porcine *OCT4* was identified on BAC clone CH242-102G9 which was used for generation of the BAC targeting vector.

First of all a modified plasmid was constructed as modification vector, which was then introduced into BAC clone CH242-102G9 via recombination, resulting in the modified BAC vector for targeting. The modification vector was assembled from the 5´-arm-fragment, the RFP-fragment, the stop-codon and the 3´-arm-fragment and cloned into the pL452 vector, which features the neomycin/kanamycin resistance (neokan^{R}) cassette shown in Figure 5.

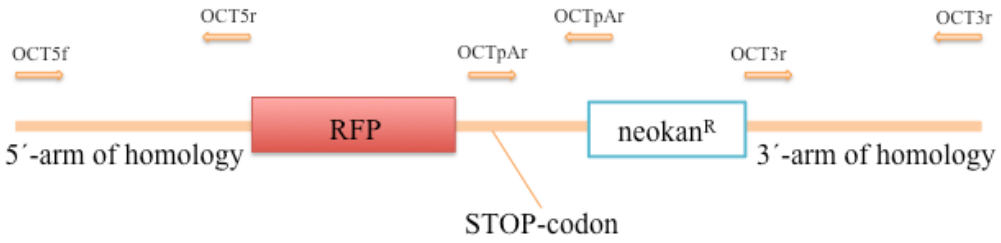


Figure 5: Design of the *OCT4-RFP* modification vector. The modification vector consists of the RFP-DNA (Evrogen), the neomycin/kanamycin resistance (neokan^R) cassette (pL452 vector), the STOP-codon, the 5´-arm of homology, and the 3`-arm of homology. The homologous arms were amplified from genomic DNA by primer pairs, indicated by the orange arrows.

2.2. Cell culture protocols

All cell culture experiments were performed under a laminar flow to prevent contamination. All media, solutions and apparatuses that have been in direct contact with the cells have been autoclaved or sterile filtered before. Cell culture media and solutions were pre-warmed to 37°C in the water bath. Culture dishes and slides were coated with Collagen R (2 mg/ml) diluted with Aqua bidest. at least three hours before use. Cells were cultured in an incubator at 37°C, 5 % CO_2 in air and humidity.

2.2.1. Primary porcine kidney cells

For transfection experiments different primary porcine cell lines were used concerning gender and breed. Female porcine kidney cells PKC0407 and PKCf and male porcine kidney cells PKCm and PKC2109 were isolated from kidneys of three and four months old Landrace pigs. These primary cell cultures have been established by Dr. Annegret Wünsch (Richter et al., 2012).

These cell cultures are a mixed primary cell population consisting of cells with flat, ellipsoid, fibroblast-like appearance and also cells with rounder, epithelial-like morphology (Richter et al., 2012). The suitability of these primary cells for genetic modification, nuclear transfer and birth of viable piglets has been tested in

our laboratory (summarized in Richter et al., 2012).

2.2.2. Cultivation and passaging

Porcine kidney cells were cultured with cell culture medium containing 10% FCS, until 80-100% confluence, which was reached after two days of culture. For passaging the medium was aspirated via vacuum pump (6-channel slide: via pipette) and the cells were washed twice with the same volume PBS as medium. The appropriate media volumes for the different wells/dishes/slides used are summarized in table 1. To calculate the appropriate cell number for the different wells/dishes/slides, we seed 1.2 $\times 10^4$ cells/cm^2 and 0.7-1$\times 10^4$ cells/channel on 6-channel slide. In order to detach the cells, they were incubated for 5-7 min with the appropriate volume of 0.4% trypsin/EDTA. When the cells had a spherical shape or even start floating around, they were completely detached and stop medium was added. Afterwards the cell suspension was transferred to a centrifugation tube and the cell number was determined using a Neubauer chamber. The appropriate cell number was transferred to a new tube for washing by centrifugation for 5 min at 180×g. After centrifugation, the supernatant was discharded and the cell pellet was resuspended in fresh culture medium and seeded on a new culture dish. If the cells were seeded on a 6-channel slide, they were first resuspended in 30 µl medium/channel and after 2-3 h, when the cells were adhered, the remaining medium was added to the channels.

Detached cells of 96-well half area plates were processed differently. Medium was added to the detached cells and directly transferred to a 96-well plate without washing.

Table 1: Overview of the cell culture plates.

Plate	Company (Catalogue No.)	Area [cm^2]	Growth medium [ml]	Trypsin/EDTA solution [µl]	Coating Collagen solution [µl]
6-channel slide	Ibidi (#80606)		0.1	30	50
96-half area	Costar (#3696)	0.16	0.05	20	20
96-well	Greiner (#167008)	0.33	0.1-0.2	30	40
48-well	Nunc (#150687)	1.1	0.35-0.5	80	100
24-well	Nunc (#142475)	1.9	1	150	200
4-well	Nunc (#176740)	1.9	1	150	200
6-well	Greiner (#140675)	9.6	2-3	400	400
60x15	Sarstedt (#831801)	21	5	750	750
100x20	Sarstedt (#831802)	58	10	1500	1500

2.2.3. Thawing and freezing

The frozen kidney cells were taken out of the liquid nitrogen tank and placed immediately in 37°C water bath for thawing. The thawed cell suspension was filled up with 10 ml stop medium and centrifuged (5 min at 180×g). Afterwards the cell pellet was resuspended in the appropriate amount of medium and seed on coated culture dishes.

For freezing, cells were harvested as described in protocol 2.1.2. After removing the cells from the plate and centrifugation, the cell pellet was resuspended in 1 ml of ice-cold cryo medium, transferred into a 1.5 ml cryo tube and slowly frozen with a rate of 1°C/min stored in a cool cell freezing container in the −80°C freezer. After at least two hours in the −80°C freezer, cryo tube was transferred to the liquid nitrogen tank.

For cryopreservation of cells growing on a 96-well plate, harvesting was performed as described in protocol 2.1.5, but the reaction was stopped using 180 µl cryo medium and the cell suspension was directly transferred to one 1.0 ml cryo tube and slowly frozen in cool cell freezing container at −80°C as described above.

2.2.4. Fixation of cells on 6-channel slides

The 6-channel slides feature the advantage, compared to the cover slip method, that less cell material is needed, the cells can be easily grown, washed, fixed and stained on the 6-channel slide. Additionally, to this much easier handling, the 6-channel slides contain a special plastic bottom improving the optical quality. When the cells had the desired confluence, the medium was removed via pipette and 100 µl 4% paraformaldehyde (Pfa) was added to each channel. Thereafter the 90 µl 4% Pfa was removed from each channel and again 100 µl 4% Pfa added to each channel. The cells were fixed 20 min at room temperature. Afterwards each channel was washed with 200 µl PBS. The nuclei of the cells were counterstained with DAPI in Vectashield by adding two till three drops to each channel and stored in darkness, sealed with Parafilm in the refrigerator until further experiments.

2.2.5. Determination of chromosome numbers

2.2.5.1. Preparation of chromosomes

The correct karyotype of the donor cell population is very important for a successful SCNT. Therefore the chromosome number of metaphase spreads of PKC0407 was determined, as described in the following section.

Cells with 60-80% confluence and many mitotic cells cells indicated by a round shape were incubated with 100 µl colcemid (KaryoMax, 10 µg/ml) for 60 min at 37°C. Colcemid blocks the formation of the spindle fibers and hence the cells arrest at metaphase. Afterwards the cells were washed, trypsinized and centrifuged as described in protocol 2.1.2. The supernatant was removed except for 1 ml medium and the cell pellet was resuspended. Subsequently the cells were treated with 13 ml of prewarmed hypertonic 75 mM KCl, which was added slowly drop by drop to the cell suspension. The hypotonic treatment is very important, because the cells get swollen and the chromosomes move from the center of the cell to a more peripheral location, where they can be spreaded easier (Claussen et al., 2002). The next step was the incubation of the cell suspension for 15 min at 37°C and followed by centrifugation at 200×g for 8 min, aspirating the supernatant and resuspending the cell pellet in 1 ml stop medium as described in protocol 2.1.2. After that the cell suspension was fixed with an ice-cold mixture of 25% glacial acetic acid and 75% methanol. During permanent tilting, 10 ml of the fixative was added slowly drop by drop to the suspension. Subsequently the suspension was stored at −20°C for at least 30 min. Thereafter the suspension was centrifuged for 10 min at 200×g, the supernatant was removed and the cell pellet was resuspended in 1 ml fixative solution and transferred into a 2 ml tube. The suspension was washed several times by centrifugation, 4 min at 1400×g at 4°C, removing the supernatant and resuspending with new fixative solution. Afterwards the suspension was stored at −20°C.

2.2.5.2. Spreading of metaphase chromosomes

The spreading of metaphase chromosomes was performed according to Henegariu et al. (2001) with minor modifications. First of all the slides were stored in the refrigerator for 1 h. Thereafter 25-35 µl of the cells suspensions in fixative solution was spread evenly on several locations on the cold slide. The distance between the pipette tip and the slide surface should be around 20 cm. When the surface became grainy, the slide was quickly passed through the steam of the water bath (37°C). Immediately afterwards four to six drops of fixative were dropped onto the slide and as soon as it covers the whole surface, the slides was held 3-5 sec over the steam of the water bath. Finally the slide was dried on the metal lid of the water bath, mounted with two drops of DAPI in Vectashield,

sealed with a cover glass and nail polish and stored in the dark in the refrigerator until analysis.

2.2.6. Analysis of metaphase chromosomes for correct chromosome numbers

Analysis for the correct chromosome set was performed using the inverted epifluorescence microscope. Digital pictures of 60 metaphases were made using the DAPI filter set 1, the CCD (charge-coupled-device)-colour camera AxioCam HR and the AxioVision SE64Rel.4.9 software. These chromosomes were counted in ImageJ and the percentage of metaphases containing appropriate chromosome numbers was calculated.

2.2.7. Generation and selection of stable transfected single-cell clones

2.2.7.1. Transfection

For the transfection of cells the Amaxa Nucleofector^{TM} device with the Primary Mammalian Fibroblast Kit from Lonza was used. In this technology electrical pulses and a cell type specific solution are applied for the transfer of foreign DNA directly into the nucleus (Hamm et al., 2002)

For transfection cells were harvested as described in protocol 2.1.2. A cell number of 0.5×10^6 cells was used for transfection and centrifuged for 5 min at 200×g after removing them from the plate. The cell pellet was resuspended in 100 µl Nucleofector solution from the Amaxa Nucleofector® Kit for Primary Mammalian Fibroblasts and mixed with 1-5 µg of endotoxine-free prepaired and linearized BAC-DNA. This mixture was transferred into an Amaxa certified cuvette and pulsed according to program U-12 of the Nucleofector^{TM} device. The cells were seeded on a 6-well culture dish containing 2 ml culture medium with 15% FCS. The medium was changed 24 h after transfection and the cells were cultured for additional 24 h until the cells had a confluence of 80-100%.

2.2.7.2. Selection and scanning

For selection of the cells, which integrated the linearized BAC-DNA containing a neomycin resistance cassette into their genome, they were cultured in selection medium containing 1.2 mg/ml geneticin (G418) for at least one week. This concentration was determined in our laboratory to be the optimal to eliminate all cells, which do not carry a neomycin resistance gene. After 48 h the transfected cells were harvested and counted according to protocol 2.1.2. In total 1000 cells per well were seeded on 96-well half-area plates. To improve the survival rate of transfected cells non-transfected cells were added to the transfected cells for selection to a final ratio of 1:3 or 1:1 or 3:1. After mixing the cells and centrifugation the cell pellet was resuspended in selection medium and seeded on the appropriate number of 96-half area plates. Every other day the selection medium was changed. After 6 days in culture the plates were started to scan via the Cellavista High End System. The plates were scanned every other day for around 1 week. The scanning over several days allows a reliable identification of wells containing single-cell colonies. The heatmap displays the cell confluence detected by the software for each well in different colors (Figure 6). For instance, a blue colored well represents that only a few cells are growing in this well and a deep red colored well indicates that a lot of cells are growing in this well., The selection filter # colony display the number of single-cell colonies per well. Each well containing cells was then check for single colonies, because only these were further proceeded by splitting with a confluence of at least 50%.

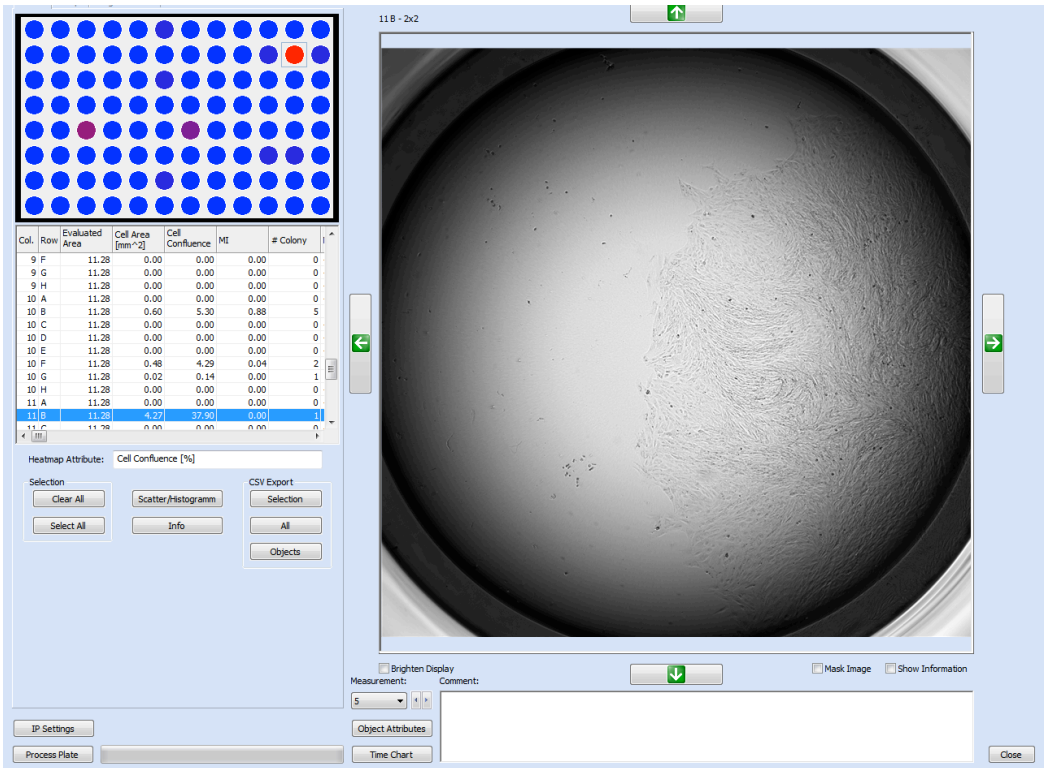


Figure 6: Systematic scanning of 96-well half-area plates for single-cell clones. The 96-well half-area plates were scanned and cell colony growth and confluence was analyzed with the Cellavista SW Workstation software to detect single-cell clones fast and accurate at the correct time point. The different colors in the heatmap reflect the cell confluence detected by the software for each well (blue: fewest cells growing in this well; deep red: most cells growing in this well).

For this, cells were passaged by washing twice with 100 µl PBS and incubating with 20 µl trypsin/EDTA for around 5 min until they detach. The reaction was stopped with 180 µl 15% culture medium and the cell clones were split on two 96 wells. Each well was filled up with 100 µl cell culture medium and medium was changed every other day. When the passaged cell clones reached 80-100% confluence one 96-well was harvested for quantitative PCR analysis and the other one was cryopreserved for SCNT (Figure 7). For qPCR analysis the cells were washed twice with 100 µl PBS, detached with 30 µl trypsin/EDTA for 5 min and transferred to a 1.5 ml Eppendorf tube. After stopping the reaction with 180 µl stop medium, the cells were pelleted by centrifugation at 7,000×g for 5 min and after removing the supernatant the pellet was first left on ice for maximum fifteen min and further stored in the −80°C until analysis. For cryopreservation the cells

were handled a described in protocol 2.1.2.

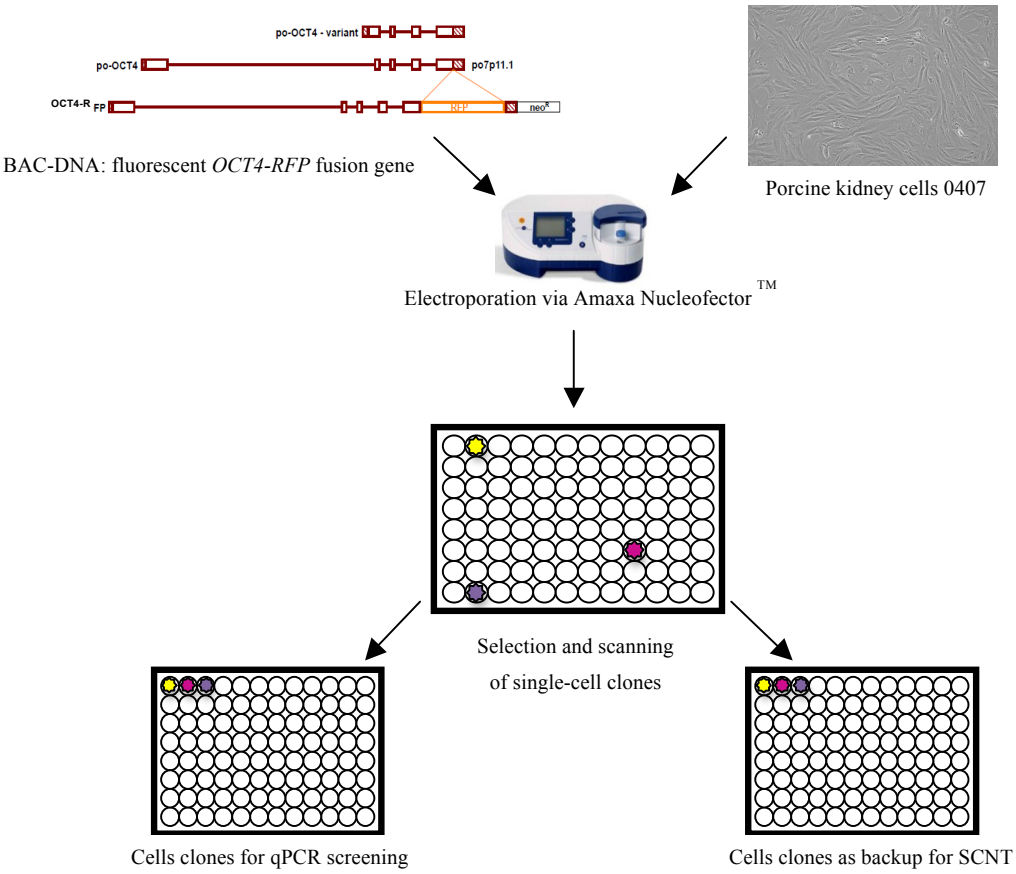


Figure 7: Generation and selection of stable transfected single-cell clones. Porcine kidney cells were transfected with *OCT4-RFP* BAC-DNA via electroporation (Amaxa Nucleofector™). After two days the cells were seeded on 96-well half-area plates in selection medium to obtain single-cell clones. The correct time for splitting of the single-cell clones on two 96-well plates was determined via scanning. One portion was screened for a correct targeting and the other one was keept in liquid nitrogen as a backup for further SCNT experiments.

2.3. Embryos

2.3.1. Production of preimplantation embryos

In vitro oocyte maturation was conducted as described in Matsunari et al. (2012). Following maturation and hyaluronidase treatment, only oocytes with regularly granulated ooplasm and an extruded first polar body were chosen for further

experiments. The production of *in vitro* produced embryos was performed by Dr. Mayuko Kurome, Dr. Barbara Kessler, Dr. Valeri Zakhartchenko and Tuna Gungör from the Chair of Molecular Breeding and Biotechnology of LMU, Munich.

2.3.1.1. Parthenogenetic embyros

Parthenogenetic embryos were produced as described in Matsunari et al. (2012). After *in vitro* maturation, the washed oocytes were activated by an electrical impulse. To suppress the extrusion of the second polar body, the activated oocytes were treated with cytochalasin B for 3 h and then cultured until the blastocyst stage.

2.3.1.2. Somatic cell nuclear transfer embyros

Single-cell clones for SCNT experiments were thawed several days before SCNT as described above (2.1.2). After centrifugation the pellet was resuspended in culture medium containing 20% FCS and seeded on a cell culture plate with an appropriate area. Two days before SCNT starvation medium was added to the cells to synchronize the cell cycle to G0/G1. Since an enucleated MII oocyte is used as the recipient cytoplast in SCNT, the donor nucleus should be in G0/G1 to maintain chromosomal diploidy of the reconstructed embryos (Campbell et al., 1996; Wilmut et al., 2002). The SCNT was performed as described in Kurome et al. (2015). In case of additional treatment with Scriptaid (500 nM), the reconstructed embryos were immediately after activation cultered with Scriptaid for 16 h to improve cloning efficiency (Zhao et al., 2010).

2.3.2. Fixation

Only good quality embryos, which showed no lysis or unevenly shaped blastomeres, were collected at specific embryonic developmental stages (8-cells, morulae and blastocysts according to Li et al., 2013) and transferred from the culture medium into 0.1% PVP-PBS to wash the embryos. Then the embryos were fixed in 1.7% Pfa for 20 min. Thereafter they were washed three times in 0.1% PVP-PBS and stored in the washing medium in 4-well dish sealed with

Parafilm in the refrigerator until further experiments.

2.3.3. Antibody staining

SCNT embryos from wild-type cells and parthenogenic embryos were stained with a fluorescent antibody binding OCT4 (Goat-anti-human Oct3/4; sc-8628), to get detailed insight into the OCT4 expression pattern. The nuclei were counterstained with DAPI in Vectashield.

For verification of the primary antibody specificity, the purified antigen was added to the primary antibody dilution. The antigen should absorb the antibody during incubation and hence the antigen prevents the binding of the antibody to the antigen on the exposed tissue or cells. Therefore the primary antibody goat-anti-human Oct3/4 (sc-8628) was diluted 1:500 with 1% BSA/0.1% Triton X-100 in PBS and relating to the primary antibody concentration, the tenfold amount of blocking peptide (sc-8628 P) was added and incubated for 2 h at room temperature. Meanwhile the fixed embryos were aspirated and permeabilized with 400 µl 1% BSA/0.1% Triton X-100 in PBS for 2 h at room temperature, to allow the antibody entering the cell. This is necessary because the OCT4 protein is located in the nucleus. The primary antibody goat-anti-human Oct3/4 (sc-8628) was diluted 1:500 with 1% BSA/0.1% Triton X-100 in PBS and stored on ice until further usage. Thereafter the embryos were incubated in 400 µl primary antibody dilution for 18 h at 4°C. A second antibody control serves as control that the binding of the secondary antibody is specific to the primary antibody. Hence the embryos were stored in 400 µl 1% BSA/0.1% Triton X-100 in PBS for 18 h at 4°C, omitting the primary antibody. On the next day the embryos were collected and washed three times for 20 min in 400 µl 0.1% Triton X-100 in PBS at room temperature. The second antibody rabbit-anti-goat–FITC (ab6737) was diluted 1:1000 with 1% BSA/ 0.1% Triton X-100 in PBS and stored on ice protected from light. From now on the embryos had to be protected from light to avoid bleaching of the fluorescence labeled secondary antibody. The selected embryos were labeled with 400 µl secondary antibody dilution for 2 h at room temperature. Subsequently they were washed three times for 20 min in 400 µl 0.1% Triton X-100 in PBS at room temperature. Afterwards the stained embryos were stored in PBS-PVP protected from light in the refrigerator up to 1 week or directly

embbeded on a slide in Vectashield containing DAPI (see protocol 2.2.6).

2.3.4. Embedding

For the analysis with the microscope, particularly the CLSM, the embryos needed to be in their natural shape. Therefore, pieces (8×15 mm) of a perforated adhesive-backed PVC-foil were stuck on a cover slip (76×26 mm). The selected embryos were transferred to one drop of mounting medium (DAPI in Vectashield) and placed separately in the holes of the foil. Then the holes were filled up with antifading Vectashield containing DAPI. Then the perforated PVC-foil was carefully covered with another cover slip (26×21 mm) and sealed with nail polish to prevent any evaporation. Finally the slides were stored in the refrigerator protected from light until the evaluation at the microscope.

2.3.5. Preparation of *OCT4-RFP* embryos

The *OCT4-RFP* embryos were protected from light, due to their fluorescence labeling and immediately fixed (protocol 2.2.4). Thereafter the fixed embryos could be stored in PBS at 4°C for the next few days until they were embedded (protocol 2.2.6) and analyzed.

2.3.6. Inverted epifluorescence microscopic analysis

The different cells and embryos were analyzed using the Zeiss Axiovert 200 M inverted epifluorescence microscope. The digital pictures were taken using a CCD (charge-coupled-device)-colour camera AxioCam HR and the AxioVision SE64Rel.4.9 software. For details see Table 2.

Table 2: The Zeiss Axiovert 200 M inverted epifluorescence microscope.

Filter set	Excitation wavelength [nm]	Emission wavelength [nm]	Label
0	365/12	LP 397	DAPI
13	470/20	LP 505-530	GFP
15	546/12	LP 590	RFP

LP= longpass.

2.3.7. Confocal microscopic analysis

The analysis by confocal laser scanning microscopy was performed by Dr. Felix Habermann from the Chair of Anatomy, Histology and Embryology of the LMU, Munich. The embryos were imaged by recording optical serial sections either using a Zeiss LSM 510 meta or a Zeiss LSM 710. For details see Table 3. The pixel size was 125 x 125 µm (LSM 510) or 115 x 115 nm (LSM 710), the z-step size was 1 µm. For processing and 3D visualization of the confocal image stacks the Zeiss ZEN lite 2011 software was used.

Table 3: The confocal laser scanning microscopes.

Microscope	Objective	Laser line [nm]	Emission filter [nm]	Label
Zeiss LSM 510 meta Zeiss LSM software v. 3.2	40x Plan-Neofluar oil immersion, NA 1.3	364	LP 385	DAPI
		488	LP 505	FITC
		543	LP 560	RFP
Zeiss LSM 710 ZEN 2009 software (black edition)	40x C-Apochromat water immersion, NA 1.2	405	410 – 556	DAPI
		561	582 – 754	RFP

LP= longpass.

V. RESULTS

1. Analysis of the WT primary porcine kidney cell line 0407 for genomic modification

The isolation of the different wild-type (WT) primary kidney cell lines PKC0407, PKCf, PKCm and PKC2109 was conducted by Dr. Annegret Wünsch as described in Richter et al. (2012). These four different WT-cell lines had to be tested, if they are suitable for genetic modification and nuclear transfer. Richter et al. (2012) analyzed several characteristics of the cell lines PKCm and PKC2109, such as cell morphology, growth potential, chromosome number, and transfection efficiency using different protocols. (Richter et al., 2012). Both cell lines were successfully used as donor cells for additive and targeted gene transfer and the production of transgenic pigs via SCNT (Richter et al., 2012). Based on the results of Richter et al. (2012) the following criteria were determined to be necessary for the evaluation of another potential cell line for targeting, namely PKC0407: (i) cell morphology, (ii) correct number of chromosomes, (iii) transfection efficiency and cell viability after nucleofection with GFP. Due to the fact that all analyzed WT-cell lines presented proper results for genetic modification, PKCf was not investigated in detail, suggesting the results would be similar.

1.1. Investigation of the cell morphology

Altogether the primary kidney cell cultures showed a mixed morphology and after twelve passages, spindle-shaped fibroblast-like cells dominated the culture (Figure 8). This kidney cell line was cultured for several weeks to test their life span and proliferation potential. At passage 47 the cell line showed slight signs of morphological senescence and reduced proliferation potential, meaning the cells should be capable for the challenging culture in a targeting experiment (Figure 8).

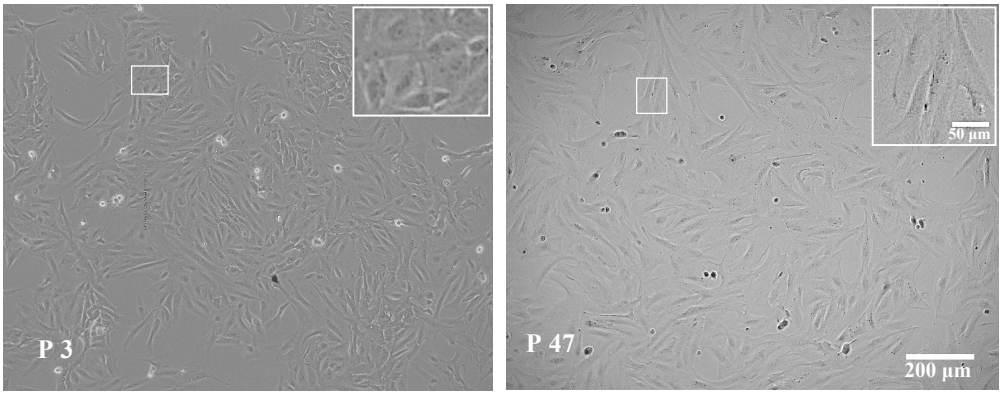


Figure 8: Morphology of PKC0407. In passage three (P3) the porcine kidney cells displayed a mixed morphology of rounde endothelial-like cells and spindle-shaped fibroblast-like cell. In passage 47 (P47) the spindle-shaped morphology dominated. The insertions demonstrate magnifications of the specified areas.

1.2. Determination of chromosome numbers

Since genomic stability of the nuclear donor cells is important for the generation of animals via SCNT, the correct number of chromosomes of the WT-cell line has been tested. Therefore cells in passage five with a confluence of 70-80% were arrested in metaphase by adding colcemid, which inactivates spindle fiber formation. The DAPI-stained chromosomes were photographed with a CCD color camera at an inverted epifluorescence microscope, and the chromosome numbers were counted with Image J. Out of 35 metaphases, 83% sustained the correct number of chromosomes (2n=38, XY) as shown in Figure 9.

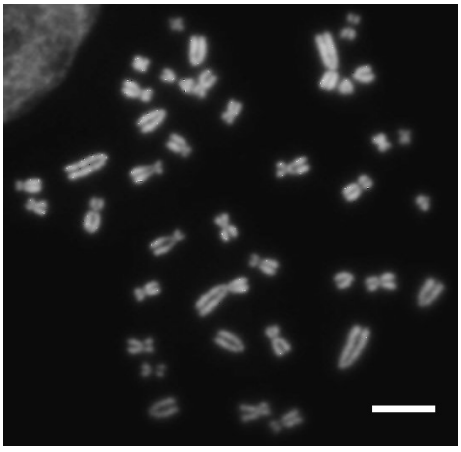


Figure 9: Determination of chromosome number. A metaphase of the cell line PKC0407 showing a correct number of chromosomes (2n=38, XY). For

counterstaining of the DNA, DAPI in Vectashield was used (scale bar = 10 µm)

1.3. Evaluation of the transfection efficiency and cell viability after nucleofection

The transfection was performed using the Nucleofector ™ II device containing programs with electrical settings programmed by the supplier. The most efficient program U12, according to Richter et al. (2012), with high transfection efficiency, good cell quality and good fluorescence signal, was used for a test transfection of PKC0407 with the plasmid pmaxGFP™ provided with the Amaxa™ Basic Nucleofector™ Kit Primary Fibroblasts. The cells were analyzed 24 h after nucleofection with an inverted epifluorescence microscope. Approximately 70% of the cells showed green fluorescence indicating sufficient transfection efficiency (Figure 10). Cell viability, evaluated by means of amount of cells in suspension and morphology of attached cells, was good compared to non-treated cells, meaning only few cells were in suspension and most of the attached cells showed no obvious signs of cell stress, such as vacuoles. Therefore the primary porcine kidney cell line 0407 could be used for nucleofection.

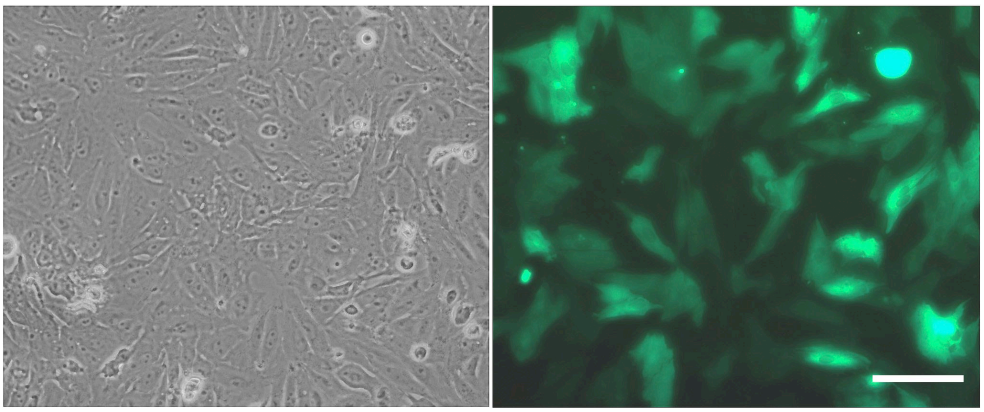


Figure 10: Evaluation of the transfection efficiency and cell viability of PKC0407. The cell line PKC0407 was transiently transfected with pmaxGFP™. Left: phase contrast. Right: fluorescence picture. 24 h after nucleofection approximately 70% of the cells exhibited green fluorescence und most of the cells showed no signs of stress. (scale bar = 100 µm).

2. Integration of the *OCT4-RFP* gene into different primary porcine kidney cells

2.1. Generation of single-cell clones

Altogether eight different transfections using four different kidney cell lines were performed. The settings for the single experiments partly varied in the passage number of cells, in the amount of transfected DNA, the number of cells seeded for selection and the numbert of WT cells added for selection (Table 4).

Selection was started 48 h after transfection using G418 as the selection antibiotic. The number of harvested cells varied from 1.3×10^5 cells (PKCm) in experiment 121211 to 8.3×10^5 cells (PKC0407) in experiment 131211. For selection, transfected and non-transfected cells were mixed at a ratio of 1:1. Subsequently 2,000 cells per well were seeded on 96-well half-area plates. In both experiments 121211 and 131211, which were transfected with 1 µg of DNA and in experiment 180112, which was transfected using program V13 instead of the usually used U12, only the transfected cells were used for selection. In the experiment 180112 program U12 was applied for transfection and only 1,000 cells per well were seeded on 96-well half-area plates.

The settings of the different transfections varied in order to improve the outcome, meaning good growing single-cell clones with a correct integration at the *OCT4* locus. Depending on the viability of the cells after transfection, the amount of DNA was reduced or a mix of transfected and non-transfected cells was seeded for selection. The mixture with non-transfected cells seemed to support the recovery of the transfected cells via the exchange of nutritive substances without increasing the risk of getting multiple cell clones per well.

Two transfections using PKCm resulted only in 12 analyzable cell clones, whereas from four transfections using PKC0407 182 cell clones could be harvested for analysis. The cell line PKCf and PKC2109 were used only for one transfection experiment resulting in 33 and 43 cell clones respectively. In summary the eight transfections of endotoxine-free prepared and linearized *OCT4-RFP* BAC-DNA into different WT PKCs resulted in a total of 473 single-cell clones which could be splitted from the original 96-well half area plate to two 96-well plates. Out of these, 271 cell clones could be harvested and further analysed by qPCR for correct integration of the *OCT4-RFP* BAC-DNA.

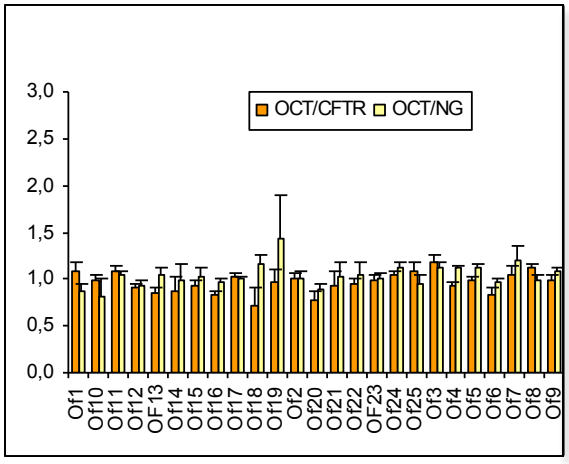
Table 4: Results of transfections with four different WT-cell lines.

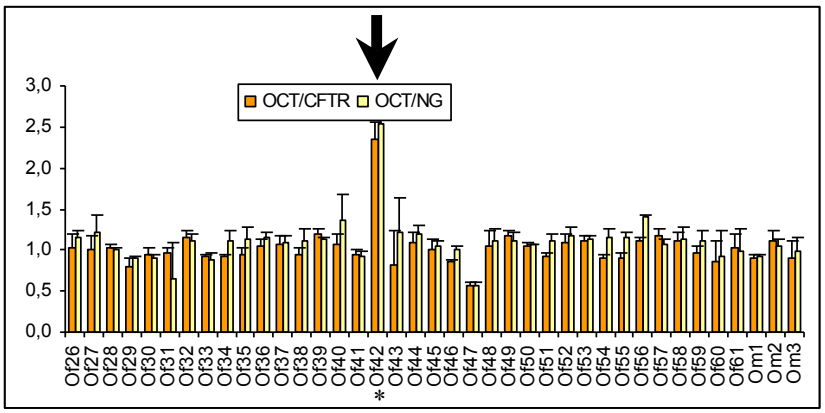
Cell line Transfection (program)	Amount of DNA (µg)	Transfected cell number per well seeded for selection (+non-transfected)	Cell number 48 h after transfection ($\times 10^5$)	Splitted single-cell clones	Harvested cell clones labeling	Correctly targeted cell clones
PKCm 121211 (U12)	5	2000	2.8	34	8 Om1-Om8	0
PKCm 121211 (U12)	5	2000	1.3	4	4 Om9-Om12	0
PKC0407 131211 (U12)	1	2000	8.3	77	24 Of1-Of24	0
PKC0407 131211 (U12)	2	1000 (1000)	6.7	87	37 Of25-Of61	Of42
PKC0407 180112 (U12)	2	1000	6.8	129	121 Of62-Of182	Of90, Of170
PKC0407 180112 (V13)	2	2000	2.2	19	0	0
PKCf 240112 (U12)	2	1000 (1000)	1.6	62	33 Oaf1-Oaf33	Oaf26, Oaf33
PKC2109 240112 (U12)	2	1000 (1000)	4.5	61	44 Oam1-Oam44	Oam32
Total (8 transfections)			34.2	473	271	7

2.2. Screening for loss of wild-type allele

In the screening for loss of wild-type allele, based on qPCR, the copy numbers of the wild-type alleles of the *OCT4* target gene and two reference genes was determined. Thereafter the ratio of the copy numbers between the target gene as well as the reference gene was calculated and compared. Cell clones with a ratio 2:2 contain the unmodified WT alleles, whereas the ratio 1:2 indicated the replacement of one wild-type allele by the *OCT4-RFP* gene at the correct integration side.

Out of the 271 collected cell clones 247 could be analyzed using the qPCR-based loss of wild-type allele screening, which was performed by Dr. Klymiuk as described in Klymiuk et al. (2014). Finally, seven clones were verified as correctly targeted containing a knockin of *OCT4-RFP* at one allee (Of42, Of90 and Of170 originated from PKC0407; Oaf26 and Oaf33 originate from PKCf; Oam32 originates from PKC2109) after confirmation in a second qPCR experiment resulting in a targeting efficiency of 2.83% (Figure 11).





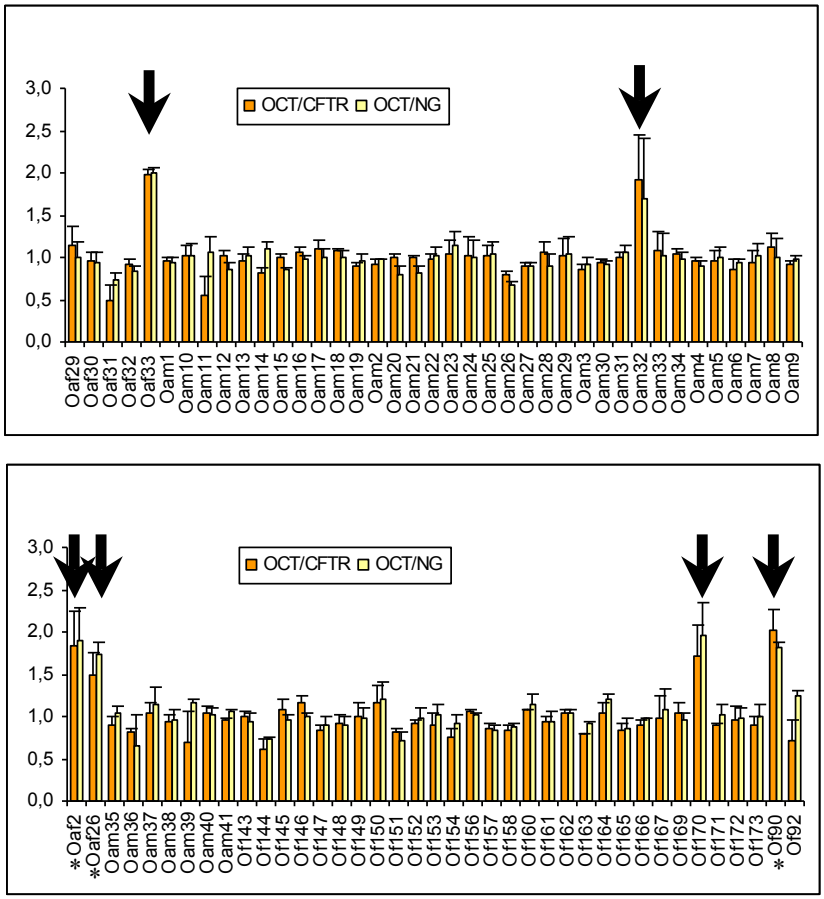


Figure 11: Results of screening for loss of wild-type allele. The ratio of the copy numbers between the target gene (OCT= OCT4) and the reference genes (CFTR and NG=NANOG) were calculated and compared. Out of 247 clones, seven correctly targeted cell clones, indicated by arrows (Of42*, Of90*, Oaf2*, Oaf26*, Oaf33, Oam32, Of170) were confirmed by qPCR, resulting in a targeting efficieny of 2.83% (*cell clones which were confirmed in second qPCR run).

3. Analysis of knockin and randomly integrated *OCT4-RFP* embryos

3.1. Analysis of blastocysts with targeted *OCT4-RFP* integration

Two different correctly targeted (knockin) cell clones from two different PKC lines were used for SCNT resulting in 12 blastocysts from Of42 (origin PKC0407) and 11 blastocysts from Oaf2 (origin PKCf). The blastocyst rate of these two cell clones was similar with 38.7% (Of42) and 35.5% (Oaf2), but higher when compared to the WT cell line PKC0407 (22.2%) as summarized in table 5. After fixation the embryos were investigated on day 5 and day 6 post activation using

an epifluorescence microscope. Blastocysts generated from PKC0407 served as control embryos. In the knockin embryos originating from the two different cell clones no specific nuclear fluorescence, besides slightly unspecific background fluorescence, could be detected.

Table 5: SCNT embryos derived from cells clones with targeted OCT4-RFP integration and WT-cells

Donor cells	Fusion rate	No. of reconstructed embryos cultured	No. (%) of reconstructed embryos developed to		No. of analysed blastocysts day 5/day 6
			2-4 cell stage	Blastocyst stage*	Epifluorescence
Of42 (PKC0407)	31/32 (96.9)	31	24 (77.4)	12 (38.7)	3/8
Oaf2 (PKCf)	31/32 (96.9)	31	21 (67.7)	11 (35.5)	3/9
wilde-type PKC0407º	18/20 (90.0)	18	13 (72.2)	4 (22.2)	2/2

* Total number of blastocyst was counted on day six.

º non transfected WT cells (PKC0407) was used as control.

The data in the table can not be compared since ovaries from different oocyte cohorts were used.

3.2. Analysis of embryos with random *OCT4-RFP* integartion

3.2.1. Initial analysis of SCNT blastocysts derived from different cell clones with random *OCT4-RFP* integration

Ten different cell clones with random *OCT4-RFP* integartion from four different PKC lines were used for SCNT in distribution on 7 different experiments. Overall 109 blastocysts could be generated - 70 from the original line PKC0407, 17 from PKCf and from PKCm, and 5 from PKC2109. The blastocyst rate varied from 11.8% (donor cell clone Of43 from PKC0407) up to 27.3% (donor cell clone Oaf13 from PCKf) as summarized in table 6. The highest blastocyst rate of 55.7% (donor cell clone Of71 from PKC0407) was achieved with additional Scriptaid

treatment of the embryos. After fixation the embryos were investigated on day 5 (4 blastocysts from cell clone Of14) and day 6 post activation using an epifluorescence microscope. Only one single blastocyst from cell clone Of71 showed a weak signal in the nuclei and a detailed analysis of the fluorescence signal was not possible. After fixation 12 blastocysts from cell clone Of71 were analyzed with the epifluorescence as well as the CLSM microscope. As a control group served blastocysts generated from the correspondent PKC0407.

Table 6: SCNT embryos derived from cell clones with random OCT4-RFP integration and WT-cells

Donor cells	Fusion rate	No. of reconstructed embryos cultured	No. (%) of reconstructed embryos developed to		No. of analysed blastocysts on day 6
			2-4 cell stage	Blastocyst stage*	Epifluorescence /CLSM
f11 (PKC0407)	71/72 (98.6)	65	40 (61.5)	14 (21.5)	10/0
f14 # (PKC0407)	24/25 (96.0)	24	21 (87.5)	3 (12.5)	2/0
f43 (PKC0407)	34/38 (89.5)	34	26 (76.5)	4 (11.8)	4/0
f71 (PKC0407)	39/46 (84.8)	39	31 (79.5)	10 (25.6)	12/12
f71^ (PKC0407)	70/75 (93.3)	70	52 (74.3)	39 (55.7)	1/0
af9 (PKCf)	37/43 (86.0)	37	34 (91.9)	6 (16.2)	2/0
af13 (PKCf)	22/24 (91.7)	22	21 (95.5)	6 (27.3)	6/0
af16 (PKCf)	19/20 (95.0)	19	14 (73.5)	5 (26.3)	4/0
m2 (PKCm)	71/74 (96)	63	53 (84.1)	11 (17.5)	7/0
m6 (PKCm)	53/54 (98.2)	46	38 (82.6)	6 (13)	4/0
am21 (PKC2109)	40/45 (88.8)	33	27 (81.8)	6 (18.2)	4/0
wilde-type (PKC 0407^º)	68/72 (94.4)	68	45 (66.2)	26 (38.2)	6/6

* Total number of blastocyst was counted on day 6.

Additional analysis of 4 blastocysts on day 5.

^ Additional treatment with Scriptaid

º non transfected WT cells (PKC0407) were used as control.

The data in the table can not be compared since ovaries from different oocyst cohorts were used.

The analysis of Of71 blastocysts with the inverted epifluorescence microscope and the CLSM showed red fluorescence signal in the nuclei. The blastocysts from the WT control showed spots of unspecific red autofluorescence in the cytoplasm, caused by extracellular fluorophores of the cell membrane in the ZP, which are excited with the same excitation wavelength as the RFP protein (555 nm).

3.2.2. Detailed analysis of SCNT embryos derived from cell clone Of71 with random *OCT4-RFP* integration

For testing whether the randomly integrated *OCT4-RFP* reporter is suitable for monitoring the porcine *OCT4* promoter activity and OCT4 protein localisation during early porcine embryogenesis a detailed analysis of SCNT embryos was performed using the randomly *OCT4-RFP* cell clone Of71. Since EGA in porcine embryos is initiated at the 4-cell stage in a stepwise manner (Tomanek et al., 1989), the embryonic *OCT4* mRNA transcription should be initiated thenceforward. Hence, interesting time points to study *OCT4* gene expression using the RFP reporter are the 8-cell, the morula and the blastocyst stage. The control embryos for the respective embryonic stage based on the WT-cell line PKC0407. In the following section, seven SCNT experiments are summarized. The analysis with the CLSM was performed by Dr. Felix Habermann.

At each stage all collected embryos were first analyzed for normal development, meaning the embryos were selected for fragmented, degenerated, unevenly sized or binucleated nuclei as well as an abnormal cleavage pattern (Figure 12). All the embryos, which showed these abnormal characteristics, were excluded from further detailed analysis.

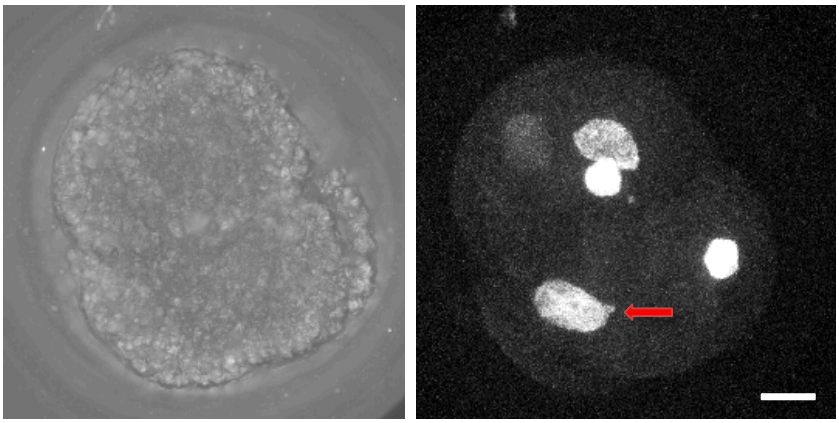


Figure 12: Abnormally developed 8-cell stage embryo (CLSM). The embryo is pictured as followed: Left: phase contrast. Right: Z-Projection of optical serial sections of counterstained nuclei with DAPI in Vectashield. An embryo, fixed 81 h post activation, presenting abnormal development. This embryo displays three large nuclei, one of them with a nuclear bleb (indicated by the red arrow) and two small nuclei (scale bare = 100 µm).

At the 8-cell stage the embryos were collected at two different time points post activation (Table 7). At the first investigated time point, 75 h post activation, 8 embryos from cell clone Of71 and 5 embryos from PKC0407 were fixed and analyzed. All the embryos showed 2-4 irregular, unevenly shaped nuclei and therefore a later time point for fixation was chosen to collect 8-cell stage embryos. At the second fixation time point (81 h post activation) 56 embryos from cell clone Of71 and 48 from the WT-cell line were analyzed for normal embryonic development. Interestingly cell clone Of71 resulted in more normally developed embryos than the WT cell line. Out of the 8 normally developed embryos from cell clone Of71, 3 embryos showed a nuclear specific red fluorescence signal (Figure 13). This fluorescence signal was detectable in several, but not in all nulei, suggesting this may be due to the stepwise activation of *OCT4* mRNA starting at the 4-cell stage. The WT-control embryos showed spots of unspecific red autofluorescence in the cytoplasm (Figure 13).

Table 7: Analysis of 8-cell stage embryos 75 and 81 h post activation derived from cell clone Of71 with random *OCT4-RFP* integration and WT-cells.

Donor cell	8-cell stage 75/81 h	Normal developed embryos 75/81 h	Normal developed embryos with RFP signal
Of71	8/56	0/8	3
PKC0407	5/48	0/3	0
	13/104	0/11	

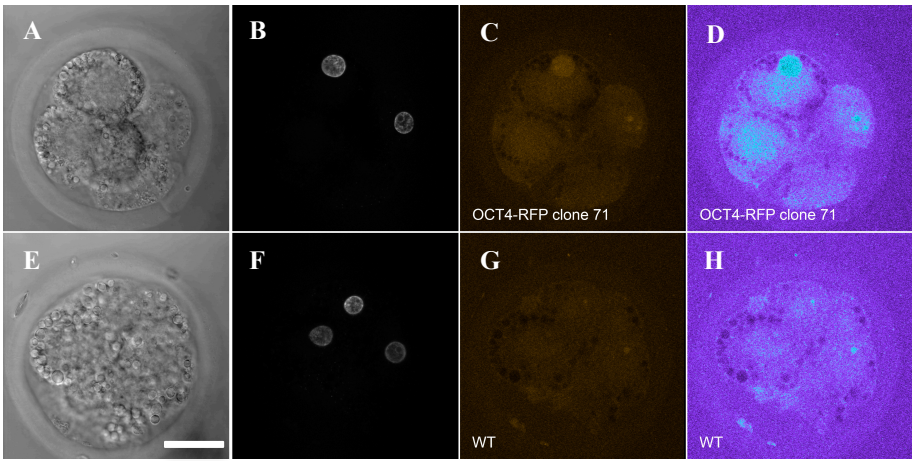


Figure 13: Analysis of SCNT 8-cell stage embryos derived from cell clone Of71 with random *OCT4-RFP* integration and WT-cells. (CLSM). The embryos are pictured in the following way: Single optical sections of: phase contrast (A, E), counterstained nuclei with DAPI in Vectashield (B, F), RFP in red fluorescence (C, G) and RFP in false color rendering (D, H) for better illustrating intensity pattern. The 8-cell stage embryo, fixed 81 h (A-D) post activation, showed a specific nuclear RFP fluorescence in two nuclei. The corresponding WT 8-cell stage (E-H), fixed 96 h post activation, showed spots of unspecific red autofluorescence in the cytoplasm (scale bar = 100 µm).

At the morula stage the embryos were collected at two different time points post activation (Table 8). At the first investigated time point, (100.5 h post activation, 8 embryos from cell clone Of71 and 12 embryos from PKC0407 were fixed and

analyzed. Thereof 17 embryos showed irregular, unevenly shaped nuclei or arrested early blastomeres and therefore an earlier time point for fixation was chosen for the collection of morula stage embryos. At the second fixation time point, 96 h post activation, 52 embryos from cell clone Of71 and 21 from the WT-cell line were analyzed for normal embryo development. Within the WT embryos no normally developed morula was found, but 2 normally developed 8-cell stage embryos. Thus embryos originating from cell clone Of71 showed more normal developed morulae. Out of the 15 normally developed embryos from cell clone Of71, 11 embryos showed a nuclear specific red fluorescence signal (Figure 14). The WT-control embryos showed spots of unspecific red autofluorescence in the cytoplasm (Figure 14).

Table 8: Analysis of morulae 96 and 100.5 h post activation activation derived from cell clone Of71 with random *OCT4-RFP* integration and WT-cells.

Donor cell	Morula stage 96/100.5 h	Normal developed embryos 96/100.5 h	Normal developed embryos with RFP signal 96/100.5 h
Of71	52/8	14/1	10/1
PKC0407	21/12	2*/2	0
	73/20	14/3	10/1

* Two 8-cell stage embryos were found.

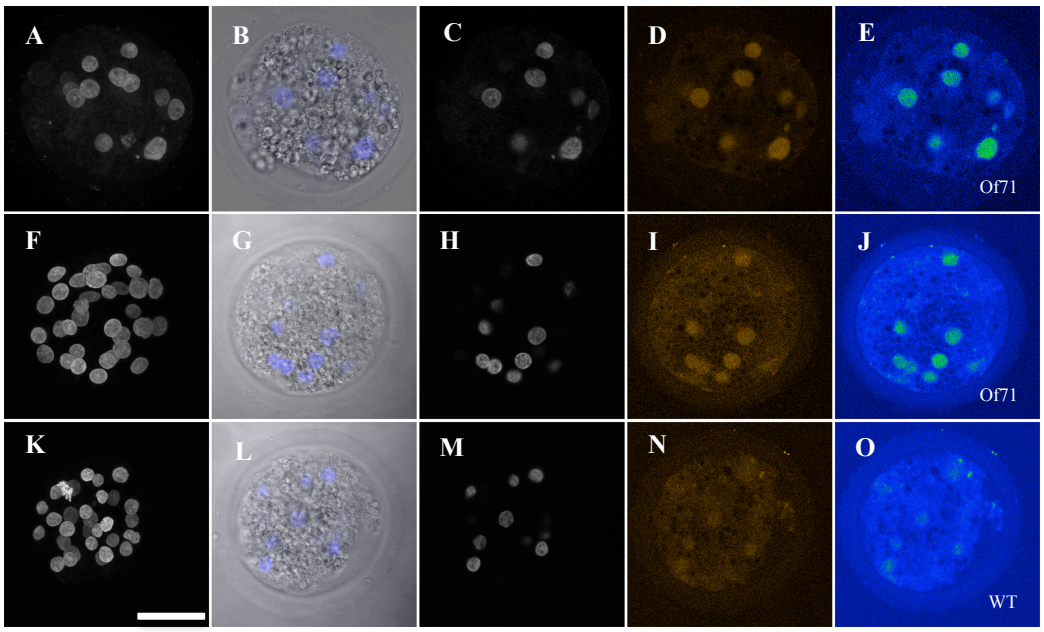


Figure 14: Analysis of SCNT morulae derived from cell clone Of71 with random *OCT4-RFP* integration and WT-cells. (CLSM). The embryos are pictured in the following way: Z-Projection of optical serial sections of counterstained nuclei with DAPI in Vectashield (A, F, K); Single optical sections of: phase contrast (B, G, L), counterstained nuclei with DAPI in Vectashield (C, H, M), RFP in red fluorescence (D, I, N) and RFP in false coulor redering (E, J, O) for better illustrating intensity pattern. Both morulae, fixed 96 h (A-D) and 100.5 h (F-J) post activation, showed a specific nuclear RFP fluorescence. The corresponding WT control morula (K-O), fixed 100.5 h post activation, showed spots of unspecific red autofluorescence in the cytoplasm (scale bar = 100 µm).

At the blastocyst stage 21 embryos from cell clone Of71 and 15 from PKC0407 (Table 9) were fixed 145.5 h post activation and analyzed for normal embryo development. The WT cell line gave rise to more normally developed embryos than the cell clone Of71. All the normally developed blastocysts from clone Of71 displayed a nuclear specific red fluorescence signal (Figure 15). Since there were not enough blastocysts with clearly identifiable ICM, we could not give a clear statement whether the RFP signal is restricted to ICM or expressed in the ICM and TE. The WT-control embryos showed spots of unspecific red autofluorescence in the cytoplasm (Figure 15).

Table 9: Analysis of blastocysts 145.5 hours post activation activation derived from cell clone Of71 with random *OCT4-RFP* integration and WT-cells.

Donor cell	Blastocyst stage 145.5 h	Normal developed embryos	Normal developed embryos with RFP signal
Of71	21	6	6
PKC0407	15	5	0
	36	12	

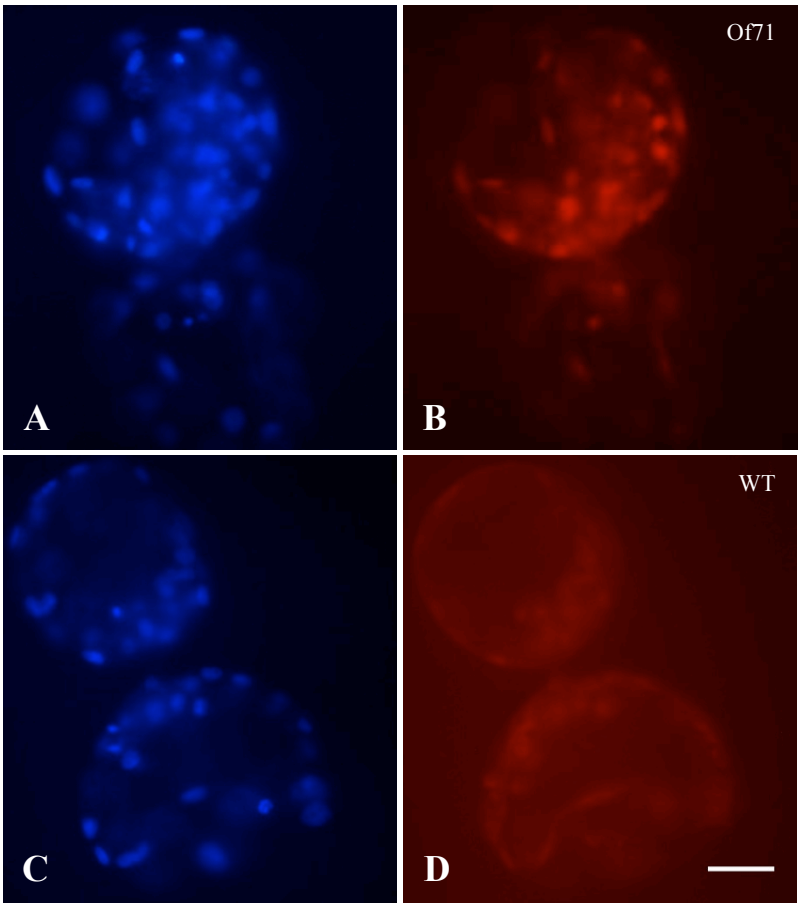


Figure 15: Analysis of SCNT blastocysts derived from cell clone Of71 with random *OCT4-RFP* integration and WT-cells with the epifluorescence microscoscope. The embryos are pictured in the following way: Left picture: DAPI counterstained nuclei (A, C). Right picture: OCT4-RFP fluorescence is displayed via red fluorescence (B, D). The blastocyst from cell clone Of71 (A, B)

showed a nuclear specific red fluorescence signal. The blastocyst from the WT cell line PKC0407 (C, D) displayed spots of unspecific red autofluorescence in the cytoplasm (scale bar = 40 µm).

3.3. Analysis of the SCNT donor cells of cell clone Of71 and WT PKC0407

3.3.1. Examination of *OCT4-RFP* expression in cell clone Of71

The *OCT4* promoter is known to be active in early embryogenesis and not in differentiated cells like fibroblasts. Therefore fibroblast cells from cell clone Of71 were investigated to confirm that the cells integrated the *OCT4-RFP* reporter construct, but do not express the construct in these differentiated cells. Both, cell clone Of71 and the corresponding WT cell line PKC0407 were fixed and analysed with the inverted epifluorescence microscope. In both cultures no RFP signal could be detected (Figure 16), indicating that the *OCT4-RFP* reporter construct was not active in the randomly integrated *OCT4-RFP* cell clone Of71.

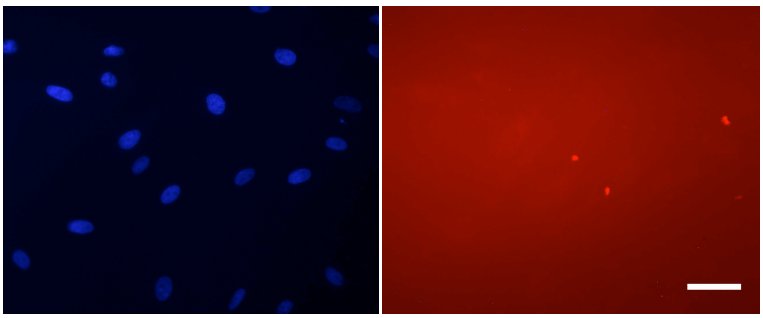


Figure 16: Analysis of the *OCT4-RFP* transgenic cell clone Of71 for RFP fluorescence. Left: DAPI counterstained nuclei. Right: fluorescence picture. The PKCs from cell clone Of71 show no RFP fluorescence signal, demonstrating that *OCT4-RFP* reporter construct was not active (scale bar=50 µm)

3.3.2. Detailed analysis of the nuclei of *OCT4-RFP* cell clone Of71 and WT cell line PKC0407

The quality of the donor cells is known to have an influence on SCNT outcome (Bureau et al., 2003). Since only a few of the produced SCNT embryos showed a normal phenotype, a closer look was taken on the nuclei of cell clone Of71 and

the wild-type porcine kidney cell line 0407. For a detailed analysis of the chromosomes a certain number of cells is necessary what is impossible to get out of a single-cell clone culture due to the limited proliferation capacity. The cells were fixed at the same passage number as used for SCNT, meaning passage 12 for clone Of71 and passage nine for PKC0407. In both cultures, cells with pyknotic nuclei have been detected (Figure 17). Pyknotic cells are charaterized by a shrunken nucleus, finally leading to cell death via apotosis or necrosis. Some cells of both cultures had two nuclei, a main nucleus and a smaller round or oval shaped nucleus, so called micronucleus (Figure 17). Micronuclei are undesired in cell cultures, because the micronucleus contains chromatin particles from abnormal mitosis (reviewed in Sabharwal et al., 2015).

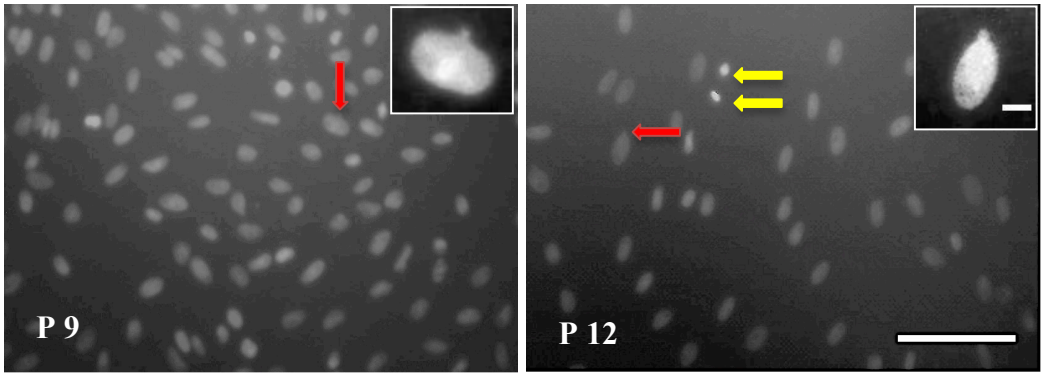


Figure 17: Analysis of the nuclei of PKC0407 and cell clone Of71. The cells of PKC0407 (P9) and cell clone Of71 (P12) were analyzed with the inverted epifluorescence microscope. The nuclei were counterstained with DAPI in Vectashield shown here in white. The yellow arrows indicate pyknotic cell nuclei. The red arrows indicate micronuclei (upper right: detail), which were present in both cell cultures (scale bars indicates: 100 µm (overview) or 10 µm (detail)).

3.4. Anti-OCT4 antibody staining in SCNT and parthenogenetic blastocysts

To confirm that the detected RFP signal in the *OCT4-RFP* blastocysts generated with the randomly integrated cell clone Of71 reflects the OCT4 localisation and *OCT4* promoter activity, *OCT4-RFP* transgenic embryos and WT blastocysts were stained on day 6 with an OCT4-specific antibody. As donor cells wild-type PCKm was used for SCNT. Furthermore parthenogenetic embryos were generated

and stained. Overall 14 blastocysts were generated via SCNT, fixed on day 6 (145 h after activation), stained and analyzed with the inverted epifluorescence microscope as well as with the CLSM. In summary the nuclei of all blastocysts showed a specific green fluorescence signal. The secondary antibody control embryos showed no signal, so a cross-reactivity of the secondary antibody could be excluded (Figure 18).

Additionally, 13 parthenotes were fixed on day 6, 142 h post activation. An earlier time point for fixation compared to the SCNT embryos was chosen, because several blastocysts were already hatched 145 h post activation, so that it was not possible to clearly identify the ICM and the localisation of OCT4 expression. All embryos were analyzed with an inverted epifluorescence microscope and a CLSM. In summary, the nuclei of all 7 stained blastocysts showed a specific green fluorescence signal (Figure 18). Both control groups (3 embryos in each group), without the primary antibody or with the secondary antibody alone, showed no sginal. The exact localisation of the OCT4 protein signal, whether it was localized in the ICM, in the TE or in both, could not be determined, due to an insufficient discrimination of ICM and TE in most of the blastocysts. In summary, the staining pattern of OCT4 in SCNT and parthenogenetic embryos was equivalent to the RFP signal detected in the nuclei of the *OCT4-RFP* blastocysts from clone Of71 (Figure 18).

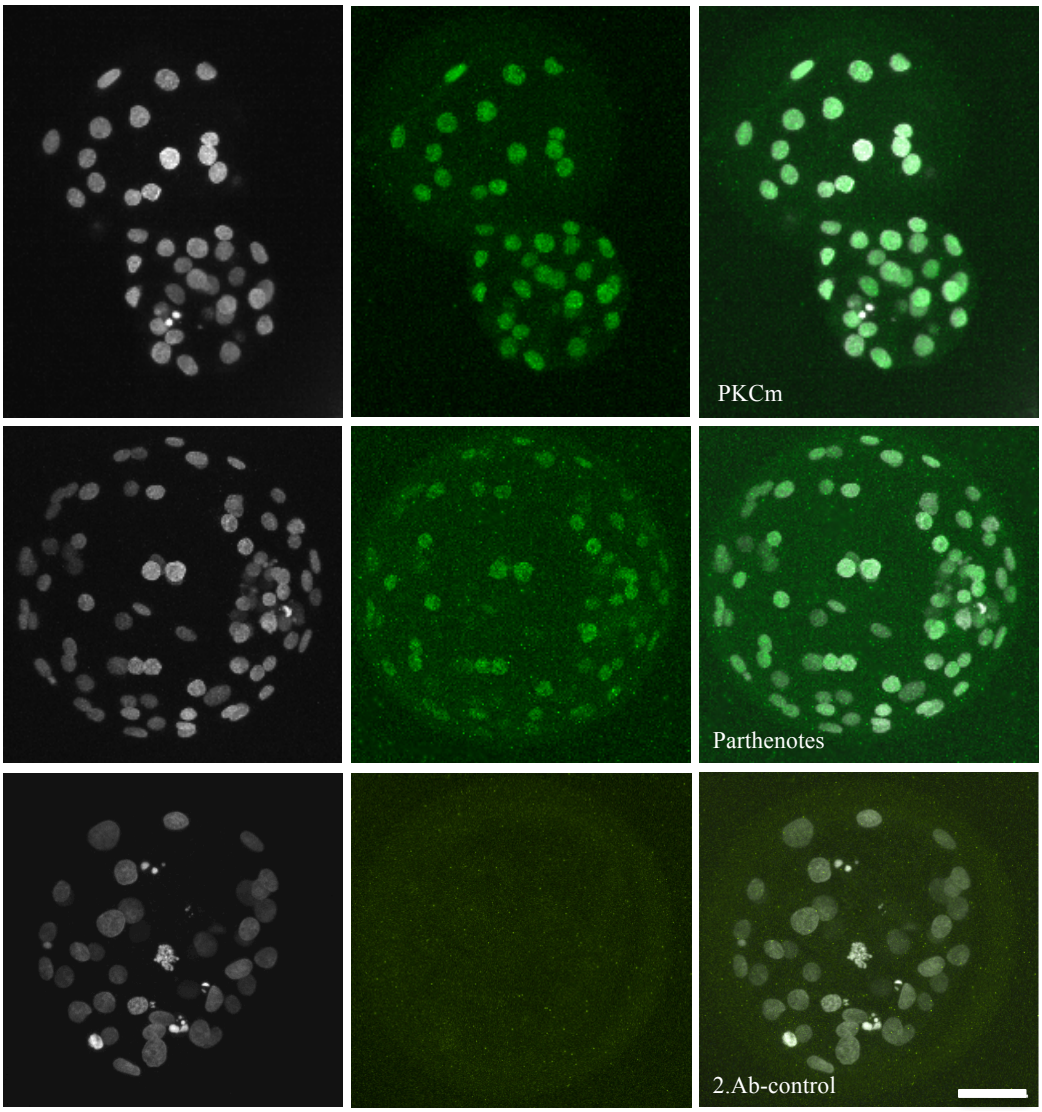


Figure 18: Antibody staining of blastocysts from PKCm and parthenotes (CLSM). The embryos are pictured in the following way: Left: Counterstained nuclei with DAPI in Vectashield. Middle: antibody staining is displayed via green fluorescence. Right: merge of both pictures. The blastocysts from the WT cell line PKCm as well as the parthenotes were stained with a goat-anti-human Oct3/4 antibody (green nuclei). In the merge all nuclei (white) displayed a nucleus specific green fluorescence, confirming the nuclear localisation of the OCT4 protein. The control blastocyst (2.Ab-control) was stained with the secondary antibody alone. In the merge, none of the nuclei (white) displayed a nucleus specific green fluorescence, confirming that secondary antibody was specific for the primary antibody (scale bar = 100 µm).

VI. DISCUSSION

1. Investigation of a new donor cell line PKC0407 and generation of single-cell clones carrying *OCT4-RFP* using different WT-PKCs

Gene targeting via homologues recombination in primary cells, generation of good growing single-cell clones and the following somatic cell nuclear transfer is challenging. Therefore donor cells have to be evaluated in advance. The WT-cell line PKC 0407 was investigated on the basis of the following criteria: cell morphology, growth potential, chromosome number, transfection efficiency and cell viability after nucleofection. As shown by Richter et al. (2012) for the cell lines PKC2109 and PKCm, in the culture of PCK 0407 spindle-shaped fibroblast-like cells dominated after twelve passages. Furthermore, this cell line could be cultured for at least 71 passages, unusual for primary cells, showing slight signs of morphological senescence and reduced proliferation potential form passage 47 on. This finding is consistent with the results of the cell lines PKC2109 and PKCm analyzed by Richter et al. (2012). The WT cell line PKC0407 showed a correct number of chromosomes in 83% of the analyzed metaphases similar to PKCm (80%) and PKC2109 (74%), as obtained in Richter et al. (2012). The transfection efficiency of 70% as well as the good cell viability after nucleofection, were comparable as described for PKC2109 (70%) and PKCm (66%) in Richter et al. (2012).

In this study different WT-PKC lines were used for transfection in order to get cell clones with different genetic backgrounds with male and female origin, since it has been already shown that donor cells of different origin can vary in targeting efficiency (Rogers et al., 2008). A missing standard protocol for gene targeting with BAC-DNA made it necessary to optimize different settings for transfection and single-cell generation, such as amount of transfectetd DNA and seeded cells per 96 well plate for selection. For a reasonble transfection efficiency the amount of DNA must be high enough to generate an appropriate number knockin single-cell clones. On the other hand too much foreign DNA could be toxic for the transfected cells (Sumiyama et al., 2010). So, due to the low cell viability after the first transfections with PKCm the amount of DNA was reduced to 1 or 2 µg BAC-

DNA. In the following experiments the usage of 2 µg DNA turned out to be appropriate for cell viability after transfection and number of viable cell clones.

Another challenging point is the generation of single-cell clones using culture conditions, which are not optimal for fibroblasts because fibroblasts normally support their growth via the exchange of nutritive substances/factors. The addition of non-transfected healthy cells for selection to support the transfected cells did not show an improvement regarding the amount and quality of single-cell clones.

For transfection different programs (offered by the supplier) can be used influencing cell viability, cell morphology and uptake of foreign DNA (Richter et al. 2012). It has been shown by Richter et al. (2012) that the program U12 and V13 were sufficient for transfection for PKCm and PKC2109. When comparing cell viability after transfection of PKC0407 using U12 and V13 in this thesis U12 was more suitable because U12 resulted in better cell viability. It has to be mentioned that only one single experiment with V13 has been conducted.

Overall 271 neomycin-resistant single-cell clones could be generated for cryopreservation and screening for loss-of-wild-type-allele. Out of these 2.83% were correctly targeted with a knockin of *OCT4-RFP* at the *OCT4* locus. This is in the same range as in other targeting experiments using BAC-DNA where efficiencies of 1.6% (Klymiuk et al., 2012) and 2.1% (Klymiuk et al., 2013) were achieved.

The efficiency of gene targeting via homologous recombination is rather low, since homologous recombination is a very rare event in mammalian cells. This limitation could be overcome by the application of molecular scissors such as zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and RNA-guided DNA endonucleases CRISPR/Cas9. The transfection efficiency for gene targeting in pig could successfully be increased in a range from 4% using ZFNs (Yang et al., 2011) up to 41% with the application of TALENs (Carlson et al., 2012).

2. Transgenic *OCT4-RFP* embryos as a reporter to monitor *OCT4* promoter activity and OCT4 protein localisation in early porcine embryos

For monitoring the *OCT4* promoter activity and localisation of the OCT4 protein on the basis of an OCT4-RFP fusion protein in embryos, single-cell clones carrying *OCT4-RFP* at the endogenous *OCT4* locus and randomly in the host genome were produced for SCNT. Two knockin and ten randomly integrated *OCT4-RFP* cell clones were used for SCNT and the resulting embryos were analyzed. Only the randomly clone Of71 showed specific nuclear RFP signal in morula and blastocyst stage embryos, whereas the embryos generated out of the two knockin and the eight other randomly cell clones showed no fluorescence signal. It could not be clarified why the RFP signal is only detectable in one cell clone with a random integration. There are different explanations for that: On the one hand RFP is not present, or on the other hand the methods to detect RFP are not sensitive enough. The following two reasons could lead to missing RFP epression:

i) The *OCT4-RFP* construct was somehow damaged and only a fragment containing at least the neomycin resistence gene was integrated into the genom. The integration at the *OCT4* locus was verified for all seven knockin cell clones using qPCR strategy based on the loss of wild-type allele. It could not be excluded the parts of the construct are missing, in both, the knockin cell clones and the cell clones with random integration. Sequencing of the generated cell clones was not performed yet.

ii) The entire reporter construct was integrated, but was not expressed. After random integration position effects caused by the surrounding local genome environment could influence the expression of the transgene (Wilson et al., 1990). These local molecular environment consists of regulatory elements, which adjust remodeling of the chromatin structure and the DNA methylation and thereby can induce silencing of the integrated gene construct. This position dependent gene silencing is quite common in transgenic animals. By integration of the transgene at a specific locus such position effects should be avoided. However, in a

recently published study from Lai et al. (2016) 21 knockin cell clones carrying a porcine *OCT4-tdTomato* construct were verified as correctly targeted via qPCR. Thereof 19 clones were used as donor cells for the generation of SCNT blastocysts and only eight cell clones resulted in blastocysts with tdTomato fluorescence signals (Lai et al., 2016). They did not discuss this observation, but just mentioned that the missing fluoresecence signal in some embryos originating from single-cell clones could be caused by non-targeted cells in the cell clones. Failure in reprogramming of differentiated somatic cells after SCNT is described, also leading to missing gene expression such as *Oct4* (Boiani et al. (2002).

In this thesis destabilized RFP has been used exhibiting a half-life of 100 min offering the possibility to monitor fast changes in expression and localisation of OCT4. But this supposed advantage could cause the problem that the existing amount of RFP is not enough to be detected by epifluorescence microscopy. This could eventually be overcome by multiple-copy integration and expression of the *OCT4-RFP* construct resulting in a detectable amount of RFP. The randomly integrated cell clone Of71 showed a reproducible red fluorescense signal in the nucleus of blastocysts probably due to integration of multiple copies, what has not been investigated.

Since the *OCT4* promoter is not active in differentiated cells like fibroblasts (Okamoto et al., 1990) cell clone Of71 has been checked for RFP fluorescence using an inverted epifluorescence microscope. No fluorescence signal was detected in cells from cell clone Of71 according to the endogenous situation.

3. Developmental competence of SCNT embryos and abnormal morphology

The blastocyst rate gives valuable information about efficiency of the experiment. In this study the blastocyst rates can not be compared, because oocyets from different ovaries cohorts were used for SCNT. In the newly published study from Lai et al. (2016) the blastocyst rate ranged from 10 up to 30% using porcine fetal fibroblast as donor cells for SCNT, indicating that the blastocyst rate in this study

lies in the normal range. To improve the blastocyst rate, the embryos were additionally treated in one experiment with Sciptaid, a histone deacetylase inhibitor, which was previously shown by Zhao et al. (2010) to support embryonic development.

A surprising finding was the high proportion of abnormally developed SCNT embryos generated with cell clone Of71 and the WT-cell line PKC0407. Many embryos showed an irregular cleavage pattern and contained a high amount of binucleated, fragmented, pyknotic or unevenly sized nuclei. SCNT is a complex procedure and therefore multiple reasons can cause abnormal embryonic development. Frequently discussed impacts are the source of the donor cell as well as aberrations of the normal chromosomal number of the used donor cell or inadequate reprogramming of the differentiated cell (Bureau et al., 2003; Daniels et al., 2001; Deshmukh et al., 2012; Enright et al., 2003; Li et al., 2014). Bureau et al. (2003) analyzed two different bovine donor cells as well as their resulting 1-4-cell stage and morula stage SCNT embryos for chromosomal aberrations. They concluded that the increased anomalies in the embryos reflect the higher number of chromosomal anomalies observed in their corresponding donor cell line (Bureau et al., 2003). In addition, Bureau et al. (2003) supposed that the SCNT process itself impacts correct chromosome segregation as well as their proper allocation during cell division. Since the karyotype analysis of single-cell clones is difficult, due to low available cell numbers and decreased growth rate, the morphology of the nuclei in the culture of clone Of71 as well as the corresponding WT cell line has been investigated. In both cell cultures pyknotic cell nuclei were partly presented and thus indicating cells that will undergo cell death. These cultures displayed partly cell nuclei with a main nucleus as well as a smaller nucleus, so called micronuclei. As a result of chromosomal damage, related to mutagenetic stress, a micronucleus can be formed, containing chromatin particles from abnormal mitosis (reviewed in Sabharwal et al., 2015). Therefore using abnormal nuclei as donor nuclei for SCNT could support abnormal embryonic development. In addition, correct epigenetic reprogramming, including chromatin remodeling and DNA methylation, is necessary for the dedifferentiation of the donor cell by the recipient oocyte and could result in faulty embryonic development. Abnormal bovine cloned morulae showed faulty methylation resembling rather methylation patterns of the differentiated donor cell

than those of the normal developed morulae (Dean et al., 2001). Deshmukh et al. (2012) recently demonstrated that porcine cloned embryos had abnormalities in chromatin remodeling beginning from the 2-cell stage compared to their *in vivo* counterparts.

Another interesting finding of this study was that only a few *OCT4-RFP* blastocysts showed a clearly distinguishable ICM and TE. This result is consistent with the previous study from Deshmukh et al. (2012) demonstrating that in early and late porcine blastocysts derived by SCNT the ICM and TE are poorly segregated and therefore the exact discrimination is almost impossible. Accordingly, porcine embryos generated via SCNT do not exactly develop as *in vivo* embryos and consequently SCNT is not the right method to get detailed insight in porcine ICM and TE segregation (Deshmukh et al., 2012).

4. Conclusions

In conclusion the specific nuclear localisation of the OCT4 protein as well as the *OCT4* promoter activation could be demonstrated in porcine embryos carrying an *OCT4-RFP* reporter system. The RFP fluorescence signal was detected in some nuclei beginning from the 8-cell stage and continued to be nuclear specific at the morula as well as the blastocyst stage. Furthermore, the investigation of the OCT4 protein localisation via anti-OCT4-antibody staining of blastocysts from various origin (SCNT, parthenotes) revealed the same nuclear specific OCT4 localisation as detected in the blastocysts carrying the *OCT4-RFP* fluorescent fusion construct. Thus, porcine embryos carrying an *OCT4-RFP* reporter construct expressing an OCT4-RFP fusion protein represent the endogenous situation and can be used as a tool for detailed investigation of *OCT4* gene expression.

However, an improvement of the tool and further investigation of the cell clones is reasonable since only one cell clone with random integration showed a RFP signal, which was however quite weak. The following points could be the reason for the weak signal:

i. The destabilized RFP variant seems to be not suitable for the detection of the fusion protein in knockin embryos, so a fluorescent protein with an enhanced brightness should be chosen. In this thesis the TagRFP with a

brightness of 148% compared to EGFP (Evrogen) was used. The tdTomato for example provides a brightness of 283% compared to EGFP (Clontech) and has already been proven to function as a fluorescent reporter protein in porcine embryos by Lai et al. (2016).

ii. The target locus in the knockin cell clones as well as the integration sites of the random cell clone Of71 should be checked for correct recombination or integration of the whole *OCT4-RFP* construct by sequencing.

VII. ZUSAMMENFASSUNG

Analyse der *OCT4* Expression von transgenen Schweineembryonen mit einem integrierten *OCT4-RFP* Reportergenkonstrukt

Der Transkriptionsfaktor OCT4 ist Teil des transkriptionellen Regulationssystems in der frühen Embryonalentwicklung und ist wesentlich an der ersten Zelldifferenzierung beteiligt. Weitere Untersuchungen der Regulation von Pluripotenz sind notwendig, nachdem noch nicht alle Fragen, insbesondere im Schwein, geklärt sind. Das *OCT4*-Gen, welches häufig als Hauptregulator zur Erhaltung der Pluripotenz bezeichnet wird, stellt demnach einen interessanten Kandidaten für detaillierte Analysen dar. Ein Reportergenkonstrukt bietet eine elegante Möglichkeit, die *OCT4*-Promoteraktivität und Proteinlokalisation gleichzeitig zu beobachten. Dafür wurden primäre Nierenzelllinien von vier verschiedenen Schweinen genetisch modifiziert, um ein *OCT4-RPF*-Fusionsprotein zu exprimieren. *RFP* wurde gezielt an das 3` Ende des *OCT4*-Locus eingefügt oder das *OCT4-RFP*-Konstrukt wurde zufällig im Genom integriert. Von insgesamt 247 analysierten Einzelklonen, wurden zwei mit einem gezielten Einbau des *OCT4-RFP*-Konstruktes am *OCT4*-Locus und zehn mit einer zufälligen Integration des *OCT4-RFP* Konstrukts für den somatischen Kerntransfer (SCNT) verwendet. Die *OCT4-RFP*-Blastozysten wurden am Tag 5 und Tag 6 nach der Aktivierung fluoreszenzmikroskopisch auf eine *OCT4*-Expression untersucht. Die Blastozysten des Zellklons Of71 mit einer zufälligen *OCT4-RFP*-Integration, zeigten ein kernspezifisches Fluoreszenzsignal. Alle anderen Embryonen, die von anderen Zellklonen abstammten, zeigten kein spezifisches Fluoreszenzsignal. SCNT-Embryonen im 8-Zell-, Morula und Blastozysten-Stadium des Zellklones Of71 mit einer zufälligen *OCT4-RFP*-Integration, wurden detailliert mit einem konfokalen Laser Scanning Mikroskop untersucht. Zusätzlich wurde die Lokalisation des RFP-Signals in *OCT4-RFP*-Blastozysten mit anti-OCT4-Antikörpergefärbten SCNT-Wildtyp sowie parthenogentischen Embryonen verglichen. Die *OCT4-RFP*-Embryonen zeigten in einigen Kernen von einzelnen 8-Zell-Embryonen ein spezifisches RFP Fluoreszenzsignal, welches sich weiterhin konstant in den Kernen der untersuchten Morula und Blastozysten darstellen lies. Zudem zeigte die OCT4-Proteinlokalisation mittels Antikörperfärbung von Blastozysten

verschiedenen Ursprungs (SCNT, Parthenoten) das gleiche kernspezifische Signal, wie die Blastozysten mit einer zufälligen *OCT4-RFP*-Integration. Ein überraschendes Ergebnis dieser Studie war, dass eine große Anzahl von abnormal entwickelten SCNT-Embryonen, sowohl vom Zellklon Of71 als auch von der Wildtyp-Zellline PKC0407, gefunden wurde. Viele Embryonen zeigten eine abnormale Teilung und einen hohen Anteil von zweikernigen, fragmentierten, pyknotischen und unregelmäßig geformten Kernen.

Zusammenfassend sind Schweineembryonen mit einem *OCT4-RFP*-Konstrukt, eine interessantes Model, um sowohl die OCT4-Proteinlokalisation, als auch die *OCT4*-Expression nachzuweisen.

VIII. SUMMARY

Analysis of *OCT4* expression in transgenic porcine embryos carrying an *OCT4-RFP* reporter construct

The transcription factor OCT4 is part of the transcriptional regulation system during early embryogenesis and critically involved in first cell lineage segregation events. Further investigations of the regulation of pluripotency are required, since not all questions are clarified, especially in the pig. The *OCT4* gene, which is often referred as the master regulator in maintaining pluripotency, is an interesting candidate for detailed analysis. A reporter gene constructs provides an elegant way to monitor the *OCT4* promoter activation and the OCT4 protein localisation in parallel. Therefore four different primary porcine kidney cell lines were genetically modified by knockin of *RFP* 3´ to the *OCT4* locus or random integration of *OCT4-RFP* for OCT4-RFP fusion protein expression. Out of the 247 analysed single-cell clones, two cell clones with a knockin of *OCT4-RFP* at the *OCT4* locus and ten cell clones with random integration were used for somatic cell nuclear transfer (SCNT). The developing *OCT4-RFP* blastocysts were investigated by epifluorescence microscopy for *OCT4* gene expression on day 5 and day 6 post activation. The blastocysts from the cell clone Of71 with random integration showed a reproducible nuclear red fluorescence, whereas none of the embryos originating from the other cell clones displayed specific fluorescence. Detailed analysis by confocal laser scanning microscopy (CLSM), using the cell clone Of71 carrying the *OCT4-RFP* at a random integration side were performed with SCNT embryos at 8-cell, morula and blastocyst stage. In addition the localisation of the RFP signal in *OCT4-RFP* blastocysts was compared with anti-OCT4-immunostained wild-type SCNT and parthenogenetic blastocysts. In the *OCT4-RFP* embryos the RFP fluorescence signal was detected in some nuclei of single 8-cell stage embryos and was continuously present in the nuclei of the analyzed morulae and blastocyst stage embryos. Furthermore, the investigation of the OCT4 protein localisation via anti-OCT4 antibody staining of blastocysts from various origins (SCNT, parthenotes) revealed the same nuclear specific OCT4 localisation as detected in the blastocysts carrying the *OCT4-RFP* fluorescent fusion construct. A surprising finding was the high amount of abnormally developed SCNT embryos with cell clone Of71 and the WT-cell line PKC0407.

Many embryos showed an irregular cleavage pattern and contained a high proportion of binucleated, fragmented, pyknotic or unevenly sized nuclei.

In summary, porcine embryos carrying an *OCT4-RFP* reporter are an interesting model for monitoring the localisation of the OCT4 protein as well as the *OCT4* expression.

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X. INDEX OF TABLES

XI. INDEX OF FIGURES

XII. ACKNOWLEDGMENT

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