

Aus dem Veterinärwissenschaftlichen Department
der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Arbeit angefertigt unter der Leitung von
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**GENETIC MODIFICATION OF PRIMARY CELLS
FOR THE GENERATION OF TRANSGENIC PIG MODELS
USING SOMATIC CELL NUCLEAR TRANSFER**

Inaugural-Dissertation
zur Erlangung der Würde eines Doktor rer. biol. vet.
der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

von
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aus Dresden

München 2012

Gedruckt mit Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. J. Braun
Referent: Univ.-Prof. Dr. E. Wolf
Korreferent: Priv.-Doz. Dr. Scholz

Tag der Promotion: 21. Juli 2012

Für Tobias

„Es kommt nicht darauf an,
mit dem Kopf durch die Wand zu rennen,
sondern mit den Augen die Tür zu finden.“
-Werner von Siemens-

During the preparation of this thesis the following paper has been published:

Klymiuk N, Mundhenk L, Kraehe K, Wuensch A, Plog S, Emrich D, Langenmayer MC, Stehr M, Holzinger A, Kröner C, **Richter A**, Kessler B, Kurome M, Eddicks M, Nagashima H, Heinritzi K, Gruber AD, Wolf E. 2011. Sequential targeting of CFTR by BAC vectors generates a novel pig model of cystic fibrosis. *Journal of molecular medicine*. 2012 May;90(5):597-608. Epub 2011 Dec 15.

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Abbreviations

BAC	bacterial artificial chromosomes
BS	blasticidine
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	bidestillata water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-MI	pronuclear DNA microinjection
DSB	double strand break
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
ES	embryonic stem cells
ET	embryo transfer
et al.	et alii (and others)
FCS	fetal calf serum
FISH	fluorescence <i>in situ</i> hybridization
G418	geneticin
GGTA1	alpha-1,3-galactosyltransferase
GFP	green fluorescent protein
hprt	hypoxanthine guanine phosphoribosyl transferase
HR	homologous recombination
ICM	inner cell mass
ICSI	intra cytoplasmatic sperm injection
iPS cells	induced pluripotent stem cells
LB	lysogeny broth
M	mole
mg	milligram
ml	milliliter

ABBREVIATIONS

MLV	Moloney leukemia virus
mM	millimolar
MSC	mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid
µg	microgram
µl	microliter
NHEJ	non-homologous end joining
nm	nanometer
PAC	P1 derived artificial chromosomes
PAEC	porcine aorta endothelial cells
PBS	phosphate buffered saline
PEF	porcine ear fibroblasts
Pen	penicillin
PFA	paraformaldehyde
PFF	porcine fetal fibroblasts
PKC	porcine kidney cells
Puro	puromycin
qPCR	quantitative polymerase chain reaction
rAAV	recombinant adeno-associated virus
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RV-GT	retroviral transgenesis
SCNT	somatic cell nuclear transfer
SDS	sodium dodecyl sulfate
SMGT	sperm mediated gene transfer
Strep	streptomycin
TALE(N)	transcription activator-like effector (chimeric nuclease)
wt	wild-type
YAC	yeast artificial chromosomes
ZFN	zinc-finger-nucleases
ZFP	zinc-finger-protein

1 INTRODUCTION

Over the last years the pig as model organism for biomedical research becomes more and more attractive due to similarities in anatomy, size, life span, physiology, metabolism and pathology with humans (reviewed in Aigner *et al.* 2010). The relatively fast maturation rate and large number of offspring in pigs can be considered appropriate and make them more suitable for research than other larger animals like cow, sheep or dog (reviewed in Lunney 2007). Furthermore, the requirements for organs for transplantation in human may be satisfied by pigs as organ donors (reviewed in Klymiuk *et al.* 2010).

The generation of transgenic pigs is achievable in different ways, including pronuclear microinjection of DNA, sperm-mediated gene transfer, retroviral transduction and genetic manipulation of cells in culture followed by somatic cell nuclear transfer (SCNT) (reviewed in Wolf *et al.* 2000 and Robl *et al.* 2007). Due to a lack of definitive embryonic stem cells in pig (reviewed in Vackova *et al.* 2007), SCNT is the method of choice for genetic engineering of pigs, especially for gene targeting. Depending on the question that is addressed to the transgenic pig model, donor cells are modified via additive gene transfer, where the modification is achieved by random integration of an expression vector into donor genome or by site-directed modification of a target locus via homologous recombination (Thomas *et al.* 1986).

The outcome of pig cloning is influenced by several parameters, such as oocyte quality and preparation, properties of donor cells, nuclear transfer protocol, embryo culture and recipient animal (Polejaeva *et al.* 2000; reviewed in Campbell *et al.* 2005). Not all parameters can be influenced properly, but donor cells with appropriate characteristics can be chosen. These are long lifespan, exhibition of a stable karyotype even after a long culture period, suitability for genetic modification, capability for *in vitro* and *in vivo* development after transfer into an enucleated oocyte and embryo transfer.

Up to now several different somatic porcine cells were used for SCNT, such as multipotent stem cells (Colleoni *et al.* 2005; Jin *et al.* 2007), fetal cells (Onishi *et al.* 2000; Kumar *et al.* 2007) and adult cells (Polejaeva *et al.* 2000; Petersen *et al.* 2008).

The genetic modification of donor cells for SCNT requires efficient transfer of DNA into cells and integration into the genome. There are different ways to transfect primary mammalian cells, for instance with chemical methods like calcium phosphate method (Graham and van der Eb 1973), lipofection (Felgner *et al.* 1987), nanofection (Orth *et al.* 2008) and with physical methods like microinjection (Liu *et al.* 1979), electroporation (Andreason and Evans 1988), nucleofection (Nakayama *et al.* 2007) as well as viral transduction (Follenzi *et al.* 2000). Since properties of primary cell types are very different, each cell type has to be characterized regarding to lifespan, stability of karyotype, proliferation capacity, transfectability and *in vitro* development competence of embryos after SCNT.

The aim of this thesis was the evaluation of primary cell cultures isolated from different tissues regarding their suitability for generation of transgenic pigs by SCNT. To follow this aim, several important characteristics of cultured porcine kidney cells (PKCs), fetal fibroblasts (PFFs) and ear fibroblasts (PEFs) had to be determined.

i) Morphology and growth potential

The cells were analyzed morphologically after isolation, during culture as well as after long periods in culture. The proliferation capacity was studied by creation of a growth curve and the growth properties of cells were analyzed by MTT assay using different coating types. In addition, stable metaphases were examined even after a long culture period. Moreover, the *in vitro* development competence of embryos after SCNT using the several donor cells were determined as well as the blastocyst rate/quality and nuclei number of obtained embryos after seven days.

ii) Determination of appropriate transfection method

The comparison of different transfection methods (lipofection, nanofection, conventional electroporation and nucleofection) was performed using PKCs in which transfection efficiency, fluorescence intensity, quality of cells and amount of dead cells, after transient transfection with a GFP expressing plasmid were determined.

iii) Comparison of SCNT experiments

Over the past four years a large number of transgenic animals was generated by SCNT at the Chair for Molecular Animals Breeding and Biotechnology. These results were compared regarding to the used donor cells and the obtained litter after embryo transfer (ET) experiments.

2 REVIEW OF THE LITERATURE

2.1 Pig models for biomedical research

Animal models are used for research of human diseases. For the selection of an animal model following points are important: aim of research, availability, costs, ease of handling as well as anatomical and physiological analogies to human. The most widely used animals in research, especially in biomedical research, are still rodents. The advantages of rodent models are low maintenance costs, high reproduction rate and well defined genetic background. In the case of mice there are a lot of transgenic and knockout animals available (reviewed in Aigner *et al.* 2010).

One disadvantage of the mouse model is the relatively short lifespan. Larger animal models allow extended observation periods, which are important for different diseases including cancer, diabetes or neurodegenerative diseases. Furthermore, larger animals are more suitable than mice for surgery, blood sampling, serial biopsies, whole-organ manipulation and a lot of different biomedical applications (Reynolds *et al.* 2009; Roberts *et al.* 2009).

Especially pigs have gained importance for translational biomedical research in recent years, because of their physiological and anatomical similarities with humans. They are closer with humans in size, life span, biochemistry and genetics than rodents or other domestic animals (reviewed in Aigner *et al.* 2010). The relatively fast maturation rate and the large number of offspring in the pig are beneficial and make them more suitable for research than other larger animals like cow, sheep or dog (reviewed in Lunney 2007 and Rogers *et al.* 2008a). Furthermore, pigs are omnivorous and their gastrointestinal morphology, digestive effectiveness and the energy metabolism are close to humans (reviewed in Miller and Ullrey 1987 and Aigner *et al.* 2010), so they are suitable for the investigation of obesity in human, metabolic syndrome and human nutrition (Spurlock and Gabler 2008). A great advantage of the pig as a biomedical model is the high DNA sequence homology and chromosome structure with humans (Wernersson *et al.* 2005; reviewed in Lunney 2007).

In addition to the general suitability of pig models for biomedical research, transgenic pigs provide great potential for human disease studies, e.g. for cystic fibrosis (CF) (Rogers *et al.* 2008b; Klymiuk *et al.* 2011a), diabetes (Umeyama *et al.* 2009; Renner *et al.* 2010), xenotransplantation (Lai *et al.* 2002; Hauschild *et al.* 2011), vaccine development for infectious agents (Mendicino *et al.* 2011), Alzheimer's disease (Kragh *et al.* 2009), breast cancer (Luo *et al.* 2011), cardiovascular disease (Hao *et al.* 2006) and atherosclerosis (Wei *et al.* 2012).

Transgenic mouse models often do not faithfully mimic the human phenotype, as seen in the gene-targeted mouse for cystic fibrosis (CF), which did not show the characteristic human pathology like abnormalities of lung, liver and other organs (reviewed in Grubb and Boucher 1999). In contrast, transgenic pig models show often similar disease progression as human patients (reviewed in Lunney 2007). Therefore, Rogers *et al.* (2008b) generated pigs either with a knock-out ($CFTR^{+/-}$) of the *cystic fibrosis transmembrane conductance regulator* ($CFTR$) gene or a knock-in ($CFTR^{\Delta F508}$). They also generated $CFTR^{-/-}$ pigs by breeding of the heterogeneous $CFTR$ knock-out animals. The $CFTR^{-/-}$ -targeted new born pigs showed several disease characteristics like new born humans with CF. These similarities of disease characteristics were further demonstrated by $CFTR^{-/-}$ pig model, which were generated by BAC-technology and homologous recombination (HR) (Klymiuk *et al.* 2011a).

Xenotransplantation is another important application of transgenic pigs; therefore organs are transplanted from other species into human, because the number of human organ donors is very limited. Normally, organ transplantation from pig to human results in hyperacute rejection which is a rapid and massive immune response against the key xenoantigen on pig cells galactosyl alpha(1-3) galactose (Platt *et al.* 1991; Cozzi *et al.* 2000). Thereby, the enzyme alpha-1,3-galactosyltransferase ($GGTA1$) plays a key role and Lai *et al.* (2002) generated the first $GGTA1$ knock-out pig. In addition to the knock-out of the $GGTA1$ gene, also tissue-specific expression of human complement regulatory proteins and other genetic factors are important for transplantation of pig tissue into humans (reviewed in Klymiuk *et al.* 2010).

2.2 Genetic engineering of pigs

The generation of transgenic pigs can be achieved in different ways. Random integration of a transgene can be obtained by pronuclear microinjection of DNA, sperm-mediated gene transfer (SMGT), viral transgenesis as well as somatic cell nuclear transfer (SCNT) using genetically modified donor cells. In addition, SCNT is suitable for generation of pigs containing a site-directed genomic modification after gene targeting in the donor cell (Fig. 1).

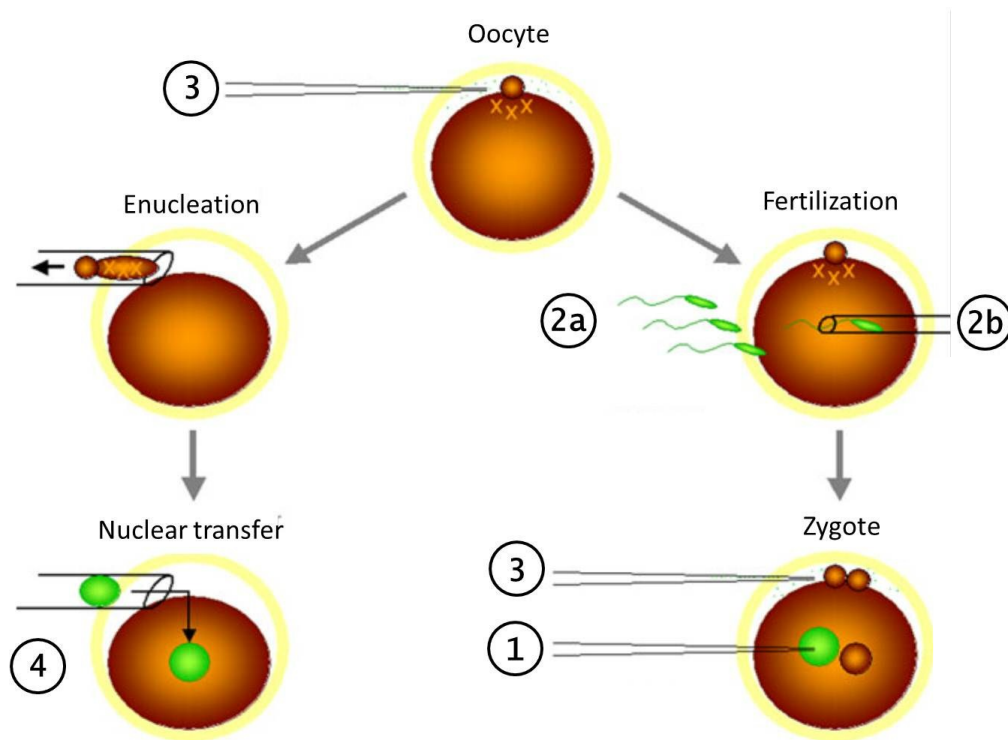


Figure 1: Different methods to generate transgenic pigs.

(1) DNA transfer via pronuclear microinjection; (2a) sperm mediated gene transfer (SMGT) and (2b) intracytoplasmic sperm injection (ICSI); (3) retroviral transgenesis can be applied by subzonal injection of viral particles into oocytes and zygotes, respectively; (4) somatic cell nuclear transfer (SCNT) of a genetically modified donor cell into enucleated oocyte. (adapted from Aigner *et al.* (2010))

2.2.1 Pronuclear DNA microinjection

Pronuclear DNA microinjection was the most widely used method to produce transgenic pigs for many years. The method has been used first successfully in mice and five years later the first transgenic pig was born after pronuclear microinjection

(Gordon *et al.* 1980; Brem *et al.* 1985; Hammer *et al.* 1985). For the implementation, purified DNA is direct injected mostly into male pronucleus of fertilized oocytes. The limitations of the method are the low efficiency and enormous cost. In mice, 1-3% of microinjected embryos become transgenic (reviewed in Houdebine 2005) and less than 1% of transgenic livestock were obtained per injected zygote (reviewed in Robl *et al.* 2007).

2.2.2 Sperm-mediated gene transfer

Another possibility to introduce foreign DNA into animal genomes is the sperm-mediated gene transfer (SMGT). Brackett *et al.* (1971) demonstrated first the transport of exogenous DNA into the oocyte via sperm cells. In pig a very efficient DNA transfer was achieved with up to 80% transgenic offspring (Lavitrano *et al.* 2002). Although, SMGT is simple and costs are low, there are some drawbacks. Robl *et al.* (2007) reviewed difficulties in reproducibility, which means lab and species-dependent variations in efficiency.

An alternative method to produce transgenic animals is the intracytoplasmic sperm injection (ICSI) mediated gene transfer which was established by Perry *et al.* (1999). They reported that incubated mouse spermatozoa with foreign DNA were microinjected into the cytoplasm of oocytes by ICSI. Kurome *et al.* (2006) demonstrated that ICSI-mediated gene transfer is an efficient and practical method to generate transgenic pigs.

2.2.3 Viral transgenesis

Retroviruses have an RNA genome, which is reverse transcribed into DNA using reverse transcriptase in host cells. Next, the DNA is incorporated into the host genome by integrase and is replicated and transcribed into mRNA. Currently, numerous transgenic animals were generated using retroviral vectors. For the creation of transgenic animals two types of retroviral vectors have been developed. One group is formed by vectors which derived from the genome of prototypic retroviruses, such as Moloney leukemia viruses (MLV). The other group are vectors which were deduced from the genome of more complex retroviruses, for example lentiviruses

(reviewed in Robl *et al.* 2007). The advantage of lentiviral vectors compared to the prototypic retroviruses is the active transport of the genome into the nucleus. Therefore, transgenesis of non-dividing cell types is possible (Follenzi *et al.* 2000). In farm animals 70% carried the lentiviral vector and 65% expressed the transgene in all tissues, including germ cells (Hofmann *et al.* 2003). The efficiency is relatively high but certainly there are several difficulties in the use of retroviruses for the creation of transgenic animals. The retroviral long-terminal-repeats (LTR) are often silencing the transgene or can interfere with mammalian promoters to inhibit or misdirect host gene expression (Jahner and Jaenisch 1985; Wells *et al.* 1999).

Additional commonly used viral vectors for gene targeting are recombinant adeno-associated viruses (rAAV), which are single-stranded DNA viruses. The *CFTR* gene was targeted in porcine fetal fibroblasts (PFFs) with efficiencies of 0.1 to 10.9% after viral transduction with rAAV (Rogers *et al.* 2008b).

Using viral vectors, disadvantages are the routine generation of high titer stocks for the generation of transgenic animals (al Yacoub *et al.* 2007) and the limited packing amount which depends on the kind of viral vector that can carry 4.5 kb - 10 kb of foreign material (Hendrie and Russell 2005; reviewed in Robl *et al.* 2007).

2.2.4 Somatic Cell Nuclear Transfer (SCNT)

Another very common method for the generation of transgenic animals is the somatic cell nuclear transfer (SCNT) with genetically modified donor cells. The process of SCNT begins with an unfertilized, haploid egg cell, of which the nucleus is extracted and replaced by a nucleus from a diploid donor cell (Wilmut *et al.* 1997). The donor cell genome can be modified by gene addition and site-directed modification before transfer into enucleated oocytes. Advantages of SCNT are the generation of non-chimeric animals that possess the genetic background of the donor nucleus. Further, it is possible to determine the sex and other characteristics of transgenic founder animals in advance and SCNT is the only method produce domestic knockout animals (Niemann and Kues 2000).

2.2.4.1 Overview

The first application of nuclear transfer in mammals was employed by McGrath and Solter (1984). They established the technique first in mice. Blastomeres were used as

nuclear donors not only in mice (McGrath and Solter 1984), but also for the production of lambs (Willadsen 1986), cattle (Robl *et al.* 1987) and pigs (Prather *et al.* 1989). The successful cloning of an entire mammal, namely “Dolly”, from a differentiated adult mammary epithelial cell was revolutionary (Wilmut *et al.* 1997). Later, a lot of different groups showed that cloning is also possible using fetal and adult somatic cells in various species, such as cattle (Cibelli *et al.* 1998; Zakhartchenko *et al.* 1999), mouse (Wakayama *et al.* 1998), goat (Baguisi *et al.* 1999), gaur (Lanza *et al.* 2000), pig (Polejaeva *et al.* 2000), mouflon (Loi *et al.* 2001), rabbit (Chesne *et al.* 2002), cat (Shin *et al.* 2002), mule (Woods *et al.* 2003), horse (Galli *et al.* 2003), rat (Zhou *et al.* 2003) and dog (Lee *et al.* 2005).

A widespread use of SCNT is the cloning of high quality farm animals, for instance in animal agriculture to improve milk production, wool quality or recreation of extinct species and conservation of endangered species (reviewed in Westhusin *et al.* 2001 and Vajta and Gjerris 2006). Moreover, the creation of genetically identical animals was suggested to be useful for the development of new vaccines (reviewed in Wolf *et al.* 2001).

Schnieke *et al.* (1997) generated the first transgenic livestock clones. They used transfected fetal fibroblasts for generation of two lambs expressing human factor IX. After this great success, a variety of transgenic animals were generated by SCNT, which can be used as bioreactors, for instance the production of medically relevant proteins (reviewed in Brink *et al.* 2000 and Houdebine 2000). Another important application is the site-directed mutagenesis to generate knock-out animals for xenotransplantation and to study human diseases (Lai *et al.* 2002; Klymiuk *et al.* 2011a).

2.2.4.2 Cloning efficiency

In general, the efficiency of SCNT is very low in mammals. Various groups calculated the cloning efficiency from the numbers of offspring per transferred embryos. The success rate between various mammals and within a species shows a wide range: pig: 0.2 to 7% (Polejaeva *et al.* 2000; Dai *et al.* 2002), cattle: 4 to 83% (Kato *et al.* 1998; Kishi *et al.* 2000), sheep: 3 to 18% (Wilmut *et al.* 1997; McCreath *et al.* 2000) and mouse: 0.5 to 3.7% (Wakayama and Yanagimachi 2001; Dai *et al.* 2002). The comparison of cloning efficiency between different animals, but also

within a species is difficult, because different donor cell types and various protocols for SCNT are used.

Furthermore, there is a low birth rate and cloned fetuses and newborns may show a variety of pathological abnormalities, for instance circulatory disturbances, malformation of kidney and brain, dysfunction of immune system as well as placenta edema and dysfunction (Hill *et al.* 1999; De Sousa *et al.* 2001; Rhind *et al.* 2003).

2.2.4.3 Factors influencing the efficiency of SCNT

SCNT consists of several steps that have an impact on the cloning success rate. On the one hand, the origin, quality and cultivation of oocytes are important points (Piedrahita *et al.* 2002). On the other hand, somatic cells are isolated from various donor animals, hence, they are differ in age (Kasinathan *et al.* 2001) and tissue origin (Kato *et al.* 2000; Wells *et al.* 2003). Other important factors are culture conditions (Zakhartchenko *et al.* 1999), cultivations time (Cho *et al.* 2004), passages (Liu *et al.* 2001) and state of differentiation of donor cells (Sung *et al.* 2006; Kumar *et al.* 2007). Moreover, cell cycle synchronization of donor cells and recipient oocytes (reviewed in Kues *et al.* 2000; Tomii *et al.* 2009) as well as epigenetic status of donor cells (Enright *et al.* 2003; reviewed in Yang *et al.* 2007) are a crucial part in successfully cloning of animals. During nuclear transfer process a lot of different factors play a role, including the method of enucleation (Vajta *et al.* 2001), activation and fusion (Galli *et al.* 2002) as well as activation and fusion time (Akagi *et al.* 2003).

Epigenetic modifications are responsible for specialization of cells during differentiation due to modifications of nucleotides and chromatin structures, but do not involve a change in the nucleotide sequence (reviewed in Surani 2001). Thereby, specific genes, like pluripotency genes are switched off and tissue-specific genes are up-regulated and need to be down regulated to achieve a totipotent state in the process called reprogramming (reviewed in Tian *et al.* 2003). The processes of reprogramming of epigenetic modifications is crucial for dedifferentiation of donor nuclei during SCNT, which affects DNA methylation, histone modification, telomere length, X chromosome inactivation as well as genome imprinting (reviewed in Shi *et al.* 2003). It is presumed that the low cloning efficiency is largely attributable to the incomplete and faulty reprogramming of epigenetic modifications, leading to abnormal patterns of DNA methylation (Bourc'his *et al.* 2001; Ohgane *et al.* 2001),

X-chromosome inactivation (Xue *et al.* 2002) and histone modifications (Enright *et al.* 2003). Furthermore, expression of imprinted and non-imprinted genes is disturbed (Bourc'his *et al.* 2001; Rideout *et al.* 2001; Dean *et al.* 2003).

There are several ways to influence epigenetic modifications. For instance Himaki *et al.* (2010) and Jeseta *et al.* (2008) demonstrated significantly higher blastocyst rate after treatment with histone deacetylase inhibitor Trichostatin A of *in vitro* cultivated porcine embryos and in SCNT embryos using PFFs as donor cells. Furthermore, in PFFs silenced transgenes were reactivated after treatment with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine and/or with Trichostatin A (Kong *et al.* 2011).

2.3 Suitable cell types for nuclear transfer in pigs

The choice of donor cells is very important for successful genetic modification and has a great influence on the efficiency of cloning by nuclear transfer. Until now, many different types of donor cells have been successfully applied as nuclear donors for nuclear transfer who can be classified into two groups: pluripotent stem cells and somatic cells, including multipotent stem cells and differentiated cells.

2.3.1 Pluripotent stem cells

Embryonic stem (ES) cells are pluripotent stem cells that are able to differentiate into cells of the three germ layers: endoderm, ectoderm and mesoderm. Though, they lack the potential to form trophoblasts and cannot develop into extraembryonic tissue, like totipotent stem cells which occur in the zygote up to 8-cell-stage of an embryo (reviewed in Leo and Grande 2006). Nuclear transplantation of inner cell mass (ICM) cells into enucleated zygote was carried out first in mice by Illmensee and Hoppe (1981). Later, donor cells of a 2-cell to 16-cell embryo were transferred into an enucleated metaphase II oocyte in sheep (Willadsen 1986), cattle (Prather *et al.* 1987), pig (Prather *et al.* 1989) and rabbit (Collas and Robl 1990).

Pluripotent definitive ES cells are isolated from morulae or inner cell mass of a blastocyst, which were first isolated in mice (Evans and Kaufman 1981; Martin 1981) and have been used successfully for cloning several times. Furthermore, it has been

demonstrated that in mice cloning efficiency using ES cells is 10- to 20-fold higher than with somatic donor cells, like fibroblasts and cumulus cells (Rideout *et al.* 2001). So far, ES cells have been isolated not only from mouse, but also from rat (Ueda *et al.* 2008), human (Thomson *et al.* 1998) and monkey (Thomson *et al.* 1995). Certainly, definitive ES cells have not yet been isolated from livestock species. Due to difficulties in pig ES cells cultivation, missing suitable feeder layers and quickly differentiation, it is necessary to look for other alternatives (reviewed in Vackova *et al.* 2007).

An alternative could be the application of induced pluripotent stem (iPS) cells. Takahashi and Yamanaka (2006) described the reprogramming of murine fibroblasts into iPS cells, which was achieved by retroviral transduction of the transgenes *c-myc*, *klf4*, *sox2* and *oct3/4*. Various laboratories generated porcine putative iPS cells and verified standard criteria for pluripotency, including the ability to differentiate along multiple tissue lineages and differentiate into teratomas composed of the three germ layers after injection into nude mice, but there was a lack in germ line competence of these cells (Esteban *et al.* 2009; Ezashi *et al.* 2009; Wu *et al.* 2009). A major disadvantage of iPS cells is their potential of tumor formation due to the reactivation of the *c-myc* transgene which was shown in mice (Okita *et al.* 2007; Miura *et al.* 2009). However, in the last years various methods were developed to avoid this negative effect of *c-myc* and *klf4* in mice and human (reviewed in Nowak-Imialek *et al.* 2011). In summary, it is difficult to predict, if iPS cells can replace embryonic stem cells and whether they are suitable for the generation of animals.

2.3.2 Somatic cells

Multipotent also called adult stem cells are found throughout the body and have the potential for self-renewal and generation of multiple but limited number of lineages (Hwang *et al.* 2005). They are very important in the repair system and maintenance of tissue homeostasis (reviewed in Caplan and Bruder 2001).

Several multipotent stem cell types were used in pigs for SCNT experiments, for instance skin stem cells (Zhu *et al.* 2004) as well as mesenchymal stem cells (MSCs) from bone marrow (Bosch *et al.* 2006) and peripheral blood (Faast *et al.* 2006).

Kato *et al.* (2004) and Colleoni *et al.* (2005) showed that bovine and porcine MSCs derived from bone marrow are suitable for genetic modifications and SCNT.

Several groups have investigated whether a less differentiated cell type can increase the efficiency of SCNT. They have shown in pig that MSCs derived from bone marrow as nuclear donor for SCNT resulted to twofold higher percentage of embryos that developed to the blastocyst stage than adult ear fibroblasts (Faast *et al.* 2006) and fetal fibroblasts (Jin *et al.* 2007; Kumar *et al.* 2007), respectively. In addition, Kumar *et al.* (2007) demonstrated in pig that MSCs derived embryos were more similar to *in vivo* embryos compared to fetal fibroblasts, regarding to expression of key embryonic genes like *OCT4/NANOG* as well as DNA methylation, histone deacetylation and additional gene expression patterns.

Usually, isolated MSCs are characterized after isolation and the multilineage differentiation potential should be shown by adipogenic, osteogenic and chondrogenic development and immunocytochemical analysis before usage (Bosch *et al.* 2006; Faast *et al.* 2006). It has to be emphasized that the culture and differentiation of these stem cells is costly due to required growth factors.

In recent years, cloning was very successful with differentiated somatic cells, although they have a limited lifespan (reviewed in Kuilman *et al.* 2010) and reprogramming after nuclear transfer is challenging. For expansion, transfection, selection and screening of genetically modified primary sheep fetal fibroblasts 45 population doublings are necessary (Clark *et al.* 2000). Therefore, not all somatic cell types are suitable for the generation of transgenic animals, because of their limited capacity for replication and consequent growth arrest, resulting in cellular senescence (Hayflick 1965). Leonard Hayflick and Paul Moorhead (1961) discovered that human diploid cell strains cannot divide indefinitely, but die after a certain number of divisions (approximately 60-80 cell divisions), which is known as the Hayflick limit.

In mammals, various laboratories achieved relatively high efficiencies in SCNT using donor cells from female reproductive system, for instance mammary gland (Wilmut *et al.* 1997), oviduct (Kato *et al.* 1998) and cumulus/granulosa cells (Wakayama *et al.* 1998; Wells *et al.* 1999). Also donor cells from male reproduction such as Sertoli cells were successfully used for SCNT (Ogura *et al.* 2000). In addition, cloned animals have been generated from kidney cells (Yin *et al.* 2002), mature B and T cells (Hochedlinger and Jaenisch 2002) as well as muscle cells (Green *et al.* 2007).

Widely used somatic cell types for cloning and generation of transgenic animals are fetal and adult fibroblasts of sheep (Schnieke *et al.* 1997), mouse (Wakayama and Yanagimachi 1999), bovine (Trounson *et al.* 1998; Kubota *et al.* 2000) and pig (Betthauser *et al.* 2000).

2.4 Genetic modifications

Depending on the question that is addressed to the transgenic pig model, donor cells are modified using additive gene transfer, where the modification is achieved by random integration of an expression vector into donor genome or by site-directed gene modification, also known as gene targeting (Thomas *et al.* 1986).

2.4.1 Additive gene transfer

The random insertion of a gene of interest into the genome is termed additive gene transfer. After transfer of a vector into a donor cell, a transient high level of gene expression can be observed (transient expression). This expression is limited due to dilution of the vector with each cell division and degradation processes.

A rare event is the integration of the vector into the genome of the donor cell (stable expression) (Yano *et al.* 1991). Stable transfected cells can be generated using a vector including a selection cassette. The addition of a gene into the genome is a random integration and consequently uncontrollable in both integration site and copy numbers of transgenes (Clark *et al.* 2000; reviewed in Robl *et al.* 2007). Multiple copies of vectors may integrate in the same location (concatemer) as well as at different loci. Moreover, a variation in expression levels may occur due to position effects. Positional effects are a result of chromatin status of neighboring DNA, which can be present in an open (for transcription factors accessible) or in a closed (not accessible) structure (Clark *et al.* 1994; reviewed in Wolf *et al.* 2000). This can also cause the variation of expression levels during development of an organism, in which high expression levels are occurred in embryogenesis, but repression of transgene in the adult organism. Further, the random integration of transgene may cause mosaicism, disruption of functional endogenous sequences and insertion mutagenesis with position effects (reviewed in Wolf *et al.* 2000 and Robl *et al.* 2007).

In the following, one possibility to generate transgenic pigs by additive gene transfer and SCNT is described and shown in figure 2. Cultured donor cells are transfected with a vector containing the gene of interest and a selectable expression vector. After selection stable transfected cell clones are pooled, used for SCNT, followed by transfer of the embryos to synchronized recipients. The resulting transgenic fetuses or offspring are individual founders with possible different expression levels due to pooling of the cell clones. Therefore, fetuses or tissues have to be investigated for integration and gene expression. After determination of the animal with the most suitable expression level of the transgene, cells of this animal are used for an additional cloning round to obtain offspring of animals with the same transgenic background.

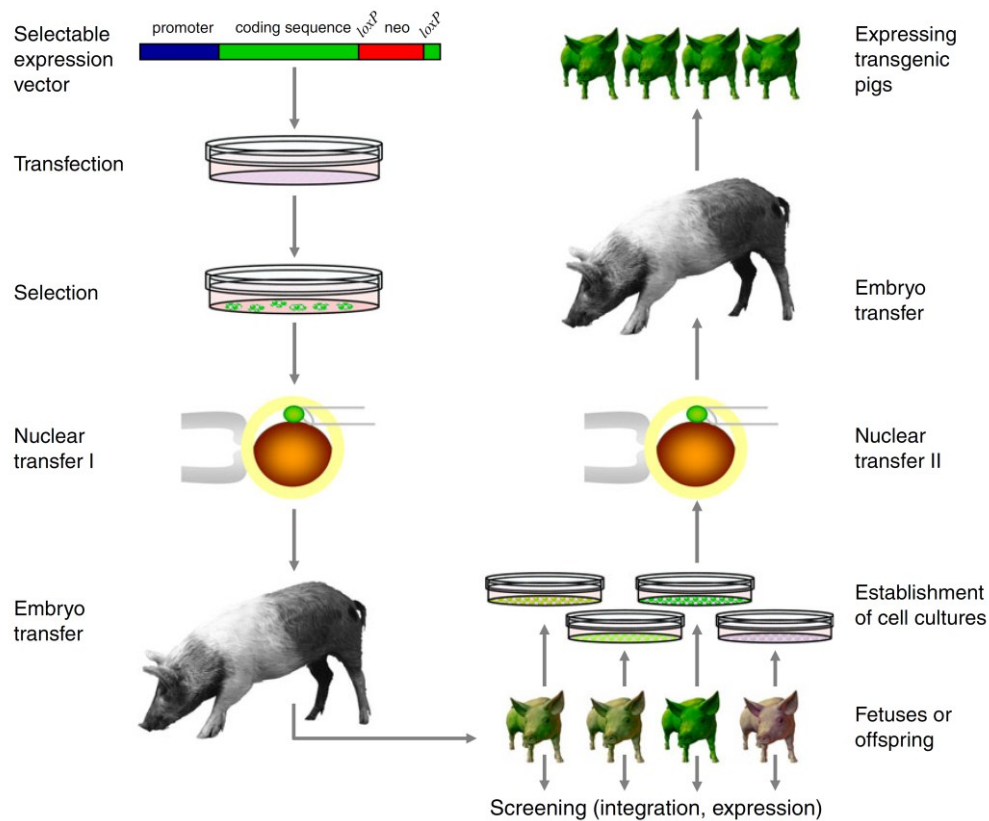


Figure 2: Generation of transgenic pigs by additive gene transfer and SCNT

The workflow for effective generation of transgenic pigs using an expression vector which was transfected and selected in donor cells is shown. These cells were used for SCNT and embryo transfer (ET). The received founder animals (fetuses or offsprings) were characterized and cells of the animal with suitable expression properties are used for recloning. (Aigner *et al.* 2010)

In addition to the widely used expression vectors for additive gene transfer, it is also possible to use mobile genetic elements, so-called transposons. Transposable elements are mobile DNA elements which were first studied by Barbara McClintock (1950). These elements can be applied as genetic vectors for genome manipulation and were successfully applied in various organisms such as drosophila (Thibault *et al.* 2004), mouse (Dupuy *et al.* 2002) and rat (Kitada *et al.* 2007). The advantages of transposon technology in comparison to conventional vectors are the efficient integration into the genome, no formation of concatemers and the possibility to integrate only one copy into the genome (reviewed in Henikoff 1998; Ivics *et al.* 2009). On the other hand, there is a wide variety of transposable elements and it is complex and time consuming to find the appropriate system and optimize them (Flutre *et al.* 2011).

2.4.2 Gene targeting

Gene targeting can be achieved in donor cells by site-directed modification which is feasible in different ways, for instance by homologous recombination (HR) with conventional targeting and BAC vectors as well as zinc-finger-nucleases (ZFN) and novel designer nucleases called (transcription activator-like effector nucleases - TALENs) or by combination of HR and designer nucleases technology.

2.4.2.1 HR using conventional vectors and BAC-technology

Gene targeting mediated by homologous recombination is a process in which a DNA molecule becomes introduced into a cell and the corresponding homologous chromosomal section is replaced by the introduced DNA molecule (reviewed in Porteus and Carroll 2005). This is a precise application to establish changes in the genome. In primary mammalian cells, gene targeting is limited by low efficiency, due to high selection effort and rare incidence of HR, while random integration occurs more frequently (Alwin *et al.* 2005). Gene targeting was first accomplished in mammalian cells at the human β -globin gene using a targeting plasmid (Smithies *et al.* 1985). Those targeting plasmids have been often used successfully in gene targeting experiments including in mice (Gordon *et al.* 1980), pig (Manzini *et al.* 2006) and cattle (Iqbal *et al.* 2009).

Large vectors with increased length of homologous arms between the DNA sequence of targeting vector and genome target locus were used to improve efficiency of HR in mouse embryo-derived stem cells (Deng and Capecchi 1992). BACs (bacterial artificial chromosomes), YACs (yeast artificial chromosomes) and PACs (P1 derived artificial chromosomes) are such large vectors which are able to maintain large DNA fragments and may be suitable for gene targeting by HR.

BACs have prevailed over the other systems, because they are single-copy plasmids, capable of maintaining 300 kbp and provide structural stability of maintained DNA, are convenient to handle and easy transfectable into cells (Shizuya *et al.* 1992; reviewed in Giraldo and Montoliu 2001). The BAC system was developed from *Escherichia coli* fertility-(F-) factor-based plasmid vectors (Hosoda *et al.* 1990).

Normally, BAC vectors were accomplished for the creation of genome libraries of an organism. Such libraries were used to sequence the genome of organisms, for example in the pig genome project (Humphray *et al.* 2007). Later it was shown that BAC vectors are suitable for various applications including additive gene transfer and gene targeting experiments. After addition of a BAC vector into the genome, the gene of interest can be expressed independently of integration site under its regulatory elements and usually keeps the native gene architecture including all cis-regulatory elements as well as exon-intron configurations (Chandler *et al.* 2007; Hofemeister *et al.* 2011).

The BAC-based site-directed mutagenesis was first successfully applied in murine ES cells (Testa *et al.* 2003; Valenzuela *et al.* 2003; Yang and Seed 2003) and seven years later also in human ES cells (Song *et al.* 2010). In 2011 the first knockout pigs using primary cells targeted with a BAC vector were generated (Klymiuk *et al.* 2011a). Certainly, genetic modifications were difficult to verify by common methods for instance PCR and Southern blot analysis, because of long homologous arms (Valenzuela *et al.* 2003). Correct integration site were verified using for instance FISH (chromosomal fluorescence *in situ* hybridization) (Yang and Seed 2003) and qPCR (quantitative PCR) (Klymiuk *et al.* 2011a).

2.4.2.2 Designed nucleases

Gene targeting by designed nucleases uses the endogenous homologous recombination machinery of the cell to repair double strand breaks (DSB). DSB can

lead to chromosomal changes or cell death and occur naturally in all cell types. The cells have many options to repair DSB. The two basic, conserved mechanisms from yeast to vertebrates are HR and non-homologous end joining (NHEJ) (reviewed in Sonoda *et al.* 2006). Normally, for repair HR uses of DSB the sister chromatid as a template for damaged DNA. NHEJ is an incomplete or faulty repair process, which frequently causes changes of DNA sequence in the region of the DSB such as deletions or insertions of nucleotides (Santiago *et al.* 2008). DSBs can also arise spontaneously by activity of reactive oxygen species (ROS) during normal metabolism, at random by ionizing radiation or specific by designed nucleases (reviewed in Wu *et al.* 2007).

In recent years, designed nucleases have become more and more popular in gene targeting next to BAC technology. These nucleases induce artificial DSB at the desired locus for site-directed mutagenesis in the genome. The repair mechanism of the cells rejoined the DSB by NHEJ, which often introduces insertions or deletions that create targeted gene knockouts. In the presence of a DNA template with homologous regions, the repair by HR could be increased and incorporation of the template into the genome could be achieved (Urnov *et al.* 2005).

One of the first artificial nucleases was the zinc-finger-nuclease (ZFN). ZFNs consist of a row of designed zinc-finger-proteins which recognize and bind to a specific DNA-sequence of <18 bp. The sequence-independent endonuclease FokI is coupled on the C-terminal site of the zinc-finger-protein (ZFP) and is able to cut the DNA (Durai *et al.* 2005; reviewed in Porteus and Carroll 2005). The development and production of appropriate ZFN is difficult and fault-prone, because sequence context and mutual influence is important (Mandell and Barbas 2006). ZFN which were not able to recognize the specific DNA target sequence, bind unspecific to the genome and generate undesired DSB. These random DSB are the cause of the cytotoxicity (reviewed in Wu *et al.* 2007). Nevertheless, site-directed mutagenesis mediated by ZFN was achieved in fruit flies (Bibikova *et al.* 2002), zebra fish (Doyon *et al.* 2008) and rats (Geurts *et al.* 2009). Moreover, this technology was applied successfully in pig (Whyte *et al.* 2011) and in addition a homozygote knockout of an endogenous gene (*GGTA1*) was succeeded (Hauschild *et al.* 2011).

Novel designer nucleases based on transcription activator-like effector (TALE) protein, which are produced by plant pathogens in the genus *Xanthomonas* and can bind effector specific DNA sequences (reviewed in Bogdanove *et al.* 2010).

Generation of site-specific DSBs were achieved by TALE chimeric nucleases (TALENs) in yeast, mammalian and plant cells (Mahfouz and Li 2011). The advantages of TALENs are their robust nuclease activity as well as low cytotoxicity (Christian *et al.* 2010).

2.4.3 Selection systems

Non-homologous recombination and random integration occurs more frequent than homologous recombination. Nevertheless, a suitable selection system is required for all methods to generate cells with stable transgene integration.

The desired selection cassette can be part of the vector containing the gene of interest or can be co-transfected using an additional vector. Positive selection is a frequent method for both additive gene transfer and gene targeting experiments. The aminoglycoside phosphotransferase gene is a common positive selection marker which confers resistance to antibiotics, for instance neomycin, kanamycin and geneticin also known as G418. Additional antibiotic resistance genes have been widely used routinely, like blasticidin, puromycin as well as hygromycin (reviewed in van der Weyden *et al.* 2002). The advantage of positive selection is the availability of various antibiotic resistance genes which can be combined in several vectors. Certainly, Pham *et al.* (1996) has evidenced that selection cassettes reduced the normal expression of multiple genes at distances greater than 100 kb from the insertion. Therefore, selection cassettes can be excised, if they are flanked by recognition sequence for site-specific recombinases such as Cre, FLPe as well as ϕ C31 (reviewed in Sorrell and Kolb 2005). Due to the possibility to remove the selection cassette, the generation of transgenic cell lines and animals without selection cassette is practicable and antibiotic resistances can be used several times and thus reach a further increase of variations.

Classical selection markers for a negative selection strategy are the herpes simplex virus thymidine kinase, diphtheria toxin A and hypoxanthine guanine phosphoribosyl transferase (hprt) (Szybalski 1992; Yagi *et al.* 1993). These genes avoid random integration of a targeting vector, because they are placed outside of one or both of the homologous sequence of the transgene. During HR the cassette is lost; otherwise the cell dies from the gene product of the negative selection cassette.

The positive negative selection (PNS) system is more effective, due the combination of an antibiotic resistance cassette in the targeted locus and a negative selection on the flank of homologous arm. In the first step, cells are selected based on the integration of the targeting vector due to positive selection and in the second step cells are selected based on the HR event and the resulting loss of the negative selection cassette. Mansour *et al.* (1988) showed a targeting efficiency of 79% with PNS in murine ES cells and 30% targeting efficiency were obtained in a rat fibroblast cell line (Hanson and Sedivy 1995). The Nobel Prize in Physiology or Medicine 2007 was awarded to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies for their discoveries of principles for gene targeting in mice including the PNS.

2.5 Non-viral gene transfer

The efficiency of additive gene transfer and gene targeting by HR with BAC technology or in combination with designed nucleases depends strongly on design of appropriate vectors, choice of cells and selection strategy as well as transfection method. The spontaneous entrance of DNA into cells is a very inefficient process. Therefore, two main categories of non-viral gene transfer techniques were developed to transfer DNA into the genome of primary mammalian cells, namely chemical and physical transfection.

2.5.1 Chemical transfection

The chemical transfection methods include calcium phosphate precipitation, lipofection (based on cationic lipids) and nanofection (combined polymers plus nanoparticle).

Calcium phosphate method is an old, easy and inexpensive transfection method discovered by Graham and van der Eb (1973). The principle is based on phagocytic absorption of a co-precipitate of DNA and calcium phosphate. Dudek *et al.* (2001) obtained transfection efficiency of 0.5% to 5% in primary neurons. A successful transfection requires a very high quality of plasmid DNA, optimization on temperature and pH (reviewed in Colosimo *et al.* 2000). A further improvement can

be achieved by treatment of cells with DMSO or glycerol (Kingston *et al.* 2001). Using a modified calcium phosphate transfection method, human chondrocytes were transfected with an efficiency of 80% (Qureshi *et al.* 2008).

During lipofection, cationic lipids forming liposomes, which spontaneously interact with negatively charged DNA and fuse with cell membrane or are absorbed by endocytosis into the cells (Felgner *et al.* 1987; Friend *et al.* 1996; Matsui *et al.* 1997). Felgner *et al.* (1987) postulated a 5- to >100-fold increased transfection efficiency of lipofection compared with calcium phosphate method depending on the cell line. Lipofection requires a thorough optimization for each given cell line, such as cationic lipid to DNA ratio, amount and size of DNA as well as incubation time to minimize cytotoxicity (Almofti *et al.* 2003; McLenachan *et al.* 2007). In general, the method is simple and easy to apply on a large scale (Yang and Huang 1997). A lot of different groups showed high transfection efficiencies in various cell types, for instance around 80% in HEK 293 cells, 30% in pig tracheal epithelial cells and 28% in pig fetal fibroblasts (Maurisse *et al.* 2010) as well as approximately 50% in mouse embryonic stem cells (McLanachan *et al.* 2007).

Nanofection is a chemical transfection method which was developed by PAA (Pasching, Austria). The transfection mechanism is based on a positively charged polymer which binds the DNA and is embedded into a porous nanoparticle. The nanoparticle protects the DNA against nucleases and degradation and further helps the DNA to deliver into the cells (PAA 2012). Using Nanofectin, Orth *et al.* (2008) obtained high transgene expression in human chondrosarcoma cell and primary cells from human fibrous dysplasia.

2.5.2 Physical transfection methods

Physical transfection methods include microinjection, electroporation, nucleofection and biolistic gene transfer.

The successful microinjection of human cells with solid glass capillaries was first shown by Diacumakos *et al.* (1970). With this method it was possible to verify mRNAs, before genes were known or cloned, for instance of interferon and thymidine kinase (Liu *et al.* 1979). Today, the mechanical transfer of DNA, mRNA, small interfering RNAs, proteins, peptides as well as drugs belongs to the wide

spectrum of molecules microinjected into single cells (reviewed in Zhang and Yu 2008a). The advantages are the high transfection efficiency, low cytotoxicity as well as precise dosage of injection material. Certainly, major drawbacks are the low number of injected cells, the high expenditure of time and laborious work (reviewed in Zhang and Yu 2008b).

Electroporation is based on the usage of an electrical impulse, which disturbs the phospholipid bilayer of the membrane and temporarily causes the formation of aqueous pores. DNA/RNA-molecules are delivered through the open membrane by diffusion into the cell. After the pulse, the cell membrane discharges, pores close and the phospholipid bilayer reassembles (Andreason and Evans 1988). For this technique, the pioneering work was done by Neumann *et al.* (1982) and Chu *et al.* (1987). For an efficient transfection many parameters must be adjusted including pulse duration and strength, capacity, DNA and cell amount, temperature and buffer solution (Baum *et al.* 1994).

A further advancement of electroporation is the nucleofection system which was developed by Amaxa Biosystem (Cologne, Germany). The optimization of nucleofection is easier compared to electroporation, because it combines preset electroporation programs with specific nucleofection solutions for the particular cell type (Maurisse *et al.* 2010). This technology is highly efficient for gene transfer into most primary cells, especially in slowly dividing or mitotically inactive cells as well as hard to transfect cell lines (Gresch *et al.* 2004). DNA/RNA is directly transported into the nucleus (Hamm *et al.* 2002). The usage of nucleofection system is easy, fast and safe, and the transfection efficiency is reproducible (Gartner *et al.* 2006). It was successfully applied to hematological and immunological cells (Lai *et al.* 2003; Martinet *et al.* 2003), primary neurons (Leclere *et al.* 2005), embryonic and adult stem cells (Lakshmipathy *et al.* 2004; Lorenz *et al.* 2004), fetal and adult primary mammalian cells (Nakayama *et al.* 2007; Skrzyszowska *et al.* 2008) as well as transformed mammalian cells and cell lines (Schakowski *et al.* 2004; Maurisse *et al.* 2010). One drawback of both electroporation and nucleofection is the need for detachment of adherent cells and the resultant stress (reviewed in Colosimo *et al.* 2000).

Biolistic gene transfer or gene gun is a particle-based transfection method, originally designed for plant cell transfection (reviewed in Klein *et al.* 1992). The DNA is linked to inert nanoparticles (commonly gold and tungsten, respectively) and is shot

directly into the nucleus of the cells. The advantages of the method are low level of cell damage, usage of small amount of DNA and target cells as well as potential application for *in vivo* transfection (Sanford *et al.* 1993). On the other hand, the method has some drawbacks, including high initial cost, poor tissue penetration and complex instrument settings such as air pressure, particle size, density as well as velocity and distance (Heiser 1994; reviewed in Colosimo *et al.* 2000). Anyway, biolistic gene transfer has been applied in a wide range of plant cells and of animal somatic tissues and cells, respectively (Gao *et al.* 2006; Zindler *et al.* 2008; Hochbaum *et al.* 2010).

3 CELLS, MATERIAL AND METHODS

3.1 Material

3.1.1 Apparatuses

Centrifuges

- Biofuge pico Heraeus, Osterode, Germany
- Rotanta 96 Hettich, Tuttlingen, Germany
- Centrifuge 5417R Eppendorf, Hamburg, Germany

Finnpipette[®] Multichannel pipette (300 µl) Thermo Fisher Scientific, USA

GFL 3031 shaker Hilab, Düsseldorf

Incubators OMEG, Schöngeising, Germany;
Mettler, Schwabach, Germany

Microscopes and Camera

- Leica DM IL Leica, Wetzlar, Germany
- Zeiss Axiovert 200 M fluorescence microscope Carl Zeiss, Oberkochen, Germany
- Axiocam HR Carl Zeiss, Oberkochen, Germany

E.coli pulser electroporation device Bio-Rad, Munich, Germany

Freezing containers YMC Co., Japan;
Thermo Fisher Scientific, USA

Microprocessor pH meter WTW, Weilheim, Germany

Neubauer counting chamber Assistant, Sondheim, Germany

Nucleofector[™] II Lonza, Basel, Switzerland

Pipettes Gilson Inc., USA,
Eppendorf, Hamburg, Germany

Gene Pulser II Bio-Rad, Munich, Germany

Spectrophotometer *Gene Quant* GE Healthcare, Munich, Germany

Steril benches Laminair[®] HB2448K, Heraeus, Osterode, Germany

HB2472

Sunrise[™] microplate reader Tecan GmbH, Salzburg, Austria

3.1.2 Chemicals

All chemicals were used in p.a. quality, if not stated otherwise.

Acetic acid (glacial) (HOAc)	Merck, Darmstadt, Germany
Agar-agar	Roth, Karlsruhe, Germany
β -Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich, Steinheim, Germany
DMSO (Dimethylsulfoxid)	Sigma-Aldrich, Steinheim, Germany
EDTA (Ethylenediaminetetraacetic acid)	Roth, Karlsruhe, Germany
Ethanol (EtOH)	Roth, Karlsruhe, Germany
Glucose	Sigma-Aldrich, Steinheim, Germany
HEPES	Sigma-Aldrich, Steinheim, Germany
Methanol	Roth, Karlsruhe, Germany
Mineral oil	Roth, Karlsruhe, Germany
Phenol red	Sigma-Aldrich, Steinheim, Germany
Potassium chloride (KCL)	Sigma-Aldrich, Steinheim, Germany
di-Potassium hydrogen phosphate (KH_2PO_4)	Roth, Karlsruhe, Germany Sigma-Aldrich, Steinheim, Germany
Sodium bicarbonate (NaHCO_3)	Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Roth, Karlsruhe, Germany
di-Sodium hydrogen phosphate (Na_2HPO_4)	Fluka, Neu-Ulm, Germany
di-Sodium hydrogen phosphate \times dihydrate ($\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$)	Sigma-Aldrich, Steinheim, Germany
mono-Sodium phosphate (NaH_2PO_4)	Sigma-Aldrich, Steinheim, Germany
Orcein	Roth, Karlsruhe, Germany
Tryptone/Peptone	Roth, Karlsruhe, Germany
Yeast extract	

3.1.3 Cell Culture

Collagenase II	Life Technologies, Karlsruhe, Germany
CollagenR	Serva, Heidelberg, Germany

DMEM (Dulbecco modified Eagle Medium) High glucose	Life Technologies, Karlsruhe, Germany
FCS (Fetal calf serum)	Life Technologies, Karlsruhe, Germany
Gelatine	Sigma-Aldrich, Steinheim, Germany
Hanks 'BSS (HBSS) with Ca ²⁺ /Mg ²⁺	PAA, Pasching, Austria
Hyaluronidase	Sigma-Aldrich, Steinheim, Germany
Karyomax (Colcemid)	Life Technologies, Karlsruhe, Germany
L-Glutamin (200 mM)	PAA, Pasching, Austria
L-Glutamin + Penicillin/Streptomycin (100x)	PAA, Pasching, Austria
Non-essential amino acids (100x)	Life Technologies, Karlsruhe, Germany
Sodium pyruvate	Life Technologies, Karlsruhe, Germany
Trypanblau	Sigma-Aldrich, Steinheim, Germany
Trypsin Difco™ 250	BD Falcon, Heidelberg, Germany
Vectashield (DAPI) Mounting Medium	Vector Laboratories, Burlingame, USA

3.1.4 Consumption and other working material

Cryotubes (1.0 ml, 2.0 ml)	PAA, Pasching, Austria Nunc, Wiesbaden, Germany
Cell culture dishes (10 cm, 6 cm, 3.5 cm)	Sarstedt, Nümbrecht, Germany
Cell Strainer 100 µm	BD Falcon, Heidelberg, Germany
Centrifuge Tubes (15 ml, 50 ml)	BD Falcon, Heidelberg, Germany
Glass pipettes	Hirschmann, Eberstadt, Germany
Multi-well-plates	
<ul style="list-style-type: none"> • 6-well, 12-well, 96-well F-bottom • 24-well, 48-well 	Greiner bio-one, USA Nunc, Wiesbaden, Germany
Parafilm®M	American Can Company, USA
Pipette tips	Eppendorf, Hamburg, Germany
Pipette tips with filter	Axygen Inc., USA
Reaction tubes (1.5 ml, 2.0 ml)	Eppendorf, Hamburg, Germany
Serological pipettes Cellstar®	Greiner bio-one, USA
SS35 50 ml centrifuge tubes	Eppendorf, Hamburg, Germany

Sterile filter

- Steritop GP 0.22 μm Express[®] plus membrane Millipore, USA
- Sterivex GP 0.22 μm Millipore, USA

3.1.5 Kits

Amaya [™] Basic Nucleofector [™] Kit for Primary Mammalian Fibroblasts	Lonza, Basel, Switzerland
Electroporation Buffer + Cuvettes (0.4 cm)	Bio-Rad, Munich, Germany
E.Z.N.A [™] Endo-free Plasmid Maxi Kit	Omega, USA
Fermentas Midi Prep DNA	Thermo Fisher Scientific, USA
Nanofection Transfection Kit	PAA, Pasching, Austria
Lipofectamine LTX, Plus SAM	Life Technologies, Karlsruhe, Germany
MTT-Cell Proliferation Kit I	Roche Diagnostics, Basel, Switzerland

3.1.6 Antibiotics and antimycotics

Amphotericin B	PAA, Pasching, Austria
Blasticidin S	PAA, Pasching, Austria
Geneticin (G418)	Life Technologies, Karlsruhe, Germany
Kanamycin	Roth, Karlsruhe, Germany
Penicillin/Streptomycin	PAA, Pasching, Austria

3.1.7 Culture media and supplements for cell culture

Millipore machine deionized water called ddH₂O was used as solvent, if not stated otherwise. Culture media, supplements including FCS for cell culture were filter-sterilized (0.22 μm pore size) before use and stored at 4°C.

Culture media for PKC, PEF and PFF

DMEM with

FCS	10 % or 15 % (v/v)
non-essential amino acids (100 x)	1 % (v/v)
L-Glutamine (200 mM) with penicillin/streptomycin (100 x)	1 % (v/v)
sodium pyruvate	1 % (v/v)
β -Mercaptoethanol	0.1 mM

Cryopreservation medium

FCS	90 % (v/v)
DMEM	10 % (v/v)

EGTA buffer

NaCl	28 mM
KCl	1 mM
NaH ₂ PO ₄	0.14 mM
Na ₂ HPO ₄ 2H ₂ O	0.12 mM
HEPES	2 mM
glucose	1 mM
NaHCO ₃	0.8 mM
phenol red	40 μ l

Chemicals were solved in ddH₂O and NaHCO₃ was added at the end. The pH of 7.2 was adjusted with 5 M NaOH. The buffer was stored at 4°C for maximum three months.

FCS (fetal calf serum)

Before use, FCS was heat inactivated. Therefore, FCS was incubated for 30 min at 56°C, sterile filtered, aliquoted and stored at -20°C.

LB medium

yeast extract	5 g
trypton/pepton	10 g
NaCl	2.5 g
Ad 1000 ml ddH ₂ O	
pH7.0 (adjust with 5 M NaOH)	

LB medium was autoclaved and stored at RT.

LB-agar plates

yeast extract	5 g
trypton/pepton	10 g
NaCl	5 g
ad 1000 ml ddH ₂ O.	

pH 7.0 (adjust with 5 M NaOH)

agar-agar	15 g
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LB-agar medium was autoclaved and cooled down to 60°C. 1 ml of kanamycin (25 mg/ml) was added to LB-agar medium. Medium was poured into culture plates, allowed to solidify and subsequently stored at 4°C.

PBS (Phosphate buffered saline, pH 7.2-7.4) w/o Ca²⁺ and Mg²⁺

NaCl	136 mM
Na ₂ HPO ₄	8.1 mM
KCl	2.7 mM
KH ₂ PO ₄	1.5 mM

After sterile filtration PBS was stored at RT or 4°C.

Starvation medium

DMEM with	
FCS	0.5 % (v/v)
non-essential amino acids (100 x)	1 % (v/v)
sodium pyruvate (100 x)	1 % (v/v)
L-Glutamine (200 mM)	1 % (v/v)

Stop medium

DMEM	90 % (v/v)
FCS	10 % (v/v)

Trypsin/EDTA

PBS w/o Ca ²⁺ and Mg ²⁺ with	
Trypsin	0.5 % (w/v)
EDTA	0.04 % (w/v)

After sterile filtration, aliquots were stored at -20°C. 0.4 % Trypsin/EDTA solution was used for PKC and 0.1 % for PEF and PFF cell cultures.

3.1.8 Plasmid

pmaxGFPTM Lonza, Basel, Switzerland

3.1.9 Bacterial strain

TOP10 Life Technologies, Karlsruhe, Germany

3.1.10 Software

Axio Vision 4.2 Zeiss, Oberkochen, Germany

WCIF ImageJ 1.34s National Institutes of Health
(<http://rsb.info.nih.gov/ij/>), Bethesda,
USA

Magellan Software Tecan Austria GmbH, Salzburg, Austria

3.2 Methods

3.2.1 Cell Culture

The cell culture work was performed under the laminar flow hood. The cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere. The media were warmed before use in a water bath at 37°C.

In general, PKCs were cultured in medium containing 10% FCS, except after transfection and selection when cells were cultured in 15% FCS. PFFs and PEFs were always cultured in 15% FCS culture medium.

All cells were cultured onto collagen coated plastic dishes. The collagen was solved 1:10 with ddH₂O and the culture dishes were incubated over night at RT.

3.2.1.1 Isolation of primary cells (PKCm, PKC2109, PEF0110 and PFF26)

The wild-type cells were isolated from different pigs (Tab.1). The isolation of PKCm and PFF26 was carried out by Dr. Annegret Wünsch.

Table 1: Wild-type cells isolated from different pigs

Name	Cell type	Breed	Age
Kidney m	PKC	German Landrace	~3 month
Kidney 2109	PKC	German Landrace	~3.5 month
PEF 0110	PEF	German Landrace	a few days
PFF 26	PFF	Swabian-Hall	abort: day 27

PKC=Porcine kidney cells, PEF=Porcine ear fibroblasts, PFF=Porcine fetal fibroblasts

Different centrifugation settings were used; PKCm, PKC2109: 5 min at 180×g; PEF0110: 10 min at 180×g and PFF26: 5 min at 140×g.

The protocols for isolation of PKC and PEF were partially the same. Tissue pieces (kidney 2×1×1 cm, ear 0.5×0.5 cm) were stored in washing buffer (PBS with 1× Pen/Strep and 1× Amphotericin B) in refrigerator or on ice. Tissue was washed twice in washing buffer, minced and suspension was washed with DMEM until the supernatant became clear. Subsequently, the protocols are distinguished between the different cell types.

The pelleted PKCm tissue was resuspended in 15 ml 0.1% (w/v) collagenase solution in HBSS and incubated at 37 °C for 2 h, while shaking once every 15-29 min. After digestion, flask was filled up to 50 ml with DMEM. Tissue pieces were allowed to settle down and supernatant was collected. The remaining pieces of tissue were digested again with 0.5% trypsin/0.04% EDTA solution in PBS for 20 min at 37°C. Afterwards, digested tissue pieces and collected supernatant were combined and filled up to 50 ml with DMEM.

In case of PKC2109 and PEF0110 pelleted tissue pieces were resuspended in 15 ml 0.1% (w/v) collagenase solution in HBSS and incubated at 37 °C while stirring. The kidney was digested 1 to 1.5 h and the ear 2 h. After incubation, flasks were filled up to 50 ml with DMEM.

Afterwards, PKCm, PKC2109 and PEF0110 cell suspensions were filtered through a 100 µm mesh and washed with DMEM until the supernatant became clear. Depending on pellet size, the amount on seeded cells varied after centrifugation: 1/6 to 1/24 of resuspended PKC were seeded per 100 mm petri dish and all resuspended PEF were seeded onto 6-well to 60 mm petri dish.

For the isolation of PPF26, the backbone of a 27 days old fetus was prepared by removing head, legs and internal organs. It was washed 3 times in PBS containing 1× Pen/Strep, minced and washed twice in DMEM. Tissue was resuspended in EGTA buffer and incubated rotating in front of infrared lamp at 37°C for 33 min. Afterwards, tissue pieces were centrifuged, resuspended in a collagenase (1 mg/ml) and hyaluronidase (1 mg/ml) DMEM solution and incubated for 20 min at 37°C. After centrifugation, tissue pieces were incubated in 1 mg/ml dispase DMEM solution and digested for 40 min at 37°C. Subsequently, tissue pieces were centrifuged, resuspended in DMEM, filtered through a tea strainer and washed twice in DMEM. After a last centrifugation step, cell pellet was resuspended in culture medium and seeded onto a 100 mm petri dish.

3.2.1.2 Subculture of cells

An overview about used culture vessels and amounts of used growth and stop medium/PBS, trypsin/EDTA and coating solution is summarized in table 2.

Table 2: Overview of used culture vessels

Description	Area [cm ²]	Growth/Stop medium/PBS [ml]	Trypsin/EDTA [μl]	Coating solution [μl]
100 mm	57	10	1500	1500
60 mm	21.5	5	750	750
6-well	9.6	2	300	400
12-well	3.9	1.5	200	250
24-well	1.9	1	150	200
48-well	1.1	0.4	80	100
96-well	0.33	0.1	30	40

At a confluence between 80-100% cells were passaged to expand the cell population. Therefore, medium was removed and cells were rinsed two times with PBS. Following, cells were incubated with trypsin-EDTA solution at 37°C for 5-7 min. Then, cells were collected with stop medium, transferred into a 15 ml tube and centrifuged at 180×g for 5 min. Finally, supernatant was removed, cells were resuspended in culture medium and a part of the cells were sowed onto new culture dishes. The split ratio of the cells was between 1:2 and 1:4 depending on confluence and growth potential of the cells.

3.2.1.3 Counting of cells

Cells were seeded with a defined cell number of $1.2-1.6 \times 10^3$ cells/cm² depending on cell type and constitution of cells onto culture dishes. Cells were counted in a Neubauer counting chamber after trypsinization.

3.2.1.4 Cryopreservation of cells

Confluent cells were frozen in liquid nitrogen at -176°C for long term storage. For cryopreservation of cells the same procedure as above (see 1.3.1.2) was carried out. The difference is that after centrifugation, cell pellet was resuspended in cryopreservation medium. Normally, up to 1×10^7 cells per ml were cryopreserved. The cells were aliquot in cryo vials, transferred in freezing container, which cools down the samples at a rate of -1°C per minute, and kept at -80°C overnight. After 24 h cryo vials were transferred to liquid nitrogen.

Cell clones prepared during gene targeting experiments (see 4.2.2.) were cryopreserved as follows: cells were washed twice with PBS, trypsinized and the reaction was stopped using 170 µl of cryopreservation medium. The cell suspensions was transferred to 1.5 ml reaction tube and put into freezing container in -80°C overnight.

3.2.1.5 Thawing of cells

Cryopreserved cells were taken out of liquid nitrogen and put for 1 to 2 min at 37°C. Cells were transferred into 15 ml tube containing stop medium and pelleted (180×g, 5 min). After removing the supernatant, cells were resuspended in an appropriate volume of culture medium and were seeded onto culture dishes.

3.2.1.6 Chromosome preparation

Cells with 60-90% confluence were arrested in metaphase by adding final concentration of 10 µg/ml colcemide and followed by incubation of 1 h at 37°C. Subsequently, cells were trypsinized and centrifuged (8 min, 180×g), medium was removed but 0.5 - 1 ml were left and cell pellet was resuspended by strong tapping. Then, 13 ml of pre warmed KCL (37°C) was added slowly to the cell suspension, gently mixed by inverting, incubated for 15 min at 37°C and were centrifuged (8 min

at 180×g). Ice-cold, freshly prepared fixative (75% methanol, 25% glacial acetic acid) was added slowly with constant gentle shaking of the tube and incubated for at least 2 h at -20°C. Following, cells were centrifuged for 10 min at 180×g, supernatant was removed until 1 ml left, and cells were resuspended and transferred to a 2.0 ml reaction tube. The suspension was washed four to five times with fixative (4°C, 4 min and 400×g) and chilled at -20°C. Slides were cleaned with ddH₂O as well as 70% ethanol and were pre warmed to 54°C. 17 µl of suspension were dropped to the slides from a height of one meter and slides were dried.

For analysis, cells were embedded by adding 2-3 drops of Vectashield with DAPI, the coverslip was sealed with nail polish and the samples were analyzed by microscopy or stored at 4°C. An inverted epifluorescence microscope (Axiovert 200M) equipped with filter sets for DAPI (#01, excitation: band-pass filter 365/12 nm, emission: long-pass filter 397 nm) was used for microscopically analysis.

PKCm (P3) and PFF26 (P5) were prepared and analyzed by Dr. Annegret Wünsch, Tanja Jäger and Pauline Fezert.

3.2.1.7 MTT-based cell proliferation assay

The MTT-Cell Proliferation Kit I determines the metabolic activity of living cells. The assay bases on color reaction to measure the viable cell number which was established by Mosmann (1983). Only in living cells, the yellow tetrazole (MTT-(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) becomes reduced in active mitochondria to purple formazan. For this, different cell numbers of PKCm and PKC2109, PFF26 and PEF0110 were cultivated in duplicates onto 96-wells with 100 µl medium per well for 48 h without coating, with collagen and gelatine coating, respectively. For collagen coating see section 3.2.1 and for gelatin coating, a 0.1% gelatin solution was used and the culture plates were incubated for 2 h at RT. A standard curve was used in each experiment for every single cell culture seven measuring points were applied: 250-50000 cells per well (PKC2109) and 2500-50000 cells per well (PKCm, PFF26 and PEF0110). For this, cells were seeded in duplicates onto the same plate 4 h before MTT treatment. Then, 10 µl of MTT (0.5 mg/ml) of the MTT-Cell Proliferation Kit I were added to each well and plates were incubated in the cell incubator for 4 h. Following, 100 µl of the solubilisation solution was added to each well and was incubated in the incubator overnight. The formation of

formazan crystals was measured by spectrophotometrical absorbance at a wavelength of 562 nm using SunriseTM microplate reader. Analysis of data was processed with the Magellan Software.

3.2.1.8 Growth curve and population doubling time

5.5×10^4 cells of PKCm, PFF26 and PEF0110 cell culture were plated onto 12-well plates and cultured under standard conditions. Over five days, three wells of each culture were trypsinized and counted every 12 h and the average was used. Population doubling was calculated in the section of the exponential growth by the following formula:
$$\frac{\log(\text{counted cell number}) - \log(\text{starting cell number})}{\log(2)}$$

3.2.1.9 Transfection of cells

At a confluence between 60-90% cells were transfected in P4. The transfection efficiency of each method was analyzed 24 h after transfection if not stated otherwise.

3.2.1.9.1 *Chemical transfection*

For chemical transfections, 3.2×10^4 of PKC2109 cells were seeded onto each 24-well the day before. The cells were transfected with the plasmid pmaxGFPTM to determine the transfection efficiency. In some experiments an additional analysis was performed after 48 h.

Nanofection

The Nanofectin Kit was used according to manufacturer's instructions. For this, 0.5 or 1.0 μg DNA was added each to 1.2, 2, 3.2 or 4 μl , and 1.5 μg DNA was added to 2 or 3.2 μl of Nanofectin solution.

Lipofection

The Lipofectamin LTX + Plus Reagents Kit was used according to manufacturer's instructions. 0.25, 0.5 or 0.75 μg of DNA was diluted in DMEM (5, 10 or 15 ng/ μl DNA) and mixed thoroughly. The optimized volume of the Plus reagent (0.25, 0.5, 0.75 or 1 μl) was added to the diluted DNA. Lipofectamin LTX was added in different ratios (1:1 – 1:4) to the diluted DNA/Plus solution.

3.2.1.9.2 *Physical transfection*

For physical transfection, cells were transfected either with pmaxGFPTM or endotoxin-free purified pmaxGFPTM. After transfection, cells were seeded onto 35 mm or 60 mm petri dishes (depending on cell number) containing pre-warmed culture medium.

Electroporation

For electroporation, PKC2109 cells were washed twice with PBS, trypsinized and counted. Then, 0.5 or 1×10⁶ cells were centrifuged for 5 min at 180×g. The pellet was washed with PBS and resuspended in 600 µl of either Gene PulserTM electroporation buffer, PBS or DMEM according to electroprotocols of BIO-RAD (2011) and electroporation buffer overview (BIO-RAD 2011). DNA (1, 5, 10 or 20 µg) was added to the cell suspension and transferred into a 4-mm gap electroporation cuvette. Cells were electroporated with Gene Pulser II using various settings (Voltage: 100 V, 230 V; High Capacity 500 µF; RT or chilled cell suspension).

Nucleofection

For nucleofection of PKCm, PKC2109, PFF26 and PEF0110 cell cultures the AmaxaTM Basic NucleofectorTM Kit Primary Fibroblasts and the Nucleofector II[®] device was used. 0.5 or 1×10⁶ cells were mixed with 1, 2, 5, 10 or 20 µg of plasmid DNA and 100 µl nucleofection solution and then nucleofected according to manufacturer's instructions using the recommended nucleofector programs A-23, T-16, U-12, U-23 and V-13 for fibroblasts.

Detection of transfection efficiency

24 h and 48 h after transfection, respectively, cells were washed twice in PBS, followed by fixation with 4% (m/v) PFA for 20 min at RT in the dark. Fixed cells were washed with PBS and covered with PBS for storage at 4°C or used for DAPI-staining. For this, cells were incubated with DAPI-Methanol (1 µg/ml) for 10 min at 37°C. Subsequently, cells were washed with methanol and PBS. Finally, the fixed cells were covered with PBS and analyzed by microscopy or stored at 4°C.

An inverted epifluorescence microscope (Axiovert 200M) equipped with filter sets for DAPI (#01, excitation: band-pass filter 365/12 nm, emission: long-pass filter

397 nm) and GFP (#13, excitation: band-pass filter 470/20 nm, emission: band-pass filter 505-530 nm) was used for microscopically analysis.

3.2.1.10 Detection of appropriate antibiotic concentration for selection

Selection procedure starts to eliminate cells without integrated construct after transfection and prevent cells toxic concentrations. To detect the appropriate antibiotic concentration, an antibiotic dilution series was tested for each given cell culture.

2×10^5 cells were seeded onto 6-wells with 15% culture medium. Antibiotics at various concentrations were added 24 h later. The selection medium was changed every other day. The optimal concentration for further experiments was those, in which all cells died within seven days.

3.2.1.11 Somatic cell nuclear transfer and embryo transfer

Cells were thawed, cultured and medium was replaced by starvation medium 48 h prior to SCNT. SCNT was performed by Dr. Mayuko Kurome, Dr. Barbara Kessler, Dr. Valeri Zakhartchenko and Tuna Güngör as described by Kurome *et al.* (2006) and Klymiuk *et al.* (2011b). The generated embryos were transferred into estrus synchronized gilts (ET) by Dr. Barbara Kessler as published (Besenfelder *et al.* 1997). Gilts were controlled regularly after ET by ultrasonic examination for conception and monitoring of pregnancy.

The development competence of cloned embryos after SCNT was analyzed using various cell types. After electric fusion and activation of oocytes and primary cells, the embryos were cultured *in vitro* seven days up to blastocyst stage. The embryos were fixed with acetic acid/methanol (1:3) and stained the nuclei of embryos with 1% orcein. The embryos were mounted onto glass slides and counted using an inverted microscope.

3.2.1.12 Statistical analyzes

χ^2 -test was used to calculated the rate of embryo development. The mean cell number of the embryos was compared using Student's *t*-test.

3.2.2 Molecular biology methods

The commercial plasmid pmaxGFPTM was amplified and purified to obtain higher plasmid concentrations and to compare the efficiency of endotoxin-free prepared plasmids with commercial generated plasmids.

3.2.2.1 Heat shock transformation

The plasmid pmaxGFPTM was transformed via heat shock into the competent *E.coli* strain TOP10. The *E.coli* cells were thawed on ice, 2 µl of 0.5 µg/µl pmaxGFPTM plasmid (diluted 1:75 with H₂O) was added and mixed carefully. Afterwards, cell suspension was incubated 20 min on ice, heated at 42°C for 45 sec and placed on ice for another 2 min. 1 ml LB medium was added to the heat shocked cells and incubated for 45 min at 37°C. Subsequently, cells were centrifuged for 5 min at 2300×g, pellet was resuspended in 200 µl of the supernatant and plated on LB agar plates in two different volumes (1/4 and 3/4) containing 25 µg/ml kanamycin. After drying, the agar plates were incubated at 37°C overnight and later stored at 4°C until endotoxin free isolation (see 3.2.2.2)

3.2.2.2 Endotoxin free isolation of pmaxGFPTM DNA

For preparation of large scales of pmaxGFPTM plasmid, a preculture (3 ml LB medium containing 25 µg/ml kanamycin) was inoculated with one picked colony and incubated while shaking at 37°C for 6 h. After incubation, the whole preculture was transferred into 150 ml LB medium (containing 25 µg/ml kanamycin) and incubated while shaking at 37°C overnight.

The pmaxGFPTM plasmid was isolated endotoxin free using the E.Z.N.ATM Endo-free Plasmid Maxi Kit according the manufacturer's protocol.

4 RESULTS

4.1 Evaluation of suitable cell sources for SCNT to generate transgenic pigs

For generation of transgenic pigs via SCNT an appropriate donor cell type is necessary which is applicable for cell isolation, propagation, transfection, selection and *in vitro/in vivo* development after SCNT. Therefore, PKC, PFF as well as PEF cell culture were analyzed according to various aspects, which are explained in the following.

4.1.1 Characterization and comparison of PKC, PFF and PEF cell cultures

PKC, PFF and PEF cell cultures were analyzed and compared regarding to their morphological properties, growth potential, correct metaphases as well as the usage as donors for SCNT and development potential *in vitro* of cloned embryos.

4.1.1.1 Morphology of cells

The morphology of various cell types was compared and cell behavior was evaluated over long periods during cultivation.

The isolation of porcine primary kidney cells as well as fetal and ear fibroblasts were straightforward, efficient and not time consuming. The first microscopically characterization of isolated cells was done 24 h after isolation. The kidney cells showed 70-100% confluence and exhibited a mixture of different cell morphologies including cobblestone and spindle shaped cells (Fig. 3). After isolation of PEFs, the yield of obtained cells after seeding is lower compared to kidney cell isolation. In PEFs culture, often cell clumps attached from which fibroblasts grow out, but also a lot of single cells adhered on the culture plate surface with a confluence between 40 to 60%. PFFs cell culture showed mainly uniform fibroblast-like morphology with a confluence between 60 to 90%.

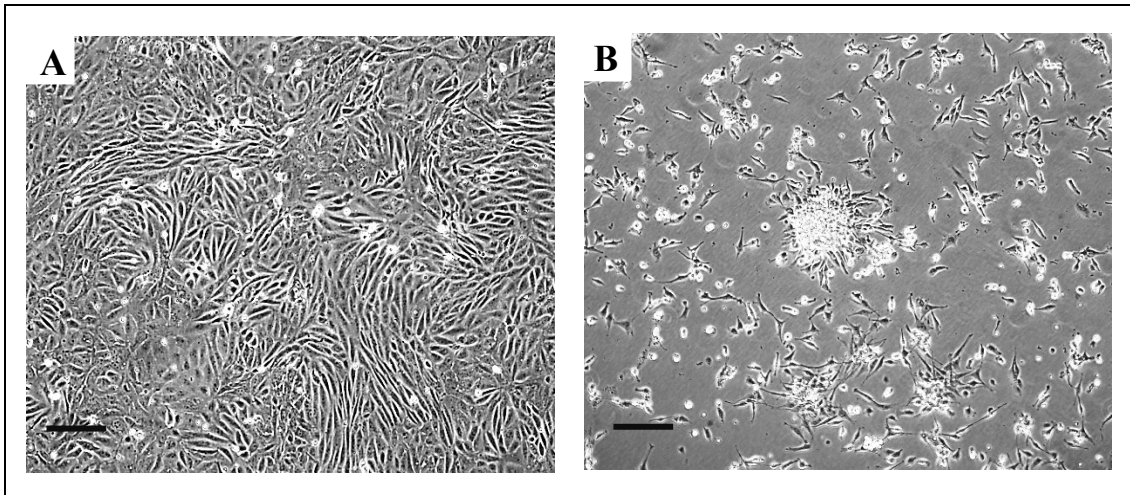


Figure 3: Primary cell culture passage 0 (P0) 24 h after isolation

(A) Various cell types were visible in a 100% confluent PKC cell culture after 24 h. (B) A typical observation after PEFs isolation was that fibroblasts grow out of detached cell clumps. bar = 100 µm

The generation of single cell clones with a porcine kidney cell culture (PKCm) in the third passage clearly demonstrated the diversity of the population. Seven days after seeding 192 cells per 10 cm dish, clear colonies had formed and showed the following characteristics (summarized in figure 5):

- i) cell morphology:
 - fibroblast-like (A, B, D, F)
 - round-shape (C)
 - epithelial-like, endothelial-like (E)
- ii) cell clone formation:
 - clearly defined (C, E)
 - frayed colonies (B, D, F)
- iii) cell distance:
 - very close (A, C)
 - small (E)
 - huge (B, D, F)

In summary, various different cell morphologies were detected. Presumably, the various detected porcine kidney cell types consisted of different types of fibroblasts, epithelial and endothelial cells.

In addition to different morphologies, variable growth rates of several cell clones were observed. The growth rates varied between very slow (B, D and F) to quick (A, C). The colonies which grew very slowly often stopped growth after a few days. On the other hand, in various gene targeting experiments it was observed that very fast

growing and strong fibroblast-like cell types, which were sometimes grown in swirls showed an acceptable proliferation rate even after splitting and freezing.

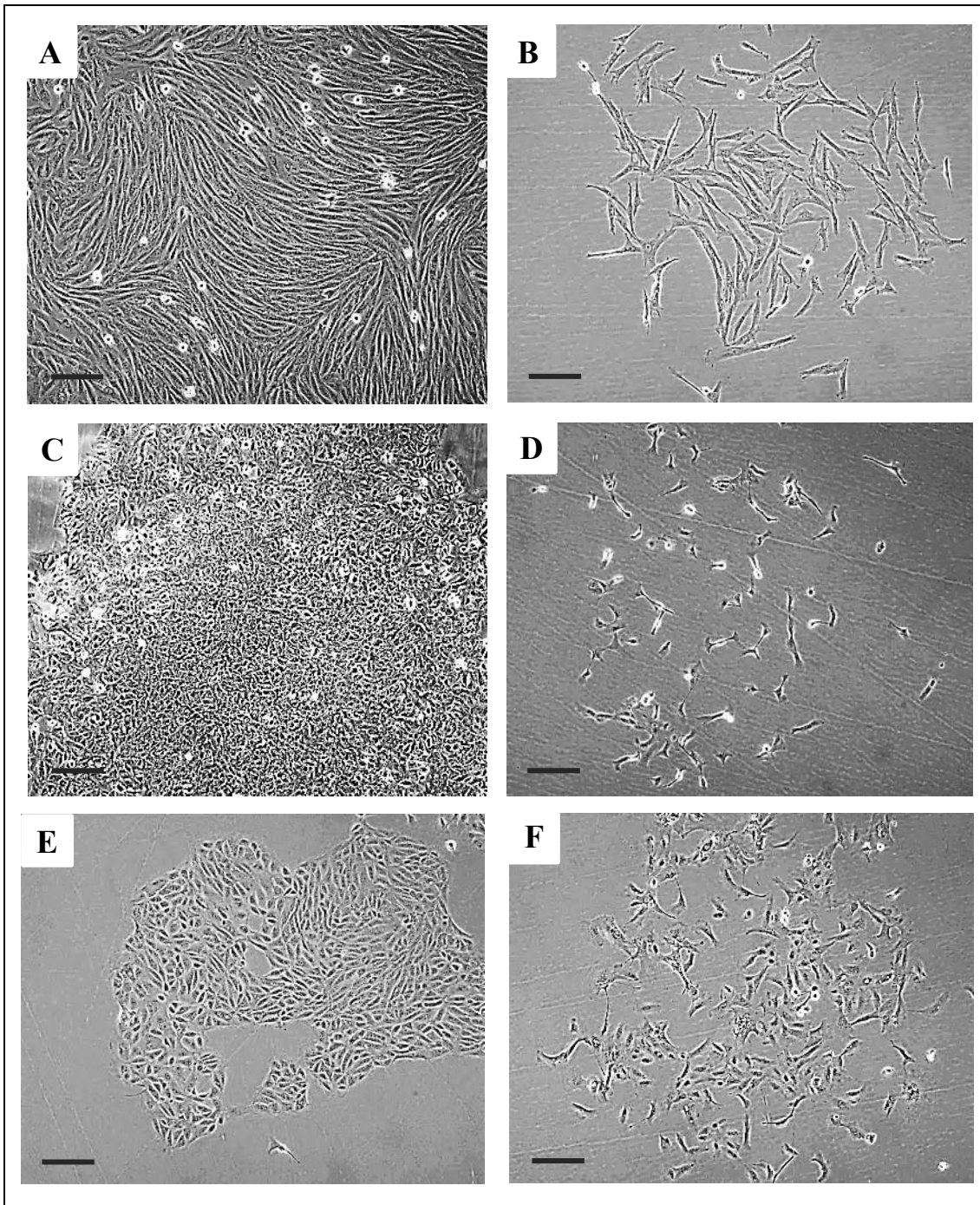


Figure 4: Detection of different colony morphologies of PKCm at P3

Single cell colonies were generated and analyzed of PKC culture. After seven days, various cell morphologies were detected. bar = 100 μ m

In addition of easy handling during isolation as well as capable detachment and growing of the primary cells on culture plates, an important role for further applications plays the behavior and changes of PKCs, PFFs and PEFs in culture over

a long period. From passage 2 to 13 it is clearly visible that cell populations of all three primary cultures became more or less homogeneous containing mostly cells with spindle shaped fibroblast-like morphology (Fig. 5).

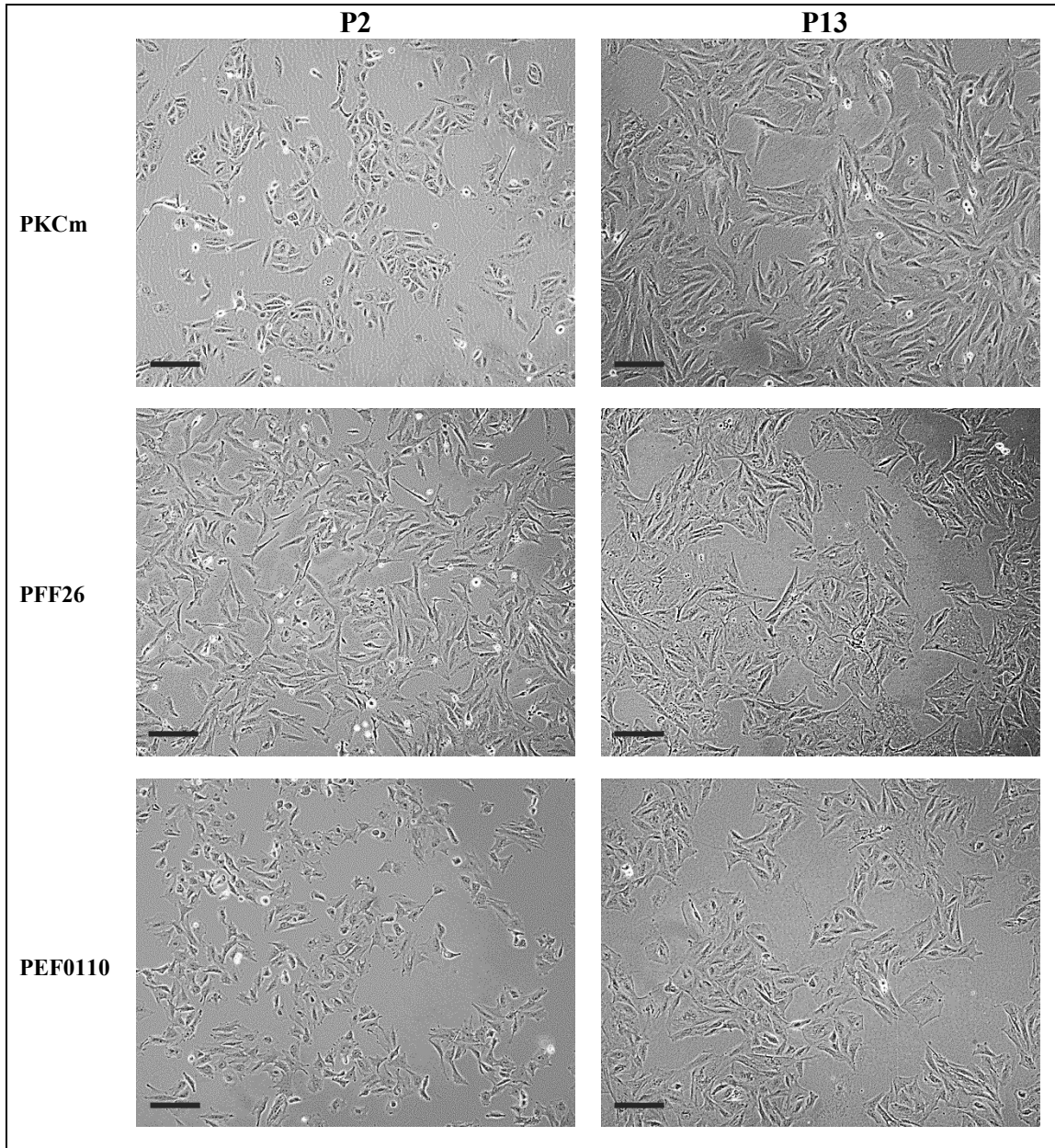


Figure 5: Morphological changes after several passages of PKCm, PFF26, PEF0110 cell lines.

Cell morphology changed from smaller and round-shaped in early passage (P2) to larger and spindle-shape morphology in later passage (P13). bar = 100 μ m

The diversity of cell types decreased over several passages probably due to the culture conditions promoting proliferation of fibroblast-like cells, which overgrew the other cell types. In general, a decreasing proliferation rate could be observed over time, since splitting intervals increased while splitting ration decreased. Furthermore,

in higher passages could be observed that cells increasing their volume in all populations. These observations are typical signs of senescence, which were described in various publications (Hayflick 1965; Oshimura and Barrett 1997).

4.1.1.2 Growth potential

For detection of growth potential, population doubling time was determined of PKCm, PFF26 and PEF0110 cell cultures at P5. For this, 5.5×10^4 cells were seeded onto 12-well plates and cultured for four days. A growth curve was created and population doublings and population doubling time were calculated (Fig. 6).

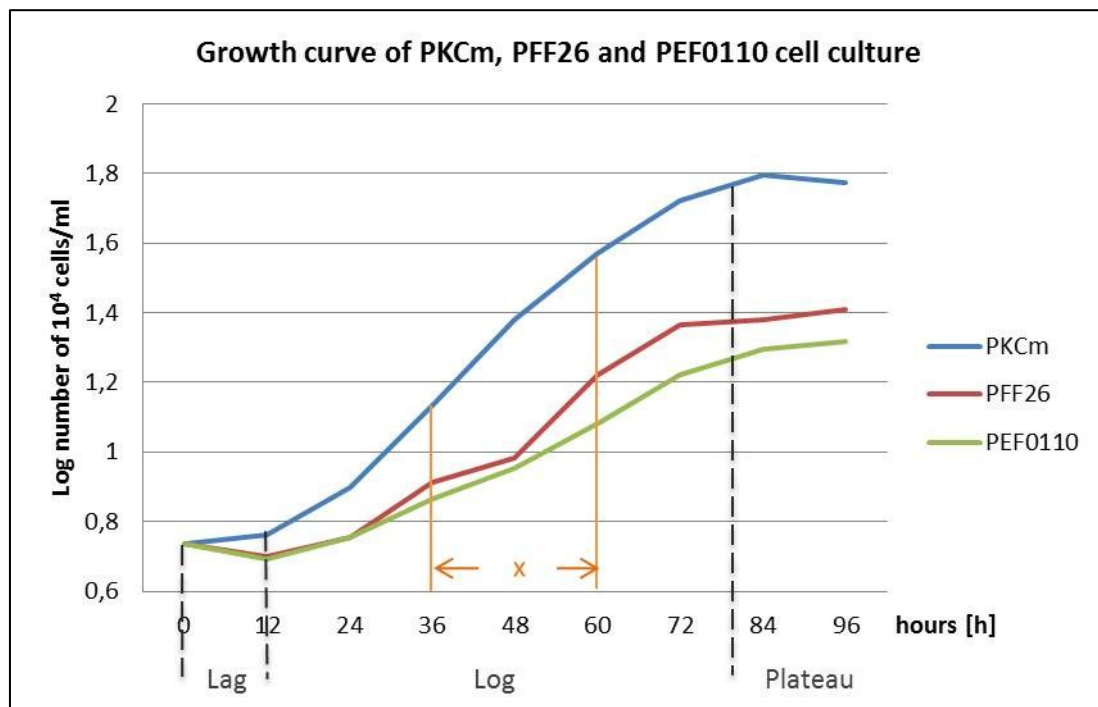


Figure 6: Growth curve of primary PKCm, PFF26 and PEF0110 cell lines

The cell numbers were plotted as Log₁₀ of 10⁴cell/ml against time [h]. Population doublings and population doubling time were calculated in the x-marked region during exponential growth phase.

The progression of obtained growth curve reflects the typical growth phases of cells. The proliferation was delayed after seeding of cells onto culture plates (lag-phase), in which the cells recover from trypsinization, attach and reenter the cell cycle. In PFF26 and PEF0110 cultures the cell number decreased in the first 12 h, while the PKCm culture showed a slight increase of cell number. In the exponential growth phase (log-phase) cells were actively dividing with constant doubling times. After a defined time, all primary cells stopped their proliferation due to confluent monolayer

resulting in contact inhibition (stationary phase - plateau). It followed last phase, the amount of cells decreased as a cause of accumulation of toxic metabolites (PKCm and 96 h).

Population doublings and population doubling time were calculated in the midpoint of the exponential growth phase that was between 36 and 60 h after seeding (marked with an x in fig. 6). The exponential growth phase increased more sharply in PKCm than in PFF26, PFF0110. In addition, PKCm and PFF26 cells showed a higher growth potential, since they needed about 22.6 h and 23.4 h for one doubling, respectively. In contrast, PEF0110 showed the lowest growth potential with 29.7 h for one doubling. In conclusion, PKC showed the shortest population doubling time. In this 24 h time period (x-marked period in fig.6) PKCm cell culture has doubled 1.1 times, PFF26 one time and PEF0110 0.8 times. Furthermore, the better growth potential of PKCm cell culture was clearly evident over the whole evaluation period of 96 h, where PKCs have doubled 3.4 times, PFFs 2.2 times and PEFs 1.9 times. From this follows that at the end of the experiment twofold more of PKCs were cultured as PFFs and PEFs. Concretely, 5.9×10^5 PKCs were counted after 4 d compared to 2.6×10^5 cells of PFFs and 2.1×10^5 cells of PEFs were detected in the same time period. The confluence of each culture was 100% and cells were tight together.

Growth potential of cells can be influenced amongst other by the usage of extracellular matrix (ECM) proteins, since they are regulating a variety of cell functions including cell proliferation, differentiation, adhesion and survival.

To determine optimal growth conditions kidney cells (PKC2109 or PKCm), fetal and ear fibroblasts (PFF26 and PEF0110) were cultured at P5 on 96-well plates without coating and coated with gelatin or collagen and analyzed via a MTT based proliferation assay. A standard curve was generated for each culture to get an indication of obtained cell numbers. The cells were seeded 4 h prior treatment, so that cells attached but did not start to proliferate. The cells were seeded with different concentrations on 96-well plates in duplicates and after 48 h the MTT treatment was started. Metabolic active cells cleaved intracellularly the added tetrazolium salt to formazan, which correlates directly to metabolic activity of cells. The formazan (yellow) became solubilized into a water-insoluble formazan dye (blue). After

overnight incubation, the emerged color intensity was measured and the obtained cell numbers were calculated (Fig. 7).

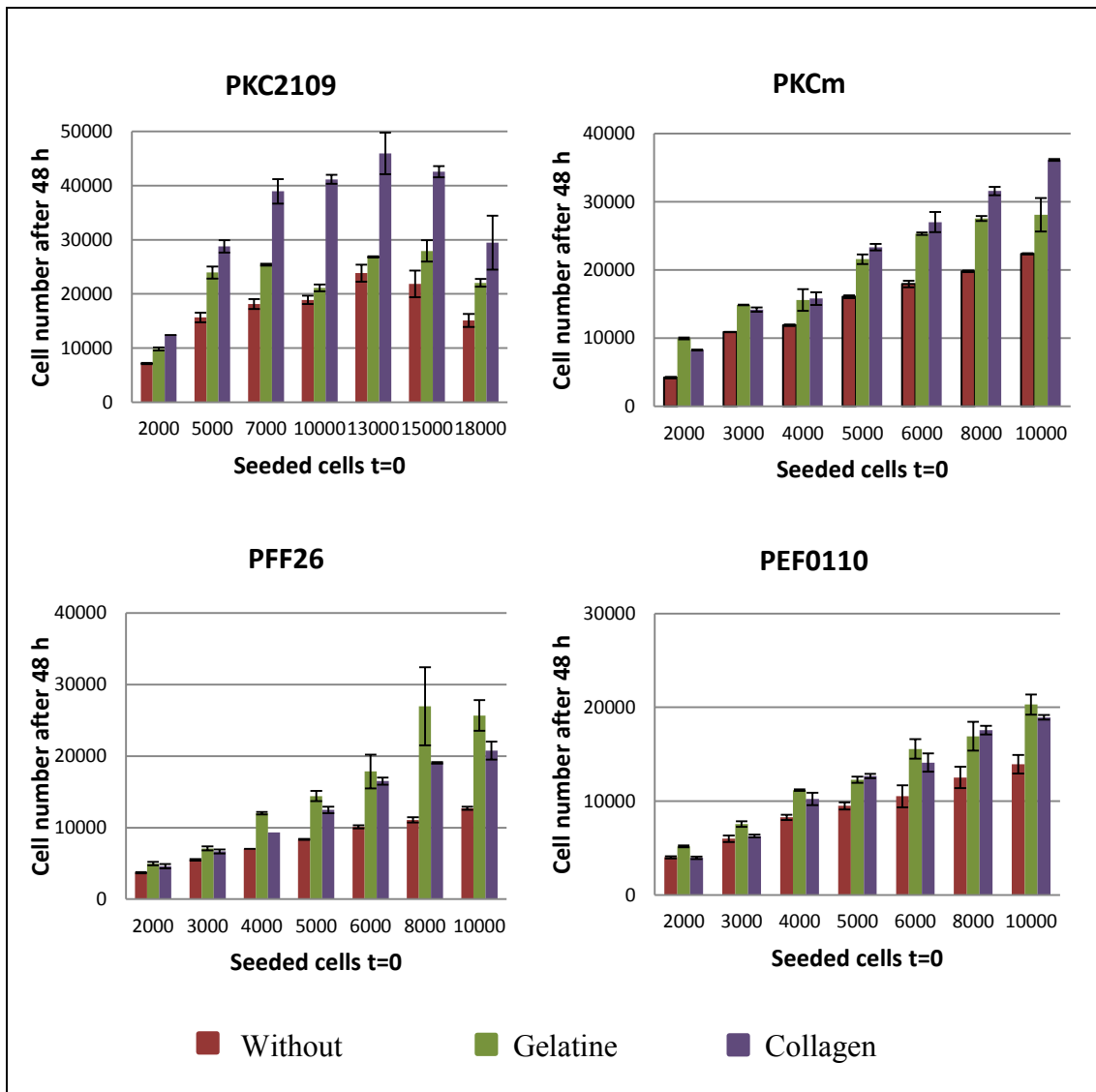


Figure 7: MTT-based proliferation assay to determine appropriate coating type of culture plates.

PKC2109, PKCm, PFF26 and PEF0110 were cultured onto plates without coating as well as plates coated with gelatin and collagen, respectively. The proliferation was detected by MTT-based proliferation assay. The assessed cell number after 48 h were plotted against seeded cells $t = 0$.

PKC2109 cells were seeded in following concentrations: 2000, 5000, 7000, 10000, 13000, 15000 and 18000 cells per well. After seeding of 2000 PKCs, obtained cell number of gelatin and collagen coated wells were similar after 48 h. If 7000 cells or a higher cell number were seeded per 96-well, around 40000 cells on collagen-coated wells were obtained after 48 h. The proliferation of PKC2109 on collagen is twofold stronger than on gelatin and without coating. Less clear results were achieved using PKCm cell culture (2000, 3000, 4000, 5000, 6000, 8000, 10000 seeded cells per

well). The cells grew better on collagen coating plates compared without coating, but similar cell number were detected with gelatin and collagen coating, respectively, starting the culture with 2000 to 8000 cells per well. Furthermore, the PKCs grew not evenly spread on plates coated with gelatin and without coating but rather in stellar islands. In addition, PKCs did not get properly confluent compared to similar cell numbers which were seeded on collagen coated wells during the time period of 48 h.

In proliferation analysis using PFF26 cell culture the differences were not decisive between the two coating types. Though, it could be shown that fetal fibroblasts grew better on coated culture plates than without coating.

The ear fibroblast cell culture PEF0110 displayed only marginal differences between gelatin and collagen coating, but a tendency of better growing compared to non-coated plates.

PKC2109 and PKCm cell culture became faster confluent and obtained a higher cell number per 96-well compared to PFF26. The ear fibroblast cell culture PEF0110 exhibited lowest cell proliferation capacity on each coating type compared to kidney cells and fetal fibroblast cell culture, which is comparable to obtained population doubling and population doubling time.

In conclusion, all cell cultures grew better on coated culture plates, whereas collagen proved to be the appropriate coating type.

4.1.1.3 Chromosome number

Besides a good proliferation rate of primary cells over a long period, a stable karyotype is crucial, especially in higher passages.

For determination of a stable karyotype, the metaphase chromosomes of several cell cultures (PKC, PFF and PEF) were counted at different passages (Tab. 3). The counted chromosomes were distinguished in correct ($2n=38$) and incorrect metaphases, which means an aberrant number of chromosomes. All tested cell cultures showed predominantly correct karyotypes ($2n=38$), whereas kidney cells exhibited a tendency of more stable karyotype.

Table 3: Chromosome number of PKCm, PKC2109, PFF26 and PEF0110 cell cultures.

	Passage	Metaphases		
		Total analyzed	2n = 38 (%)	Incorrect
PKCm	3	35	28 (80)	7
PKC2109	71	19	14 (74)	5
PFF26	5	14	9 (64)	5
	13	25	17 (68)	8
PEF0110	13	28	21 (75)	7

Chromosomes of metaphases were counted in different passages of various cell cultures. Karyotype were distinguished in correct (2n = 38) and incorrect, which means more or less chromosomes.

Beside the good growth potential, kidney cells were capable to be passaged up to P71, whereas PEFs and PFFs already showed signs of senescence and can hardly passaged longer than 15-20 times. Surprisingly, in P71 PKC2109 cell culture 74% on the metaphases showed a correct number of chromosomes, which is similar to PEF0110 cell culture in P13.

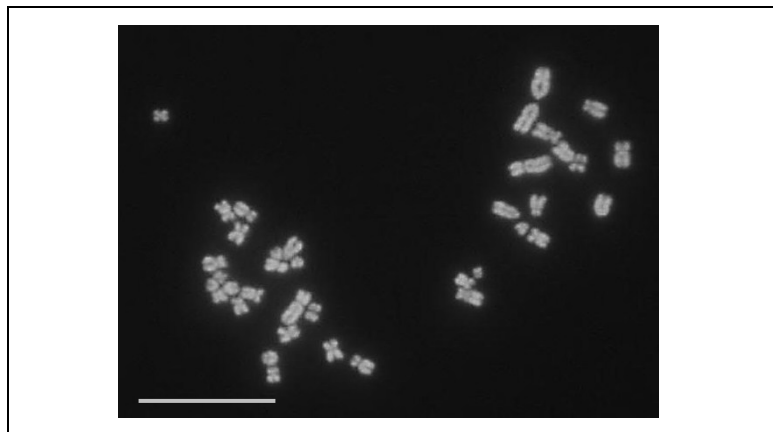


Figure 8: Metaphase of PKC2109

The correct number of chromosomes (2n=38) of PKC2109 at P71. The cells were treated with colcemid. bar = 10 µm

The karyotype analysis shows that primary kidney cell cultures have not only the potential to grow much longer than “conventional” fibroblasts, but also show a stable karyotype after a long culture period including several cell divisions.

4.1.1.4 Somatic cell nuclear transfer – *in vitro* development competence of cloned embryos

A further characterization step to define an appropriate cell culture, which is capable for generation of transgenic animals, is the determination of the *in vitro* development

competence of cloned embryos. PKCm, PFF26, PEF0110 were cultured and treated with serum starvation medium for 48 h. Subsequently, cells were detached and cell pellets were given for SCNT (see 3.2.1.11). The cells were used in three independent experiments for SCNT and Dr. Mayuko Kurome determined the fusion rate between enucleated oocytes and donor cell (Tab. 4) and counted the number of reconstructed cultured embryos, which means successfully fused oocytes, embryos developed to 2-4 cell stage and blastocyst stage. Further, the quality of blastocysts was classified as +++ = hatched blastocysts (very good quality), ++ = blastocysts (normal quality) and + = poor blastocysts (poor quality) cultured *in vitro* after seven days. Subsequently, Dr. Mayuko Kurome stained the blastocysts with orcein and introduced me in counting the cell nuclei of blastocyst derived from various cell types (Fig. 9).

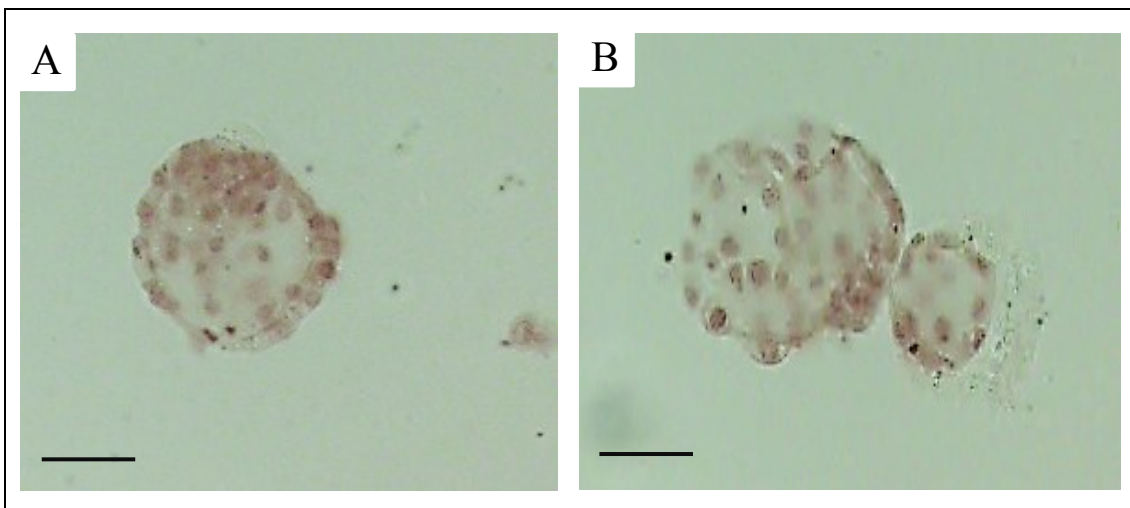


Figure 9: *In vitro* cultivated blastocysts seven days after SCNT using PKCm as donor cells

A = blastocyst, B = hatched blastocyst. bar = 50 μ m

A high fusion rate of 94.4% was obtained using PKCm. Furthermore, 81 reconstructed embryos could be cultured of which 57 embryos achieved 2-4 cell stage (70.4%) and 17 embryos (21%) developed to blastocyst stage. These results were significant better compared to PEF0110, where a fusion rate of 82.4% was obtained and only three embryos out of 70 reconstructed cultured embryos (4.3%) developed to blastocyst stage. Using PFF26 a higher fusion rate of 86% was obtained compared to PEF0110, but lower rate of embryos developed to blastocyst stage than with PKCm as donor cells.

Although percentage of embryos developed to blastocysts were significantly different, the quality and development into hatched-blastocyst was acceptable using

all cell types. Using PKCm and PFF26 as donor cells for SCNT, day seven blastocysts contained a higher number of nuclei (43.5-56.1) compared to PEF0110 donor cells, which is sign for better blastocyst quality (reviewed in Kikuchi 2004; Zhu *et al.* 2004).

Table 4: Overview of *in vitro* development competences of cloned embryos using PKCm, PFF26 and PEF0110 cell cultures as donor cells.

Donor cells	Fusion Rate (%)	No. (%) of reconstructed embryos cultured	No. (%) of reconstructed embryos developed to		Quality of blastocysts	No. of cells in blastocysts [mean+/-SEM]
			2-4 cell stage	Blastocyst stage		
PKCm	85/90 (94.4) ^a	81 (90)	57 (70.4)	17 (21.0) ^a	+++ : 10	43.5+/-4.2
					++ : 6	
					+ : 1	
PFF26	74/86 (86.0)	73 (84.9)	46 (63.0)	7 (9.6)	+++ : 5	56.1+/-9.5
					++ : 2	
PEF0110	70/85 (82.4) ^b	70 (82.4)	48 (68.6)	3 (4.3) ^b	+++ : 2	23.5+/-7.5
					++ : 1	

SCNT was performed by transferring nuclei of different cell cultures (PKCm, PFF26 and PEF0110). Embryos were cultivated *in vitro* for seven days up to blastocyst stage. Quality of blastocysts: +++ = hatched blastocysts, ++ = blastocysts, + = poor blastocysts. Values with different superscript were significantly different (P<0.05), SEM = standard deviation.

4.1.2 Transfection of porcine kidney cells - comparison of different methods

For transfection of primary mammalian cells many commercial kits and reagents are available. Since there was no report in the literature about transfection of primary PKCs, four different transfection methods, which were used successfully for transfection of fibroblasts, were tested. The aim was to find a method resulting in good (>50%) transfection efficiency, viable cells of good quality, but causing low cytotoxicity after transfection. For this chemical (lipofection, nanofection) and physical (electroporation and nucleofection) transfection methods were compared.

To determine the transfection efficiency, either the commercial vector pmaxGFPTM or endotoxin-free purified pmaxGFPTM plasmid were used. Transfection efficiency was calculated 24 h post transfection on fixed cells using an epifluorescence microscope as follows: $\frac{GFP \text{ expressing cells (green fluorescence)}}{\text{total cell number (DAPI stained nuclei)}} * 100$

During lipofection and nanofection, some experiments were analyzed after 48 h. The cell quality including cell morphology was assessed using a grading system ranging from 1-5. Fluorescence intensity and number of dead cells were valued by a score

system: lowest (-) up to highest (+++) intensity or cell number. Dead cells were those swimming in the medium.

4.1.2.1 Chemical transfection by nanofection and lipofection

To determine optimal lipofection conditions 3.2×10^4 cells (PKC2109) were seeded per 24-well. Then, different DNA concentrations (0.25-0.75 μg) and lipid to DNA ratios (1:1 – 4:1) were tested as well as varying amounts of Plus reagent (0.25-0.75 μl) (Tab.5).

The transfection efficiency results showed a wide range from 0.5 to 54% depending on the amount of DNA and plus reagent as well as the lipid to DNA ratio. A low amount of DNA (0.25 μg) and Plus reagent (0.25 μl) and low ratio of lipid to DNA resulted in good cell quality, which is comparable to the control (rating: 1 to 2) without vacuoles, no dead cells and a confluence between 70-100%. Certainly, in these experiments transfection efficiency was very low (<4%) and marginal fluorescence intensity (score: +) was detectable. A higher ratio of lipid to DNA (4:1) resulted in a higher transfection efficiency (26%) and fluorescence intensity (score: ++), but also in poor cell quality with stressed and enlarged cells containing many vacuoles and lipids. Hence, in following experiments such high ratios were not applied. The application of higher amount of DNA resulted in higher fluorescence intensity (score: ++), but led also to stressed and enlarged cells (rating: 3 to 5) which contained vacuoles and lipids. To determine if lipid drops and vacuoles appeared only directly after transfection, particular transfection approaches were analyzed after 48 h or splitted 1:3 after 24 h and analyzed 24 h later, respectively. Similar transfection efficiency and fluorescence intensity compared to analysis after 24 h could be observed by analysis of cells after 48 h. On the other hand, less vacuoles and lipids were detected, though cells appeared stressed and enlarged. In addition, more dead cells were observed, although toxic lipid vesicles were washed away with PBS after 24 h and medium was changed. In the other approach, in which cells were splitted, similar results could be observed, beside a lot of cells died after passaging.

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Table 5: Lipofection results

DNA amount [μg]	Plus reagent [μl]	Ratio lipid to DNA	Quality	Confl. [%]	Dead cells	Fluor. intensity	Transfection efficiency [%]
0.25	0.25	1:1	1	100	-	+	0.6
		2:1	2	80-90	-	+	0.5
		2,5:1	2	80-90	-	+	3.1
		3:1	2	70-80	-	+	3.8
		4:1	4	80-90	-	++	26
0.5	0.5	1:1	2	80-90	-	+	2.6
		2:1	4	70	+	+	26
		2,5:1	3	70	+	++	50
		2,5:1*	5	50-60	+	++	43
		2,5:1*	4	70-80	+	++	38
		2,5:1 ^{a)}	3	40	++	++	30^{a)}
		2,5:1 ^{b)}	3	<20	+	++	18^{b)}
		3:1	4	60	++	++	38
		3:1*	4	60-70	+	++	44
		3:1*	5	40-50	++	++	52
		3:1 ^{a)}	3	40	++	++	43^{a)}
		3:1 ^{b)}	3	<20	+	++	24^{b)}
0.5	1	2,5:1	4	60	+	++	24
		2,5:1*	4	70	++	++	50
		2,5:1*	4	60	+	++	24
		2,5:1 ^{a)}	3	50	++	++	24^{a)}
		2,5:1 ^{b)}	3	<20	+	++	16^{b)}
		3:1	4	40-50	++	++	45
		3:1*	3	70	+	++	53
		3:1 ^{a)}	4	50	++	++	54^{a)}
3:1 ^{b)}	3	<20	+	++	17^{b)}		
0.75	0.75	1:1	4	80-90	-	++	28
		2:1	5	70-80	+	++	37
		2,5:1	5	60-70	+	++	28
		3:1	5	60	+	++	21
Control 0	0.5	2.5:1	1-2	70-80	-	-	-

Analysis of lipofection approaches after 24 h. ^{a)} Analysis after 48 h ^{b)} Cells were splitted 1:3 after 24 h, analysis was performed after additional 24 h, * repeat the previous experiment at a different time point. Scale for the assessment of cell quality: **1**-excellent to good; **2**-no vacuoles and lipids detectable, cells are bit stressed (stretched); **3**-partially vacuoles and lipids, stretched cells; **4**-many vacuoles and lipids, stressed cells, partially enlarged cells; **5**-a lot of large vacuoles and lipids, altered morphology, stressed and enlarged cells; Confl.= Confluence after transfection, Fluor. = Fluorescence.

Best results were obtained using 0.5 μg DNA, 0.5 to 1 μl Plus reagent and a lipid to DNA ratio of 2.5:1 or 3:1. Thereby, transfection efficiency alternated between 38 to 54% and acceptable fluorescence intensity (score: ++) and number of dead cells (score: + to ++) were observed. However, there was no improvement of transfection efficiency, fluorescence intensity as well as cell quality by increasing the volume of Plus reagent. The detected confluence correlated to the amount of dead cells.

In order to optimize the transfection efficiency of nanofection, 3.2×10^4 cells (PKC2109) were seeded per 24-well and DNA concentration (0.5-1.5 μg) as well as

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nanofectin amounts (1.2-4 μ l) had to be set up (Tab.6). The transfection efficiency using nanofectin ranged from 2 to 25%, therefore being less efficient than lipofection. Independently of nanofectin concentration, low DNA amount resulted in excellent to good cell quality, which is comparable to the control (rating: 1), low transfection efficiencies (10-16%), fluorescence intensity (score: +) as well as dead cell number (score: - to +). Stressed cells (rating: 3) containing partially vacuoles and lipids were only detected after using very high DNA (1-1.5 μ g) and nanofectin amount (2-4 μ l). Certainly, fluorescence intensity was lower (score: + to ++) compared to lipofection using same (0.5 μ g) amount of DNA.

Table 6: Nanofection results

DNA amount [μ g]	Nanofectin [μ l]	Quality	Confluence [%]	Dead cells	Fluor. intensity	Transfection efficiency [%]
0.5	1.2	1	80	-	+	10
	2	1	80	-	+	16
	3.2	1	80	+	+	16
	4	1	80	+	+	15
1	1.2	2	80-100	+	+	23
	1.2*	2	70	+	+	25
	1.2 ^{a)}	2	80	+	+	13^{a)}
	1.2 ^{b)}	2	40	+	+	4^{b)}
	2	2	80-100	+	+	17
	2*	3	70	+	+	17
	2 ^{a)}	2	80	+	+	7^{a)}
	2 ^{b)}	2	40	+	+	2^{b)}
	3.2	2	80-100	+	++	21
	3.2*	3	70	+	+	21
	3.2 ^{a)}	2	80	+	++	17^{a)}
	3.2 ^{b)}	2	40	+	+	5^{b)}
1.5	4	3	50-60	+	+	20
	2	3	70	+	+	7
Control	3.2	3	50	+	++	11
	0	1	80	-	-	-
0	3.2	1	80-100	-	-	-

Analysis of nanofection approaches after 24 h. ^{a)} Analysis after 48 h ^{b)} Cells were splitted 1:3 after 24 h, analysis was performed after additional 24 h, * repeat of previous experiment at a different time point. Scale for the assessment of cell quality: **1**-excellent to good; **2**-no vacuoles and lipids detectable, cells are bit stressed (stretched); **3**-partially vacuoles and lipids, stretched cells, Fluor. = Fluorescence

Also with nanofectin transfection, cell quality after cultivation of cells for 48 h and splitted cells after 24 h and following cultivation for 24 h was analyzed. In both approaches, no vacuoles and lipids were detected compared to analysis after 24 h, but cells appeared a bit stretched and stressed. Furthermore, fluorescence efficiencies were reduced and fluorescence intensities as well as number of dead cells were similar after 48 h.

Best transfection efficiencies (20 and 25%) were obtained using 1 µg of DNA and 1.2 or 3.2 µl of nanofectin. In these approaches, cell quality and estimated number of dead cells was good.

Both, lipofection and nanofection showed increased amount of lipid drops and vacuoles after transfection with increasing amount of both DNA and transfection reagent (lipofectamin or nanofectin).

4.1.2.2 Physical transfection by electroporation and nucleofection

For conventional electroporation of cells (PKC2109) using Bio-Rad Gene pulser II, different DNA concentrations (1, 5, 10, 20 µg) and cell numbers (0.5×10^6 , 1×10^6 cells) were tested. In addition, several other parameters, like resuspension buffer (electroporation buffer from Bio-Rad, DMEM and PBS) and voltages (100 V or 230 V) were used to obtain the optimal electroporation conditions (Tab. 7).

Table 7: Electroporation results

Cell number	DNA amount [µg]	Buffer	Pulse duration [ms]	Quality	Confl. [%]	Dead cells	Fluor. intensity	Trans. efficiency [%]
0.5×10^6	1	EB	35,2	3	70-80	+	+	28
	1*	EB	29,8	4	60	+	+	25
	5	EB	34	3	60-70	++	+	46
	5*	EB	27,9	4	80	++	+	38
	5*	EB	30,3	2	50-60	+	+	41
	10	EB	31,9	3	50-60	++	++	47
	10*	EB	29,4	4	50	+	++	45
1×10^6	1	EB	29,1	4	70-80	++	+	24
	5	EB	27,2	4	60-70	++	+	22
	5*	EB	28,9	3	60-70	++	+	30
	5*	EB	29,2	4	50-60	+	++	46
	5	EB ¹⁾	48,8	2	70	++	+	15
	5	PBS	8,4	2	70-80	++	+	9
	5	DMEM	7,7	2	90-100	+	+	5
	5	DMEM	7,5	2	90-100	+	+	7
	10	EB	26,4	4	60	++	++	30
	10*	EB	29,7	4	60	++	++	57
20	EB	28,4	5	40	++	+++	54	
Control 1×10^6	0	EB	28,2	4	60-80	+	-	0,4

Analysis of electroporation approaches after 24 h, 230 V and high capacity of 500 µF; *previous experiment was repeated at a different time. ¹⁾chilled buffer. Endotoxin-free purified pmaxGFPTM plasmid was used. Scale for the assessment of cell quality: **1**-excellent-good; **2**-cells are ok, but they are bit stressed/stretched; **3**-stressed cells with vacuoles; **4**-stressed cells with very spindle-shaped morphology **5**-extreme spindle-shaped and stressed cells with vacuoles. Confl. = Confluence, Fluor. = Fluorescence, Trans. = Transfection.

Initial experiments (5 or 10 µg DNA and 0.5×10^6 cells) were carried out using electroporation buffer from Bio-Rad and 100 V. 24 h after electroporation, low number of dead cells (score: +) and acceptable cell quality were achieved with only a few stretched cells, but cells showed no GFP fluorescence. Therefore, following experiments were accomplished using a higher voltage (230 V).

In the control experiment, 1×10^6 cells were transfected without DNA which resulted in stressed cells with very spindle-shaped morphology (rating: 4), but less dead cells. The application of chilled electroporation buffer as resuspension buffer resulted in a transfection efficiency of 15%, reduced fluorescence intensity (score: +), high amount of dead cells (score: ++) and cells at good quality (rating: 2). Chilled electroporation buffer resulted to the longest pulse duration with 48.8 ms. In contrast, using PBS or DMEM as resuspension buffer the pulse duration was the shortest (7.5-8.4 ms) compared to other results. In addition, transfection efficiency of only 5-9% was achieved with marginal fluorescence intensity (score: +), but cells were detected with good quality (rating: 2). Using PBS, the number of dead cells was increased (score: ++) in opposite to DMEM as resuspension buffer (score: +).

In comparison of several resuspension buffers for electroporation, the most appropriate one was the electroporation buffer from BIO-RAD. Best transfection efficiency with around 40 to 50% was achieved using 0.5×10^6 cells with 5 or 10 µg DNA and 1×10^6 cells with 10 or 20 µg DNA. Further, fluorescence intensity varied from weak to strong (score: + to +++), but many cells died after electroporation and surviving cells showed signs of stress including extreme spindle-shape morphology and vacuoles.

Generally, increasing DNA concentration improved fluorescence intensity and efficiency, but very high amounts of DNA (20 µg) resulted in very poor cell quality (rating: 5) with extreme spindle shaped and stressed cells containing vacuoles. Surprisingly, large variations of fluorescence efficiencies could be detected in repeated experiments using 1×10^6 cells. The efficiency ranged from 22% to 46% using 5 µg DNA and 30 to 57% with 10 µg DNA.

In summary, cells transfected with conventional electroporation method exhibited acceptable transfection intensity and efficiency, but showed signs of stress including spindle-shaped morphology and vacuoles.

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An advanced electroporation procedure called nucleofection developed by Amaxa (Lonza) with a kit especially designed to transfect mammalian fibroblasts including an established protocol was applied. Nevertheless, optimization of experimental settings is necessary to a limited extent (Tab. 8). For this, in an initial experiment 0.5×10^6 cells of PKC2109 were transfected with 2 μg DNA using different preset programs (A-24, T-16, U-12, U-23 and V-13) recommended in the manufacturer's protocol.

Table 8: Nucleofection results

Cell Number	DNA amount [μg]	Program	Quality	Confl. [%]	Dead cells	Fluor. intensity	Transfection efficiency [%]
PKC2109							
0.5×10^6	2	A-24	2	70-80	+	+	28
	2	T-16	2	80	+	+	54
	2	U-12	1	80	+	++	63
	2	U-23	2	50	++	+	60
	2	V-13	1	70	+	++	59
0.5×10^6	1	U-12	1	90	+	+	36
	5	U-12	1	90	+	++	70
	10	U-12	1	80	+	+++	77
	20	U-12	2	70-80	+	++	89
1×10^6	5	U-12	1	90	+	++	68
	10	U-12	1	70-80	+	+++	88
	20	U-12	2	50-60	++	+++	83
0.5×10^6	5 ¹⁾	U-12	1	80-90	+	++	64²⁾
	10 ¹⁾	U-12	1	80-90	+	++	65
	20 ¹⁾	U-12	3	60-70	+	++	82
Control							
0.5×10^6	0	U-12	1	80-90	+	-	0
PKCm							
0.5×10^6	2	U-12	1	90-100	+	++	49
	2	T-16	1	90	+	++	50
	2	V-13	2	90	+	++	66
PFF26							
0.5×10^6	2	U-12	1	90	+	+	53
	2	T-16	1	90	+	+	50
	2	V-13	2	80-90	++	++	77
PEF0110							
0.5×10^6	2	U-12	1	90-100	+	+	15
	2	T-16	1	90	+	+	11
	2	V-13	2	70	++	+	34

Analysis of nucleofection approaches after 24 h. Scale for the assessment of cell quality: 1-excellent-good; 2-cells are ok, but they were bit stressed/stretched; 3-stressed cells with vacuoles; 4-stressed cells with very spindle-shaped morphology 5-extreme spindle-shaped and stressed cells with vacuoles. Confl. = Confluence; Fluor. = Fluorescence; ¹⁾ other DNA batch (1 $\mu\text{g}/\mu\text{l}$) ²⁾ error message during nucleofection.

24 h after transfection, cells looked very good or a bit stretched (rating: 1 to 2). Nucleofection with the program A-24 resulted in the lowest transfection efficiency of

28% with low fluorescence intensity (score: +) and few dead cells (score: +). Using program U-23, the highest rate of dead cells (score: ++) was determined concurrent with high transfection efficiency (60%). The best fluorescence intensity was achieved with U-12 and V-13 (score: ++) exhibiting a transfection efficiency of 63% and 59%, respectively and cells of excellent quality (rating: 1). Due to this results and the fact that U-12 resulted in less dead cells (score: +) than V-13 (score: ++), U-12 was used for further experiments with various cell numbers and DNA concentrations. Different DNA concentrations (1, 5, 10, 20 μg) were tested with 0.5×10^6 and 1×10^6 cells, respectively. The obtained transfection efficiency ranged from 36% to 89%. In a control experiment, using 0.5×10^6 cells and nucleofector program U-12 without DNA, best cell quality (rating: 1) and less amount of dead cells (score: +) was obtained.

The best transfection efficiency (89%) was achieved with 0.5×10^6 cells and 20 μg DNA. Additionally, the fluorescence intensity (score ++), low number of dead cells (score +) and good quality of cell morphology was detected (rating: 2). Similar effects were also seen with endotoxin-free prepared pmaxGFP.

In comparison with conventional electroporation, a similar trend is recognizable: high DNA-concentration led to an increase of dead cells as well as fluorescence intensity and efficiency, respectively. If it is desired to transfer higher amounts of DNA into the nucleus, plasmid DNA up to 20 μg could be transfected without tremendous decrease in cell quality and vitality.

Moreover, nucleofection was suitable for transfection of PKCm culture as well as fetal and ear fibroblasts. After transfection of 0.5×10^6 cells with 2 μg DNA using different programs (U-12, V-13 and T-16) best transfection efficiency and fluorescence intensity results were obtained using program V-13. However, the number of dead cells after treatment of PFFs and PEFs with V-13 was higher (compared with U-12 and T-16) and cells showed signs of stress. Overall PEFs showed the lowest transfection efficiencies (11-34%) compared to PFF (53-77%) and PKC (49-66%) in this approach. The nucleofector programs U-12 and T-16 are the most suitable for PKCm, PFF26 and PEF0110 transfection regarding to amount of dead cells, quality of surviving cells, fluorescence intensity and transfection intensity.

In summary, comparison of different transfection methods using cell cultures originating from different somatic tissues indicate that nucleofection is the most

suitable transfection technique to generate genetically modified cells. Moreover, the nucleofection of PKCs and PFFs with this system is highly efficient and results in viable cells of good quality. The settings for efficient transfection of PEF cell cultures could be improved in further experiments. In consequence of all obtained results, nucleofection program U-12 was applied for the following additive gene transfer and gene targeting experiments.

4.2 Generation of transgenic animals using PKC, PFF and PEF cells as donors for SCNT

Over the past four years a large number of transgenic animals were generated by SCNT at the Chair for Molecular Animal Breeding and Biotechnology using different lines/populations of PKCs, PFFs and PEF as donor cells. The generation of large animal models requires a high degree of expertise.

4.2.1 Additive gene transfer, cloning and recloning using different cell populations

The method of nucleofection was used to transfect 0.5×10^6 to 1×10^6 cells in P2-P4 with 1-3 μg endotoxin-free purified and linearized plasmids. Dr. Nikolai Klymiuk was responsible for vector design and construction as well as endotoxin-free preparation of plasmids. After transfection, cells were plated either onto one 35 mm or 60 mm petri dish depending on cell number. After 24 to 48 h, selection was started with following antibiotic concentrations: PKC: 10 $\mu\text{g}/\mu\text{l}$ blasticidin S, 0.6 or 1.2 mg/ml G418, 3 $\mu\text{g}/\text{ml}$ puromycin; PEF: 4 $\mu\text{g}/\mu\text{l}$ blasticidin S; PFF: 0.4 or 0.6 mg/ml G418. Selection proceeded at least one week, medium was changed every other day and cells were splitted once in this time. After selection, cells were cryopreserved or used directly for SCNT after starvation. Dr. Annegret Wünsch was responsible for generation of most transgenic cell lines.

In the following, data of the last four years were evaluated. In table 9 and 10, various SCNT experiments were compared regarding to the used cell source, total number of

born and stillborn piglets as well as litter rates, which were calculated as follows:

$$\frac{\text{number of obtained litters}}{\text{number of performed ETs}} * 100.$$

SCNT after first round of transfection

For generation of transgenic animals by additive gene transfer, which results in random integration of gene constructs, various primary wild-type cell cultures of PKCs and PFFs were used (Tab. 9). In a first round eight different gene constructs were stable transfected into three different primary PKC cell cultures, (PKCm, PKC2109 and PKC9753). After selection, a mixed population of stable transfected PKC clones was used for a total of 29 ETs and resulted in twelve litters (41%) with 45 animals (including seven stillborn).

Using stable transfected PFFs with twelve different gene constructs 29 ETs were conducted and resulted in 17 litters (59%) with 68 animals overall, of which ten animals were stillborn. The efficiency of obtained litters was higher using PFFs whereas the number of born piglets per litter was nearly comparable.

For selection procedure of the first transfection round, either blasticidine (BS) or G418 resistance were used. In all experiments there were no clear differences identified in efficiency of obtained animals between BS and G418 antibiotic resistances.

In conclusion, three different wild-type kidney cell cultures and two wild-type fetal fibroblast cell cultures were capable for production of genetically modified pigs.

Table 9: SCNT using stable transfected cell population

Cell type	No. of transgene cell lines/ populations*	Passage	ET	No. of litters (%)	Total piglets/ Stillborn
1st transfection					
PKC ¹⁾	8	6-8	29	12 (41)	45/7
PFF ²⁾	12	6-8	29	17 (59)	68/10
2nd transfection					
PKC ³⁾	5	6-8	19	10 (53)	38/12
PEF	1	7	5	1 (20)	1/1

For additive gene transfer PKC and PFF cell lines were transfected with various constructs. To obtain double or triple transgenic animals, PKC and PEF cell cultures were used for an additional transfection round. *mixed population: after additive gene transfer; cell line: cell isolation from transgene animals.

¹⁾PKC m, PKC2109, PKC9753, ²⁾ PFF14, PFF26, ³⁾ four different cell cultures were used.

SCNT after a second round of transfection

Since a mixed population of transfected and selected cell clones was used for SCNT, the generated animals had to be analyzed for transgenesis and expression level in desired organs. Animals with best transgene expression were used for breeding, recloning or an additional transfection with a further gene construct.

Hence, it is possible to generate multitransgenic animals by serial transfection and cloning. For this, different transgenic PKC cell cultures were used as donors for additional five different gene constructs. Out of 19 ETs, we obtained ten litters (53%) with 38 animals (of those twelve were stillborn).

PEFs were used in one experiment for a second additive gene transfer but only one litter with one stillborn animal was obtained out of five ETs.

Using transfected cells of a transgenic animal, the number of stillborn animals increased, because 31.6% of the animals were stillborn after SCNT. No living animal was born using PEF cell culture of a transgenic animal. In opposite, using transfected wild-type cell cultures 15.6% (PKCs) and 15.7% (PFFs) of animals were stillborn.

Recloning experiments

Cloned transgenic animals with the appropriate expression levels in the desired organs were reproduced by breeding if the animals were still alive or in recloning experiments if the animals had to be killed for expression analysis. Isolated and propagated cells of best expressing animals were applied directly for SCNT without any further genetic modification.

Using PKC cell cultures obtained from six transgenic animals with partially different transgenic backgrounds, 35 ETs resulted in 16 litters (46%) with overall 48 animals including eight stillborn animals (Tab. 10). One PFF cell culture was used for three ETs resulting in one litter with two stillborn animals. Using PEF cell culture only five of 19 ETs (26%) led to ten piglets (including five stillborn).

In summary, recloning of PKC cell culture is satisfying compared to PFF and PEF cell cultures. However, the strong variations in cloning efficiency have to be considered when using PEF cell cultures.

Table 10: Using donor cells for recloning and cloning SCNT experiments

Cell type	No. of transgene cell lines/	Passage	ET	No. of litters (%)	Total piglets/ Stillborn
Recloning					
PKC	5	2-7	35	16 (46)	48/8
PFF	1	2-3	3	1 (33)	2/2
PEF	3	2-7	19	5 (26)	10/5
Cloning					
PFF	2	3-4	24	5 (21)	29/3
PSF	1	5	2	0	0

PKC, PFF and PEF cell cultures were compared regarding to their cloning and recloning usability. Cell line: cell isolation from transgene animals. PSF = porcine skin fibroblasts.

Cloning experiments

In addition, cell cultures of bred animals established in another laboratory containing three different constructs were cloned (Tab. 10). Often, it is not possible to exchange animals between collaborating laboratories; nonetheless shipping of cells is possible. These cells were utilized for cloning experiments without further modification.

Embryos generated with PFF cell cultures were transferred to 24 recipients, resulting in five litters (21%) and 29 animals (including three stillborn animals). Using porcine skin fibroblasts (PSF) no pregnancy was achieved after two ETs.

In general, the efficiency of cloning experiments was low using PFF and PEF cell cultures. However, these cells were not isolated and cultured at the beginning in our laboratory and therefore it is difficult to compare these results with the other SCNT results.

In conclusion, large differences in efficiency of obtained litters and litter rates could be observed between several experiments using different donor cell types and passage number as well as additive gene transfer constructs.

4.2.2 Gene targeting

BAC-vectors were used for site-directed mutagenesis which were designed and constructed by Dr. Nikolai Klymiuk. Endotoxin-free purification and linearization of these vectors were performed by Dr. Nikolai Klymiuk, Katinka Burkhardt as well as Katrin Krähe. The BAC-vectors were nucleofected using 0.5×10^6 or 1×10^6 PKCs in

P2-P4 and 0.25 , 0.5 or 0.75×10^6 cells of PFF26 in P3. Subsequently, cells were cultured onto 6 mm or 10 mm petri dishes, respectively in culture medium for 24 to 48 h. In contrast to additive gene transfer, it is not possible to use a mixed population of cell clones for SCNT. To obtain single cell clones, cells were counted and seeded onto 96-well plates for selection 24-72 h after nucleofection (Fig. 10). In some experiments transfected cells were mixed (1:1 – 1:3) with non-transfected cells. The seeded total number of cells varied between 2000-4400 cells per well. Following antibiotic concentrations were used: PKC cultures 1.2 mg/ml G418 or 6 μ g/ml blasticidine S and fetal cell culture 0.6 mg/ml G418 or 9 μ g/ml blasticidine S. The medium was changed three times per week. After seven days, 96-well plates were screened for single cell colonies.

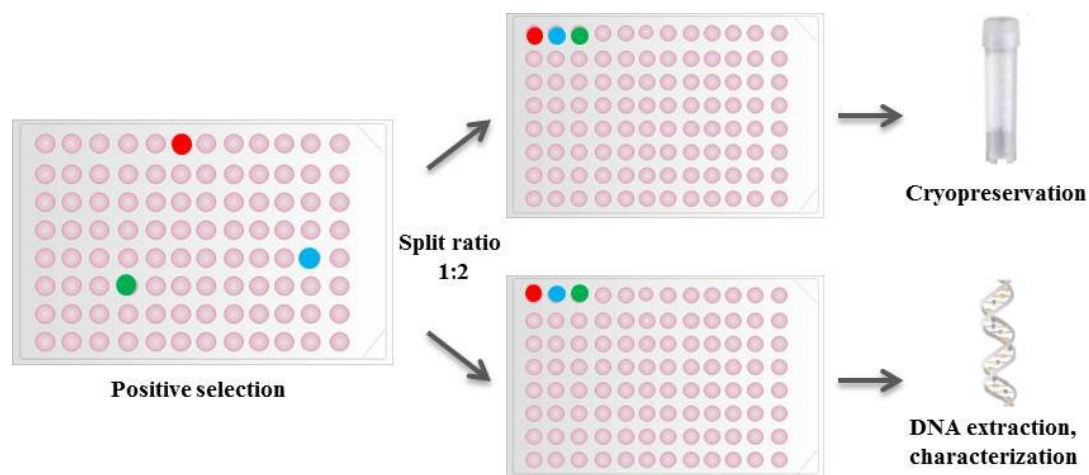


Figure 10: Generation of single cell clones in a gene targeting experiment.

Stable transfected single cell clones were generated during gene targeting experiments onto 96-well plates. When reaching confluency of 50-80%, cell clones were propagated by splitting 1:2 to obtain cells for cryopreservation and DNA extraction and characterization.

Cell clones were splitted 1:2 onto new 96-well plates, if they have reached a confluence of 50-80%. Again, when reaching 80-100% confluency, cell clones were harvested. The cells of one 96-well were centrifuged ($330 \times g$ for 10 min), supernatant was removed and the pellet stored at -80°C for analysis. The cells of the other 96-well were cryopreserved (see 3.3.1.3) for SCNT.

Obviously, cell cultures used for these procedures need a high proliferation capacity and a long lifespan. Therefore, generally cells were used with low passage number (P1-P3) for a targeting experiment. After transfection, selection and propagation, the

stable transfected single cell clones were used as donors for SCNT in P7 to P10. In the following, data of the last two years were evaluated.

Using PFF26 cell culture, it was not possible to generate targeted cells after ten different gene targeting experiments using different BAC vectors, DNA-concentrations (1-10 μg) as well as cell numbers ($0.25 - 0.75 \times 10^6$ cells). In concerning of the difficulties using PFF cell cultures for gene targeting experiments, the primary PKCs were used for targeting in four different experiments.

After targeting one allele, the cell clones were used for SCNT after transfection and selection. In summary 19 ETs resulted in seven litters (37%) with a total of 28 animals (including one stillborn) and two terminated pregnancies (at day 58 and 61, respectively) containing 17 fetuses.

Variations in efficiency (38% - 66.7%) of obtained litters after ETs were observed also in gene targeting experiments using different BAC-vectors.

For sequential targeting of the second allele of one locus, isolated primary PKCs from heterozygote animals were used. After transfer of embryos to four recipients, two litters (50%) with 11 animals (two stillborn) were obtained (Klymiuk *et al.* 2011a).

In conclusion, PKCs are useful for gene targeting and these cells were more suitable than PFFs and PEFs. Further, targeted PKCs cell clones showed an acceptable rate of pregnancies and litter size after SCNT.

5 DISCUSSION

Genetically engineered pigs offer great benefits in biomedical research as models for human diseases and as organ donors for xenotransplantation (reviewed in Aigner *et al.* 2010). SCNT is used often for the generation of transgenic pigs, due to of several advantages such as generation of non-chimeric animals that possess the genetic background of the donor cells and it is the only method to introduce site-directed mutagenesis in domestic animals (Ross *et al.* 2009). Thereby, donor cells can be modified genetically by additive gene transfer and particularly gene targeting. The choice of donor cell type, usage of appropriate transfection method and selection are crucial for successfully transgenesis and generation of a pig model.

5.1 Morphology and proliferation of primary cell cultures

In the last years, various porcine cell types were used as donor cells for SCNT such as MSCs (Jin *et al.* 2007; Kumar *et al.* 2007), kidney cells (Yin *et al.* 2002; Hoshino *et al.* 2005), ear fibroblasts (Faast *et al.* 2006), adipocytes (Tomii *et al.* 2009) and granulosa-derived cells (Polejaeva *et al.* 2000) and most frequently wild-type and transgenic PFFs (Betthausen *et al.* 2000; Onishi *et al.* 2000). Until today, genetic modified PKCs have not been used for the creation of transgenic pigs.

The isolation of porcine primary kidney cells as well as fetal and ear fibroblasts was simple to perform, beneficial and not laborious compared to other common cell isolations such as porcine lung epithelial cells (Blickwede and Borlak 2005), bovine muscles cells (Green *et al.* 2007) as well as isolation of porcine MSC derived from bone-marrow and following differentiation into osteocytes (Colleoni *et al.* 2005).

After isolation, the primary cells have to adapt to an artificial environment, which include changes in O₂-level, concentration of nutrition and growth factors as well as absence of extracellular matrix constituent and surrounding cell types (reviewed in Kuilman *et al.* 2010). Various types of cell morphology were detected in the initial kidney cell culture passage 0. Presumably, these cells are different types of fibroblasts like cortical and inner medullary fibroblasts (reviewed in Grupp and Muller 1999). In

addition, other cell types were verified such as epithelial cells, dendritic cells, macrophages and lymphocyte-like cells (reviewed in Kaissling *et al.* 1996; Yin *et al.* 2002). Probably, PFF and PEF cultures consist of different types of fibroblasts and other cell types as well, but it was not clearly visible in passage 0 and after single cell clone generation. Various cell types were determined after single cell cultivation of PKCs. Thereby, very fast growing and marked fibroblast-like cell types were detected also after gene targeting experiments followed by single cell clone generation. It was shown that these cells were particularly suitable for splitting, cryo preservation and SCNT. It would be a possibility to characterize such well growing cell types and adjust the isolation process by, for example gradient centrifugation or antibody protein affinity and magnetic bead purification to obtain only the desired cells. On the other hand, colonies which grew very slowly often stopped growth after a few days. In addition, in long-term analysis it was seen that the diversity, especially in PKCs decreased after several passages, because cells with less growth potential were lost and cells with high proliferation capacity were enriched. Thereby, PKCs showed an increased growth potential compared to PFFs and PEFs also in higher passages. The focus is on cells that are cultivatable over several passages for additive gene transfer and especially gene targeting experiments, and thus are suitable for SCNT. Furthermore, it has to be emphasized that the kidney cells (PKC2109) were cultivatable for at least 70 passages after isolation offering a great potential for further proceedings. PFFs and PEFs are widely used for genetic modification and due to their proliferation and reprogramming properties stopped growing around passages 15-20 in distinct experiments. The reasons for this could be that somatic cells succumb to cellular senescence and thus differ from cancer cells (Hayflick 1965). The Hayflick limit (Hayflick and Moorhead 1961) describes a phenomenon where human diploid cell strains cannot divide indefinitely, but die after a certain number of divisions (approximately 60 to 80 cell divisions). The reason for cellular senescence also called replicative senescence is the progressively shortening of telomeres (reviewed in Kuilman *et al.* 2010). DNA polymerase fails to completely replicate the lagging strand, which is called “end replication problem” and contributes to telomere shortening (Olovnikov 1971; Watson 1972). The proliferation is arrested due to critical minimal length of telomeres and results in activation of DNA damage response proteins and cell cycle proteins (reviewed in Kuilman *et al.* 2010). PFFs and PEFs seem to reach this limit much earlier than PKCs. It was possible to passage the

PKC2109 cell culture more than 70 times, which implies a cell division rate over 150 times and is more as the Hayflick limit would allow. Presumably, the cells changed spontaneously to become a permanently growing (immortalized) cell line during cultivation to P71, although they exhibited a stable karyotype, density-dependent inhibition of proliferation (contact inhibition) as well as similar proliferation capacity as in early passages. Moreover, cellular senescence can be caused by reactive oxygen species (ROS) as shown on human fibroblasts (Chen and Ames 1994; von Zglinicki *et al.* 1995). PKCs, PFFs as well as PEFs are growing under standard conditions (21% O₂, 5% CO₂) (reviewed in Simon and Keith 2008). Probably, this means oxidative stress for the cells and could contribute to stress-mediated telomere damage and resulted in telomere shortening (von Zglinicki *et al.* 2000). As reviewed by Yegorov and Zelenin (2003) a reduced oxygen concentration in culture results in an increase in cell life, because the telomere shortening rate and the rate of replicative aging can be either increased or decreased by a modification of the amount of oxidative stress (von Zglinicki *et al.* 2000; reviewed in Yegorov and Zelenin 2003). Presumably, PKCs are less sensitive to oxidative stress in comparison to PFFs and PEFs and can be passaged therefore over a longer period.

A growth curve was created to determine the population doublings and population doubling time of PKCs, PFFs and PEFs. In the first 12 h after seeding, a decreasing cell number of PFFs and PEFs was observed, in opposite to the PKC population which increases minimally. The proliferation was delayed after seeding of cells onto culture plates (Lag-Phase), because cells must adapt to the new growth conditions and recover from trypsinization process. Trypsin was used to subculture the cells, certainly due to the proteolytic activity, cell surface proteins were cleaved, which leads to faulty regulation of cell functions (Huang *et al.* 2010). It seems that this adjustment and recovery proceeded faster in PKCs than in PFFs and PEFs.

Population doubling time was calculated at the midpoint of the exponential growth phase between 36 and 60 h after seeding. PKCm and PFF26 cells showed similar population doubling times, since they needed about 22.6 h and 23.4 h for one doubling, respectively. I assume that PFFs would need longer for one doubling regarding to the whole growth progression. However, the curve did not retained constant in this interval, probably due to faulty detachment or counting of cells. After a defined time, nutrients are depleted and there was no more of space for proliferation, cells achieved the stationary phase and stopped their growing.

Furthermore, PFFs and PEFs need more time to double their population in comparison to PKCs. A total of 5.9×10^5 PKCs were counted after 4 d, which is more than twice the number of PFFs (2.6×10^5 cells) as well as PEFs (2.1×10^5 cells). Though PKCs were slightly earlier confluent and entered the last phase of the growth curve after 4 d. This implies that PKCs could be smaller compared to PFFs and PEFs, because more cells were counted during at 100% confluence. This result indicates that PKCs grew much better and faster than the other cell types. The diversity of various cells in a PKC culture could influence the growth of cell population in a positive way.

In summary, PKCs, PFFs and PEFs were very different regarding their morphology and growth potential. PKCs showed higher morphological diversity and grew faster compared to PFFs and PEFs. Presumably, PKC are more resistant to oxidative stress. Moreover, their sensibility to antibiotic concentrations was different (PKC: 10 $\mu\text{g}/\mu\text{l}$ BS, 0.6 or 1.2 mg/ml G418; PEF - 4 $\mu\text{g}/\mu\text{l}$ BS; PFF - 0.4 or 0.6 mg/ml G418) as well as to trypsin concentrations (PKCs: 0.4% and PFFs/PEFs: 0.1%).

5.2 Promoting of proliferation capacity using various coatings

Various factors are important for a well growing cell population e.g. medium composition, growth density, contact of cells to each other, factors produced by the cells themselves and furthermore the coating of culture plates.

The coating of culture plates with adhesions factors such as fibronectin, poly-lysine, laminin, gelatin as well as collagen influences the cell growth. Collagen can be divided into 29 various subtypes. Of these, collagen type I is a prevalent substrate for the culture of, for instance human fibroblasts (Yashiki *et al.* 2001), porcine fetal fibroblasts (Rogers *et al.* 2008b) and rat epithelial cells (Strom and Michalopoulos 1982). Gelatin is a denatured collagen and was used in cell attachment and growth of porcine and human fibroblasts and endothelial cells (Wisseemann and Jacobson 1985). The MTT-based proliferation assay was used to detect optimal coating conditions. The assay bases on a color reaction to measure the number of viable cells. Therefore, it is possible to detect metabolic active cells, but it does not provide evidence about proliferation.

In the initial experiment, 2000-18000 PKC2109 cells were seeded per 96-well and 250-50000 cells were seeded for the standard curve corresponding to (Mosmann 1983). The differences in the achieved cell number between collagen and gelatin coating as well as without were marginal after 48 h. The progression of obtained cell numbers was similar to the generated growth curve. Since more than 13000 cells were seeded per 96-well, cells were in the death phase after 48 h and a decreased number of metabolic active cells could be measured, due to the lack of nutrition and space. Further, there were problems during measurement of standard curve in the values of 250 and 500 cells per well. The MTT-proliferation assay was not sensitive enough for such small amounts of cells. Presumably, this could be the reason for the high difference between collagen and gelatin as well as without coating in comparison to PKCm. For this reason, lower number of cells was used in the experiment and the standard curve started at 2500 cells per 96-well in the following experiments. Moreover, the stated cell numbers are only indicative, because it is difficult to determine a correct cell number after 48 h. The reason therefore is the reference to the standard curve, which is sensitive to various factors, including counting and dilution of cells as well as treatment of MTT after 4 h, which can be freely determined. In growth curve analysis it has been shown that in PFFs and PEFs cell cultures the cell number was reduced after subcultivation in the first 12 h, in contrast to PKC cultures. That implies a variation of seeded cells after 4 h for determination of standard curve, but gave evidence about obtained cell numbers after 48 h.

In summary, the obtained results showed that all investigated cells grew better on coated compared to non-coated plates.

5.3 Comparison of non-viral gene transfer efficiencies

Numerous methods have been applied to introduce exogenous DNA into porcine fibroblasts, including lipid based delivery (Hyun *et al.* 2003), electroporation (Watanabe *et al.* 2005) and viral delivery (Rogers *et al.* 2008b). Each of these methods has been used successfully to generate transgenic piglets. Comparing the transfection methods lipofection, nanofection, conventional electroporation and nucleofection for the genetic modification of PKCs, showed that nucleofection resulted in best transfection efficiency and cell quality.

In lipofection and nanofection experiments the ratio of DNA to lipid is important for efficiency and cellular toxicity (Felgner *et al.* 1987). The adjustment of lipofection parameters was difficult, because amount of DNA, Plus-reagent and lipofection solution had to be adapted to PKCs. Vacuoles and vesicles were detected with increasing amount of DNA concentration and transfection solution and could have caused cytotoxic effects, which were also reported in rat skin fibroblasts with high amounts of lipofectin (Nishiguchi *et al.* 1996). I suggest that the vesicles were formed of big DNA-lipid conglomerates, and the vacuoles probably originate from cell stress during transfection. After splitting, the transfection efficiency was reduced, probably due to stressed and damaged cells which disappeared and untransfected cells which have a better proliferation capacity in comparison to transfected cells. The use of lipofection for transfection of PKCs resulted in a maximum transfection efficiency of ~40 to 50%, whereas the quality of the cells was not acceptable because of high amounts of DNA and lipofection. Results achieved by lipofection are very heterogeneous due to combination of numerous parameters which had to be tested due to a lack of established protocols for transfection of kidney cells. Cells with good quality (rating: 1 to 2) after transfection were obtained after usage of low amount of DNA (0.25 µg), but in these experiments the transfection efficiency was very low (0.5 to 3.8%). Colosimo *et al.* (2000) reviewed that transfection efficiency is dependent on cell number, growth state and their state of differentiation, because those factors influences the total net charge. It has been shown that kidney cell population consists of various cell types with different growth potential, which could influence the efficiency. Further, the transfection efficiency depends on cell division, high rate of endocytosis as well as presence of serum (Ross and Hui 1999; Gresch *et al.* 2004). Not only small DNA constructs, but also BAC-vectors should be transfectable with lipofection technology for site-directed mutagenesis, but McLenachan *et al.* (2007) demonstrated in mouse embryonic stem cells that the nuclear delivery was reduced with increasing plasmid size and linear DNA was transfected with lower efficiency than circular DNA.

The transfection efficiency of nanofection was very low (20-25%) in PKC. To my knowledge only Orth *et al.* (2008) published nanofection efficiencies on various human and mice cell lines. They determined high transgene expression by chemiluminescence in human chondrosarcoma cells, primary cells from human fibrous dysplasia as well as murine fibroblasts (NIH 3T3) but also very low

transfection efficiency were achieved in human osteosarcoma cells and murine skeletal myoblasts.

In comparison of lipofection and nanofection results, no convincing transfection efficiencies were achieved in nanofection experiments. Acceptable transfection efficiencies (40 to 50%) were obtained in PKC using lipofection. However, the liposomes stressed the cells and had cytotoxic effects, but no long-term study was done to assess how cells behave over time.

In addition to the tested chemical transfections, cells are also frequently transfected by physical methods. Using conventional electroporation we achieved a maximum transfection efficiency of 57% in PKCs, but stressed cells as well as high toxicity were observed. Similar cell quality was found in the control transfection without DNA. The reason for this could be the usage of high voltage to achieve DNA transfer into the cells, because with lower voltage (100 V) successfully transfection was not possible. Ross *et al.* (2010) demonstrated in PFF that elevating voltage (100 to 350 V) increased transfection efficiency, while survival of cells decreased. Furthermore, heterogeneous results could be explained by pulse duration of electrical impulse which was clearly visible by using different buffers. Increasing pulse duration resulted in increased permeabilization of mammalian cells and improved transfection efficiency (Wolf *et al.* 1994), but could not be adjusted using Gene Pulser II (Bio-Rad). Moreover, cells were stressed using high amount of DNA (20 µg), probably due to cytotoxic effects of DNA (Sumiyama *et al.* 2010), and general cytotoxicity of electroporation method, which reflects the control approach without DNA.

Nucleofection was applied successfully for transfection of various cell types including difficult transfectable cells, such as primary neurons (Leclere *et al.* 2005) and leukemia cell (Schakowski *et al.* 2004). Furthermore, for many human primary cells nucleofection was more convenient than other chemical transfection methods (Hamm *et al.* 2002). A transfection efficiency of 40% was achieved in human renal epithelial cells using program T-13/T-20 and 3 µg of DNA (Lonza 2012). Nucleofection of PKCs resulted in 82-89% transfection efficiency using program U-12 and 20 µg of DNA, whereas the quality of cells was marginal reduced and amount of dead cells increased exiguous. To evade negative effects of high DNA

amounts, 1-5 μg of DNA was nucleofected $0.5\text{-}1 \times 10^6$ cells with program U-12 resulted in transfection efficiencies between 36-70%. Similar results were achieved in PFF using 10 μg pmaxGFP and nucleofector program U-23 (Nakayama *et al.* 2007). Maurisse *et al.* (2010) reported a transfection efficiency of 90% in PFFs using nucleofector program U-20 and 2 μg of pmaxGFP plasmid. The nucleofection efficiency in PFFs as well as porcine adult dermal fibroblasts were nearly 100% using 3.5 μg GFP-plasmid and nucleofector programs U-20 and U-23, respectively (Skrzyszowska *et al.* 2008). Nucleofection of PFFs resulted in 50 to 53% transfection efficiency using program U-12 and T-16, respectively and 2 μg of DNA. In comparison, higher efficiency of 77% was obtained using program V-13, but including higher amount of dead and stressed cells. Low transfection efficiencies (11 to 34%) were obtained using PEFs regardless of the used nucleofection program. In general, it is necessary to choose the best conditions to obtain acceptable transfection efficiencies but at the same time cells with good quality. Using nucleofection, low cytotoxicity and nearly no change in morphology was detected in transfected PKCs, PFFs and PEFs. Previous studies using the nucleofection technology confirmed no effects on cell properties, such as alteration of cell morphology, response to chemicals and pattern of gene expression (Lakshmiathy *et al.* 2004; Hagemann *et al.* 2006). Furthermore, purification of expression vector influences transfection efficiency (reviewed in Colosimo *et al.* 2000), but there were no differences detectable using commercial pmaxGFPTM plasmid and endotoxin-free prepared pmaxGFPTM plasmid. The transformed pmaxGFPTM plasmid was purified endotoxin-free, because removal of bacterial endotoxins or lipopolysaccharides resulted in increased transfection efficiencies in cultured cells lines such as HeLa cells (Weber *et al.* 1995).

In conclusion, transfection efficiency with 57% was good using electroporation in PKC, but cells appeared extremely stressed. The result after comparing various non-viral transfection methods strongly suggests that nucleofection enables highly efficient gene transfer into PKCs as well as PFFs and PEFs. In addition, nucleofection has no limitations concerning safety, insert size and immunogenic reactions such as viral transduction (Gresch *et al.* 2004; Hendrie and Russell 2005).

5.4 Efficiency of *in vitro/in vivo* SCNT

5.4.1 Effects of donor cell source on *in vitro* development of SCNT embryos

PKCs, PFFs and PEFs were used as donor cells for SCNT to analyze *in vitro* development of SCNT embryos by determination of blastocyst rate and quality which is ascertain by the mean number of cells of an embryo in blastocyst stage.

Table 13 compares the usage of several porcine donor cell types for SCNT and *in vitro* analysis. In various publications the blastocyst rate using PFFs and genetically modified PFFs varied from 7% (Betthausen *et al.* 2000) to 31.2% Onishi *et al.* (2000), whereas in our experiments a blastocyst rate of 9.1% was obtained. Using PKCs as donor cells the blastocyst rate with 21% was higher in comparison to the report of Yin *et al.* (2002) with 4% blastocyst rate after six days of embryo *in vitro* culture. Faast *et al.* (2006) described a blastocyst rate of 18% using PEFs as donor cells, which is four times higher compared to our results (4.3%). A much higher rate (37.2%) was obtained using adult fibroblasts (Colleoni *et al.* 2005) which belongs to the best results next to the application of MSCs as donor cells.

Using bone-marrow derived MSCs for SCNT experiments, very high blastocyst rates between 29.5% (Faast *et al.* 2006) to 64% (Kumar *et al.* 2007) were achieved, in comparison to MSCs derived from peripheral blood in which a blastocyst rate of 18.1% was obtained. The high efficiencies could be explained with better reprogramming properties which were analyzed by Kumar *et al.* (2007). They reported that MSCs derived from bone-marrow were more similar to *in vivo* embryos compared to fetal fibroblasts, regarding to expression of key embryonic genes like *OCT4/NANOG* as well as genes involved in DNA methylation (*DNMT1/DNMT3a*), histone deacetylation (*HDAC2*) and additional gene expression patterns (Kumar *et al.* 2007). On the other hand, a major drawback of MSCs is the verification of differentiation capacity by adipogenesis, osteogenesis and chondrogenesis as well as immunocytochemical analysis after isolation (Bosch *et al.* 2006; Faast *et al.* 2006). Moreover, the differentiation verification is laborious and causes high costs for required media and growth factors.

Table 11: Overview about blastocyst rates using various donor types for SCNT in pig

Cell type	Blastocyst rate [%]	Reference
PFF	7 ⁽¹⁾	Bethhauser <i>et al.</i> (2000)
	6 ⁽¹⁾	Nakayama <i>et al.</i> (2007)
	11.9	Nakayama <i>et al.</i> (2007)
	12.8	Kurome <i>et al.</i> (2008)
	20	Zhu <i>et al.</i> (2004)
	23	Jin <i>et al.</i> (2007)
	26	Hyun <i>et al.</i> (2003)
	31	Kumar <i>et al.</i> (2007)
	31.2 ⁽¹⁾	Onishi <i>et al.</i> (2000)
PEF	18	Faast <i>et al.</i> (2006)
PKC	4*	Yin <i>et al.</i> (2002)
Adult fibroblasts	37.2	Colleoni <i>et al.</i> (2005)
MSCs – BM	29.5	Faast <i>et al.</i> (2006)
	44.7	Colleoni <i>et al.</i> (2005)
	47	Jin <i>et al.</i> (2007)
	64	Kumar <i>et al.</i> (2007)
MSCs – B	18.1	Faast <i>et al.</i> (2006)
Osteocytes	33	Colleoni <i>et al.</i> (2005)
PSOS	27	Zhu <i>et al.</i> (2004)
Preadipocytes	33.7-39	Tomii <i>et al.</i> (2009)
pSGP	27.7	Kurome <i>et al.</i> (2008)

The embryos were cultured *in vitro* for seven days. PFF: porcine fetal fibroblast; PEF: porcine ear fibroblast; MSCs: mesenchymal stem cells derived from bone marrow (BM) and peripheral blood (B), respectively; PSOS: fetal porcine skin-originated sphere stem cells; pSGP: salivary gland-derived progenitor cells; ¹genetically modified donor cells; *embryos were cultured *in vitro* for six days.

Colleoni *et al.* (2005) achieved a blastocyst rate of 33% using osteocytes as donor cells, which were differentiated *in vitro* from MSCs. In general, the efficiency of blastocyst rate was very high with 27-39% using fetal porcine skin-originated sphere stem cells (Zhu *et al.* 2004), preadipocytes (Tomii *et al.* 2009) and salivary gland-derived progenitor cells (Kurome *et al.* 2008) as donor cells for SCNT. However, these cell types are expensive both in isolation and due to the need of growth factors as well as unknown transfectability.

The efficiencies of various donor cell types for SCNT are contradictory. In pig Colleoni *et al.* (2005) demonstrated similar blastocyst rates that were reconstructed with MSCs-BM (44.7%) and adult fibroblasts (37.2%), whereas Faast *et al.* (2006) reported much higher percentage of blastocyst cloned from MSCs-BM (29.5%) than those derived from adult fibroblasts (18%). Unlike, Sung *et al.* 2006 described in mice that cloning efficiency increased over the differentiation hierarchy, because they have reached greatest cloning efficiency using differentiated granulocytes (34.5%)

compared to hematopoietic progenitor cells (10.6%) and hematopoietic stem cells (4.1%-7.9%).

In conclusion, it is difficult to compare cloning efficiencies between different laboratories, because a plethora of parameters influence the outcome of SCNT experiments. Though, in our hands PKCs were more suitable for generation of cloned embryos compared to PFFs and PEFs. Only the comparison of *in vitro* development competence of several different PKC, PFF and PEF cell cultures over time would give a more precise picture of the suitability of different donor cell types.

Further, the amount of cells in a blastocyst stage embryo is a possibility to detect the quality of *in vitro* cultivated embryos (Zhu *et al.* 2004). 200-300 cells/blastocyst were counted of *in vivo* developed blastocyst after seven days (Hunter 1974; Papaioannou and Ebert 1988). Kikuchi (2004) reviewed that the mean cell number per blastocyst was significant different between *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) oocytes (74.4 cells per blastocyst) compared to *in vitro* cultivated embryos after six days (38.4 cells per blastocyst). Further, *in vitro* cultivated embryos did not show hatching or hatched blastocysts compared to IVM/IVF blastocysts which developed also to hatching blastocysts (134 cells per blastocyst) and hatched blastocyst (215 cells per blastocyst), equal to that of *in vivo* matured oocytes (reviewed in Kikuchi 2004).

Using PKCm and PFF26 as donor cells for SCNT, a mean cell number between 43.5 and 56.1 cells per blastocyst was obtained. This cell number is significantly smaller compared to *in vivo* developed blastocyst, but similar to results obtained by other laboratories after SCNT using PFF - 53.5 cells per blastocyst (Kurome *et al.* 2008) and 66 cells per blastocyst (Betthausen *et al.* 2000) after seven days of *in vitro* cultivation. Using PEF0110 as donor cells, the obtained cell number of 23.5 cells per blastocyst was very low. The lower amount of cells in blastocyst stage is probably due to higher amount of dead cells during development. Presumably, at least one arrested and/or dead blastomere of the first four divisions could be responsible for the variations of cell number in the blastocyst stage (Leidenfrost *et al.* 2011).

Taken together, the quality of blastocysts was better using PKCs and PFFs as donor cells compared to PEFs. Certainly, the detection of blastocyst cell number as well as

blastocyst rate allows no conclusion about the development competence *in vivo* after embryo transfer.

5.4.2 An overview of cloned transgenic piglets originating from different donor cells

In the last years the Chair for Molecular Animal Breeding and Biotechnology was able to generate different transgenic pigs using several types of porcine primary cells (PKCs, PFFs, and PEFs) transfected with the nucleofector technology.

Transfection for additive gene transfer or homologous recombination has been done with conventional DNA plasmids that were linearized to remove the backbone which was important for propagation in bacteria. In BAC vectors the risk to achieve undesirable DNA molecules generated by random linearization within the BAC construct prior to the integration is reduced (Giraldo and Montoliu 2001).

In additive gene transfer experiments, litter rates between 41% and 50% were achieved using transgenic PKCs and PFFs, respectively, after the first transfection as nuclear donor cells. In opposite, Hyun et al. (2003) achieved litter rates between 6.7% and 7.7% using untransfected and genetically modified PFFs for SCNT. The cells of several cloned transgenic animals were used for a second transfection. Here, the litter rates were highly different between PKC (52.6%) and PEF (20%) as donor cells, taking into account that only one transfected PEF line was used for SCNT and ET, whereas five different PKC populations were used. As expected, the obtained efficiency after first and second transfection of PKCs as donor cells was similar. In recloning experiments, a similar trend was observed in obtained litter rates using PKCs (45.2%), PFFs (33.3%) and PEFs (26.7%) as donor cells, which is comparable to the *in vitro* development competence of embryos using several donor cells. In addition, also a low efficiency was obtained by cloning of untransfected PFFs with 21%, whereas no litter was achieved after two ETs using PSF. Lai and Prather (2003) got similar cloning success rates between 20-30% using untransfected PFFs. Certainly, there were differences in achieved litter rates using transfected (50%), recloned (33.3%) and cloned PFFs (21%). This could belong to different stages of development and quality of fetuses. In recloning and cloning experiments, cells of animals were used which were already generated by SCNT.

Classical HR is a widely used method for site-directed modifications in the mammalian genome, certainly, the principal limitation of this classical strategy is the low targeting efficiency (reviewed in Vasquez *et al.* 2001). In mice HR in somatic cells is less efficient than in ES cells (Arbones *et al.* 1994; reviewed in Wang and Zhou 2003). Due to the fact, that no true ES cells are available in pigs, gene targeting of somatic cells results in generation of a high number of cell clones and is laborious and costly.

Rogers *et al.* (2008b) verified that homologous recombination depends on donor cells. They targeted fibroblasts from several fetuses obtained from the same uterus at the same time. After targeting which was done by the same people and with same reagents, they achieved targeting frequencies between 0.07 to 10.93% (Rogers *et al.* 2008b). Using PFFs, we were not able to knock-out a gene on cell culture level, probably, due to their reduced growth potential, resulting in low number of cell clones after transfection and selection. On the other side, four different loci could be targeted using PKCs. SCNT with these successful targeted PKC cell clones resulted in cloning efficiencies from 37-50%.

Overall, these data prove that primary PKC as well as PFFs are highly sufficient for generation of genetically modified pigs by additive gene transfer. PEFs as donor cells were unsuitable for SCNT, because of low efficiency of obtained litter rates. Only with PKCs site-directed mutagenesis experiments were successfully.

5.5 Outlook

During this thesis, several donor cell sources were characterized and compared for generation of transgenic pigs using SCNT. This is a very important point, but merely one way to increase the efficiency of generating transgenic piglets. In future projects, the focus could be on manipulation of the reprogramming processes of epigenetic modifications, because this is a crucial part for dedifferentiation of donor nuclei during SCNT. It is presumed that the low cloning efficiency is largely attributable to the incomplete and faulty reprogramming of epigenetic modifications (Bourc'his *et al.* 2001; Xue *et al.* 2002; Enright *et al.* 2003). There are several ways to influence epigenetic modifications, for instance the chemical treatment of donor cells, oocytes as well as embryos for more efficient epigenetic reprogramming. In pig several

studies showed higher blastocyst rate after treatment with histone deacetylase inhibitor Trichostatin A of *in vitro* cultivated porcine embryos and in SCNT embryos using PFFs as donor cells (Jeseta *et al.* 2008; Zhao *et al.* 2009; Himaki *et al.* 2010). Moreover, Kong *et al.* (2011) demonstrated the reactivation of silenced transgene after treatment with DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine and/or with Trichostatin A in PFFs.

Another possibility would be the improvement of the selection system for site-directed mutagenesis. The used BAC-vectors which were applied for site-directed mutagenesis contain a positive selection cassette such as blasticidin or geneticin resistance gene. The combination of a positive-negative-selection (PNS) strategy could increase the efficiency of obtained targeted clones. Cells are selected on both integration of the targeting vector due to positive selection and on the HR event as well as the resulting loss of the negative selection cassette. In murine ES cells 79% targeting efficiency was achieved with PNS strategy (Mansour *et al.* 1988) and 30% targeting efficiency was obtained in a rat fibroblasts (Hanson and Sedivy 1995).

6 SUMMARY

Genetic modification of primary cells for the generation of transgenic pig models using somatic cell nuclear transfer

The generation of transgenic pig models is increasingly being established for selected human diseases, particularly because of physiological and anatomical similarities with humans. Somatic cell nuclear transfer (SCNT) technique was applied to create number of genetically modified pigs. Thereby, the type of employed donor cells plays a crucial role and should comply with many criteria including efficient isolation, simple culture conditions, high proliferation capacity and genetic stability over long time culture as well as good *in vitro* development competence of cloned embryos, high transfection efficiencies in combination with low cytotoxicity.

The aim of this study was the characterization of porcine kidney cells (PKCs) as novel donor cell source for generation of transgenic pigs via SCNT and the comparison of these cells with widely used porcine fetal fibroblasts (PFFs) as well as porcine ear fibroblasts (PEFs). PKC cultures demonstrated large morphological variations of cell types and were capable to be passaged up to P71, whereas PFFs and PEFs could hardly passaged longer than 15-20 times. Moreover, kidney cells exhibited a higher proliferation rate and a twofold higher blastocyst rate was obtained after SCNT and *in vitro* culture. Therefore, PKC culture was used to established optimal transfection protocols using lipofection, nanofection, conventional electroporation as well as nucleofection. The cells were transfected with green fluorescent protein (GFP) as reporter gene. The highest transfection efficiencies (70-89%) were obtained using the nucleofection procedure. Moreover, cells showed high fluorescence intensity, low cytotoxicity and a good proliferation, with almost no signs of stress. Furthermore, nucleofection resulted in acceptable transfection efficiencies in both PFF (77%) and PEF (34%).

Using PKCs as donor cells for SCNT, as well as PFFs and PEFs, our lab accomplished successful the generation of various genetically modified pigs. In conclusion, PKC showed better proliferation rate, growth capacity, transfection efficiency and blastocyst rate after SCNT compared to PFFs and PEFs. Furthermore

SUMMARY

primary porcine kidney cells are highly sufficient for production of genetically modified pigs by gene targeting and additive gene transfer.

7 ZUSAMMENFASSUNG

Genetische Veränderung von primären Zellen zur Erzeugung transgener Schweinemodelle durch somatischen Kerntransfer

Die Generierung von transgenen Schweinemodellen gewinnt zunehmend an Bedeutung für die Erforschung und Behandlung verschiedener Krankheiten des Menschen, vor allem wegen ihrer physiologischen und anatomischen Ähnlichkeiten. Die Technik des somatischen Kerntransfers (SCNT) wurde verwendet, um transgene Schweine zu erzeugen. Hierbei spielt die Art der verwendeten Spenderzellen eine entscheidende Rolle und sollten folgende Kriterien erfüllen: effiziente Isolierung, einfache Handhabung während der Kultivierung, stabiler Chromosomensatz auch in hoher Passagenzahl, hohe Transfizierbarkeit in Verbindung mit geringer Zytotoxizität sowie eine gute Entwicklung der Embryonen *in vitro* nach dem SCNT.

In dieser Arbeit werden Nierenzellen aus dem Schwein (PKCs) als neuartige Spenderzellen für den Kerntransfer beschrieben, charakterisiert und mit fetalen Fibroblasten (PFFs) und Ohrfibroblasten (PEFs) aus dem Schwein, welche breite Anwendung für den SCNT finden, verglichen. PKCs zeigten in Kultur große zellmorphologische Unterschiede und konnten bis zur Passage 71 kultiviert werden, wohin gegen PFFs und PEFs kaum mehr als 15 bis 20mal passiert werden konnten. Zudem zeigten PKCs eine höhere Proliferationsrate und die Entwicklungsrate von Blastozysten *in vitro* war doppelt so hoch nach der Verwendung der Zellen für den SCNT als mit PFFs und PEFs. Aus diesem Grund wurden für die PKCs die optimalste Transfektionsmethode aus Lipofektion, Nanofektion, der herkömmlichen Elektroporation und Nukleofektion bestimmt. Dazu wurden die Zellen mit dem grün fluoreszierenden Protein (GFP) als Reportergen transfiziert. Unter der Verwendung der Nukleofektion wurde die höchste Transfektionseffizienz (70 bis 89%) ermittelt, sowie eine hohe Fluoreszenzintensitäten und geringe Zytotoxizität erzielt. Die Nukleofektion eignete sich ebenfalls für die Transfektion von PFFs (77%) und PEFs (34%) mit denen akzeptable Transfektionseffizienzen erreicht wurden.

Unserem Labor gelang unter anderem die erfolgreiche Generierung von transgene Schweinemodellen unter der Verwendung von PKCs als Donorzellen für den SCNT.

Zusammenfassend zeigten die Nierenzellen aus dem Schwein bessere Proliferationsraten, Transfektionseffizienzen und bessere Blastozystenentwicklung *in vitro* nach dem SCNT als vergleichende Analysen mit PFFs und PEFs. Darüber hinaus wurden die PKCs sehr erfolgreich für additiven Gentransfer und das Einbringen gezielter Mutationen verwendet, welches Grundvoraussetzungen für die Generierung transgener Schweine sind.

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11 ACKNOWLEDGMENT

First of all I would like to thank Prof. Dr. Eckhard Wolf for giving me the great opportunity to perform my doctoral thesis at the Chair for Molecular Animal Breeding and Biotechnology (LMU-Munich) as well as for his support, motivation, helpful discussions throughout my work and great enthusiasm for science.

I would like to thank my supervisor Dr. Annegret Wünsch for suggestions, her scientific and non-scientific support and helpful discussion. Thank you for mentoring me and spending lot of time during this thesis.

Many thanks also to all my colleagues from the Chair for Molecular Animal Breeding and Biotechnology, especially Dr. Mayuko Kurome, Dr. Barbara Kessler, Dr. Valeri Zakartschenko and Tuna Güngör for carrying out the nuclear transfer, help, nice conversations and good atmosphere. Moreover, thanks a lot to Dr. Simone Renner for her kindness, support, fruitful discussion and someday we will go by dogsled through the wilderness ;-) and Eva-Maria Jemiller.

Thanks to my fellow doctoral students Katinka Burkhart, Katrin Krähe, Lisa Streckel, Christina Braun, Stephanie Sklenak, Jens Popken, Mai Le, Marieke Matthiesen, Sudhir Kumar and Andrea Beck for their input, exchange of ideas and friendship. Hugh thanks to Pauline Fezert for the great time, moral support, jogging and tea time breaks.

My deepest gratitude goes to my husband Tobias Richter for his love, continues support, motivation and that you always believed in me. Especially at a time, both of us were working on our doctoral theses. You are the best husband one can imagine.

Ich liebe Dich!

A special thanks to my family, who have accompanied me all the time, supported and believed in me and have given me the opportunity to develop myself.

Huge thanks to Julia Vulprecht for your unlimited joy, patience, inspiration and particularly long phone calls, which rebuilt and encouraged me.