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Inducible expression of RANKL in transgenic pigs under the control of the Tet-On system

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zur Erlangung der tiermedizinischen Doktorwürde

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ABBREVIATIONS

°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
AC	alternating current
AD	Alzheimer's disease
AHXR	acute humoral xenograft rejection
ALP	alkaline phosphatase
APP	amyloid precursor protein gene
bGH	bovine growth hormone
Bla	blasticidin
BMD	bone mass density
BSA	bovine serum albumin
BSD	blasticidin-S deaminase
CAG	CMV early enhancer and chicken beta-actin promoter
CB	cytochalasin B
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator (CFTR)
CMV	cytomegalovirus
COCs	Cumulus-oocyte complexes
DC	direct current
DNA	deoxyribonucleic acid
Dox	doxycycline

e.g.	for example
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
END	early neonatal death
eNOS	endothelial cell nitric oxide synthase
et al.	and others
ET	embryo transfer
ExperiMed	Experimental Surgery and Regenerative Medicine
FBS	fetal bovine serum
Fig.	Figure
FSH	follicle-stimulating hormone
g	gram
GFP	green fluorescent protein
h	hour(s)
HAR	hyperacute rejection
hCG	human chorionic gonadotropin
ICSI	intracytoplasmatic sperm injection
IU	international unit
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
JNK	c-jun N-terminal kinase
l	liter
LH	luteinizing hormone
Lox P	locus of X-over P1

LTR	long terminal repeat
mg	milligram
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
MODY3	maturity-onset diabetes of the young type 3
mRNA	messenger RNA
MSC	mesenchymal stem cell
NCSU-23	North Carolina State University medium-23
NF-kappaB	nuclear factor kappa beta
NO	nitric oxide
NT	nuclear transfer
OPG	osteoprotegerin
ORF	open reading frame
OVX	ovariectomy
pA	(polyA) polyadenylation signal
PBS	phosphate buffered saline
Pcmv	minimum promoter from human cytomegalovirus
PEF	porcine ear fibroblast
PFF	porcine fetal fibroblast
PGE2	prostaglandin E2
PKC	porcine kidney cell
pmol	picomolar
pRANKL	porcine RANKL

PSEN	presenilin gene
PTH	parathyroid hormone
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidon
PZM	porcine zygote medium
RANK	receptor-activator of nuclear factor kappa beta
RANKL	receptor-activator of nuclear factor kappa beta ligand
RNA	ribonucleic acid
RRE	Rev-responsive element
rtTA	reverse tetracycline-controlled transcriptional activator (Tet-On)
SCNT	somatic cell nuclear transfer
SD	standard deviation
SMGT	sperm mediated gene transfer
sRANKL	soluble RANKL
Src	sarcoma (proto-oncogenic tyrosine kinases)
TA	transcriptional activator
TALP	Tyrode´s albumin lactate pyruvate
TARE	Tet-advanced transactivator response element (TRE-Tight)
Tet	tetracycline
TetO	tetracycline operator
TetR	tetracycline repressor
TG	transgenic
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor family

TRE	tetracycline response element
tTA	tetracycline-controlled transcriptional activator (Tet-Off)
TU	Trächtigkeitsuntersuchung (pregnancy control)
vs.	versus
WHO	World Health Organization
WT	wild-type
αGal	α-1,3-Galactosyl
αGalT	α-1,3-galactosyltransferase

I. INTRODUCTION

Before going into detail of the purpose and the need of laboratory animal models, it should be clarified how to define this term. One example for defining gives the U.S. National Research Committee on Animal Models for Research on Aging, which reads as follows: “an animal model in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal”. Most of the times when the term “animal model” is used, the true meaning is modeling humans. Consequently it would be more correct to speak of “human model” on this context. Recently, just as well as in the past 150 years, the majority of laboratory animal models are developed and used to study the cause, nature, and cure of human disorders (SVENDSEN & HAU, 1994). The successful development of transgenic animal models for human diseases has led to remarkable breakthroughs that have a major impact on the diagnosis, treatment and intervention in human diseases. Furthermore they have helped to clarify our understanding of disease mechanisms, as well as the onset and course of disease pathology. Because no single animal model meets all necessary requirements to exactly mimic the human circumstances, it is the obligation of researchers to identify the best and most appropriate animal model that is adequate for a specific study (CHAN, 2004; CHAN & YANG, 2009). The question is which animal on the one hand fits best these requirements of modeling the human conditions and on the other hand is feasible in financial and practical terms. In the long period of domestication, presumably 9000 years (LARSON et al., 2005), the pig was not only selected for meat quality but also for high fertility. The pig is used as a livestock species in most parts of the world and people accept killing of swine for human nutrition. Livestock pig breeds and miniature pig breeds are already commonly used in various fields of research. In human medicine they are used for different purposes for example in surgery, internal medicine, nutritional medicine and infectious diseases (SMITH & SWINDLE, 2006; LUNNEY, 2007). Pigs are contributing not only to research but also to therapy: bioprostetic heart valves originating from pigs are commercially available and routinely used for transplantation to human patients (BROWN et al., 2009). Through this long time of experience in usage of

pig in the laboratory many technical procedures have been already established, like for example pig handling, husbandry, anesthesia, analgesia and perioperative care, and are now available for non-transgenic and transgenic pig models. Reproductive technology and techniques of genetic modifications have considerably advanced in the last years (WOLF et al., 2000; HOUDEBINE, 2005). In contrast to traditional laboratory animals like rodents, pigs have a relatively long lifespan and are more similar to humans concerning their organ size, anatomy and physiology (VAJTA et al., 2007). Especially for chronic diseases, where it will take years until the specific phenotype will show, a long lived animal model is needed. For all these reasons our choice fell on the pig to establish a transgenic animal model for human osteoporosis, based on osteoclast deregulation. The excessive resorption of bone tissues should be stimulated by a regulated expression of RANKL (receptor-activator of nuclear factor kappa beta ligand). One major limitation in transgenic pig production is the efficiency in somatic cell nuclear transfer. Most of the transferred SCNT embryos do not develop full term, so that the number of born transgenic SCNT piglets still is quite low (VAJTA et al., 2007; KEEFER, 2008). The subject of this thesis was the elucidation of the generation process of transgenic pigs via SCNT exemplified by the generation of RANKL transgenic pigs. Nuclear transfer and embryo transfer data from our laboratory of the years 2006 until 2010 were examined to identify additional features that influence overall SCNT efficiency. As an essential part of the DFG research group “Mechanisms of fracture healing and bone regeneration in osteoporosis” (DFG 793/ project 2: http://ufbweb.medizin.uni-ulm.de/dfg793/), this project created the basis for the first transgenic large animal model for osteoporosis, which will be used for fracture healing studies by other research group partners.

II. LITERATURE

1. Osteoporosis in general

1.1. Pathophysiology of osteoporosis

1.1.1. Definition

The chronic skeletal disorder osteoporosis is described by low bone mass and microarchitectural deterioration of bone tissue, which leads to an increased fracture risk (REINWALD & BURR, 2008; RAHMANI & MORIN, 2009). The World Health Organization (WHO) defines osteoporosis in humans as a bone mineral density that is 2.5 standard deviations (SD) below the mean value of healthy young adults (*T*-score) (EGERMANN et al., 2005; SIPOS et al., 2009). Additionally, osteoporosis changes the course of fracture healing in terms of diminished amount and speed of callus formation (EGERMANN et al., 2005). The homeostasis of bone formation is disturbed through either too much absorption or too less build-up of bone tissue.

1.1.2. Bone remodeling

The most important cell types of bone are osteoblasts and osteoclasts. In the organ system bone, there is a life-long state of repairing, adapting bone constitution and maintaining calcium and phosphorus level (KEARNS et al., 2008; WRIGHT et al., 2009). Bone homeostasis is maintained by a balance between bone-forming cells, the osteoblasts, and bone-resorbing cells, the osteoclasts (NANES & KALLEN, 2009). Osteoclasts are derived from hematopoietic progenitors, whereas osteoblasts differentiate from mesenchymal stem cells. In a physiological state there is a balance between activity of osteoblast and osteoclast cells, but in osteoporotic patients this balance is disturbed. This balance is regulated through various hormones and cytokines. Complete osteoclastogenesis and osteoclast activity can only be performed if RANKL is present (YAVROPOULOU & YOVOS, 2008).

1.1.3. Clinical aspects

There is a clear relationship between aging and the incidence of osteoporosis. Women are affected more often than men, due to the postmenopausal estrogen

deficiency (RAHMANI & MORIN, 2009; SIPOS et al., 2009). Typically the disease proceeds without any clinical symptoms and doesn't become apparent until a bone fracture occurs. Screening for individual fracture risk is still not feasible in usual clinical settings. To estimate the progression of the disease, the following factors have to be taken into account: bone architecture and geometry, mineralization, microdamage accumulation, and properties of the collagen and mineral matrix (NANES & KALLEN, 2009). Around the age of 30 years adult people reach their peak bone mass, and then bone density continues to fall in both sexes, with loss accelerating in women after menopause. The rate of loss is similar in older men and postmenopausal women (NANES & KALLEN, 2009). Physical activity has a major impact on incidence of osteoporosis. Weight and bone mass index are strongly aligned to bone mineral density in women and elderly men. Additionally weight affects bone mineral density as a load factor (FELSON et al., 1993). Dietary patterns and especially protein intake as well have an impact on bone mineral density (HANNAN et al., 2000; TUCKER et al., 2002).

1.1.4. Classification

There is a basic classification into primary and secondary osteoporosis. The primary form shows in a decrease of bone mineral density during the course of aging and the subsequent sex hormone changes. Bone loss in primary osteoporosis is caused by estrogen deficiency in postmenopausal women and aging men (senile osteoporosis) (SIPOS et al., 2009). Most frequently the cause of secondary osteoporosis is glucocorticoid medication. But there are also a variety of medical conditions and other medications that can affect bone turnover. Prevalently primary osteoporosis holds the biggest fraction of all cases (BONURA, 2009).

1.1.5. Fragility fractures and health risk

A normal human being ought to be able to fall from standing height without breaking bones, therefore a fragility fracture indicates pathologic weakness of the skeleton (NANES & KALLEN, 2009). Epidemiological studies showed that 2 million fractures or even more occurred every year in the United States caused by osteoporosis (BURGE et al., 2007; NANES & KALLEN, 2009). After a fragility fracture, caused by osteoporosis, the risk of subsequent fractures is significantly elevated. The study accomplished by Bliuc et al. demonstrated that there is an increased mortality following all major types of osteoporosis related fractures and

even after minor fractures with older age. Mortality risk was highest in the first 5 years following all types of fractures (BLIUC et al., 2009). Especially hip fractures had a fatal follow-up, which displayed in mobility loss that most of the times will never fully reach previous status and finally ended in the requirement of long term care (BONURA, 2009).

1.2. Economic burden

The prominence of osteoporosis is expected to increase rapidly in the close future, because of the demographic changes. The group of Häussler et al. examined cases of osteoporosis during the year 2003 in Germany. They calculated the total direct cost resulting from osteoporosis and the outcome was 5.4 billion Euros. They also estimated the average cost of an osteoporotic patient with a fracture to 9962 Euro and to 281 Euro without a fracture. The Central Bureau of Statistics in Germany figured that about 4 to 6 million people are affected by osteoporosis. It was predicted that in the year 2013 the number of patients will grow up to 990 million persons. The study shows that osteoporosis imposes a considerable economic burden for the healthcare system in Germany (HAUSSLER et al., 2007). In the United States costs to the health care system aligned to osteoporosis and related fractures were estimated at $17 billion in the year 2005 (BURGE et al., 2007; BONURA, 2009). This setting is probably due to impaired fracture healing in the osteoporotic bone, leading to an increased failure rate of implant fixation. There have been reported failure rates of up to 50% for osteoporotic patients, mostly due to a pull-out or cut-through phenomenon (EGERMANN et al., 2005).

2. Large animal models for osteoporosis

2.1. Need for large animal models

When animal models were applied for the study of osteoporosis some difficulties could be sidestepped which occur in human study groups, such as the variability seen in both genetic background and environmental influences and the inability to obtain human tissues for molecular analysis (LIND & SEMSARIAN, 2006). In addition, results of in vitro studies of human tissues can be difficult to extrapolate to the in vivo situation (PEARCE et al., 2007). Cell cultures and other in vitro assays might help to investigate the reaction of normal and osteoporotic cells subsequent to different kinds of stimuli, but they lack the effects of central control of bone turnover and the feedback mechanisms involved in physical activity

(EGERMANN et al., 2005). One obstacle to the understanding of postmenopausal osteoporosis is that this disease is restricted to humans and does not naturally occur in other species. Due to the chronically progressive nature of the disease it is necessary to employ long-time studies of several years duration, resulting in a protracted progress in research. Accordingly, collection of data costs a lot of time and preservation of study groups can be very difficult (TURNER, 2001). Individual behavior like smoking, alcoholism, inadequate nutrition, and insufficient physical activity were reported to have an effect on the incidence of osteoporosis (BONURA, 2009). As a consequence creating a homogenous study group is problematic and data is impaired by relatively high variance. The gain of using an animal model is the availability of more uniform experimental material and the possibility of extensive testing of potential therapies. Large animal models provide also the possibility of testing new prosthetic devices which than can be optimized to fulfill all requirements of the human osteoporotic bone. Drug therapy trials and orthopedic implant testing in large animal models can be accomplished at a level of experimental control impossible in human clinical research (TURNER, 2001). Regulatory guidelines for preclinical evaluation of new experimental drug therapies to treat or prevent postmenopausal osteoporosis require the use of two species to assess bone safety: the rat, because it is well characterized, and a second large long-lived animal model with intracortical bone remodeling (THOMPSON et al., 1995; SMITH et al., 2009). The often used rodent models have the disadvantage of dissimilar anatomy and physiology of bone compared to human, limitation of size, and their short life-span. In consequence it is impossible to test human implants, gather greater amount of tissue samples, and to perform long-time studies of several years duration (PEARCE et al., 2007). Rodents show no intracortical bone remodeling, they lack structures as Haversian canals, and they have a different form of fracture healing when compared with humans (REINWALD & BURR, 2008).

2.2. Sheep as animal models

Adult sheep have a similar body weight to humans and the size of their long bones is suitable for human implants and prostheses. It is described that sheep predominantly have a primary bone structure on the contrary to humans with mainly secondary bones. Sheep also display a notably higher bone density and consequently a higher strength when compared to humans (PEARCE et al., 2007).

Due to their body size it is possible to implant prosthetics and to obtain large amounts of blood and urine samples as well as bone biopsies (SIGRIST et al., 2007). Older sheep display Haversian bone remodeling (ZARRINKALAM et al., 2009). Sexual cycles of different sheep breeds are generally seasonally polyestrous, but some breeds have an almost continues cycles (e.g. Merino). Seasonal changes in the bone density have been observed in the sheep, which could be caused by the periods of anestrous linked to the changing photoperiods throughout the year (TURNER, 2001). The sheep is a quadruped and herbivore animal, which leads to a different physiology when compared with humans (REINWALD & BURR, 2008). Aged sheep showed 6 months after ovariectomy an 8-10% loss of cortical bone and <1% loss of cancellous bone at the distal tibia (LILL et al., 2000; REINWALD & BURR, 2008). Thus in the sheep a maximum of 10% bone mass reduction could be achieved through estradiol withdrawal and aging, whereas in osteoporotic humans this value is at least fourfold higher (ROSS, 1996; SIGRIST et al., 2007). When osteoporosis was induced with ovariectomy, additional low calcium diet and steroid injection over 6 months, the bone mineral density decreased by more than 25% (ZARRINKALAM et al., 2009). To study the long-term effects of ovariectomy on bone metabolism, the group of Sigrist et al. operated six ewes and observed them over a period of 18 months. They could show that most of the bone loss (-13%) occurred during the first 4 months. Between 7 and 9 months the bone mass appeared to stabilize at the mentioned osteopenic level. Afterwards the bone mass returned to pre-ovariectomy levels and remained at this value for the rest of the study. Further on their studies revealed that there was a significant drop of systemic estrogen levels after ovariectomy, followed by increasing values until they finally reach again the basic level same as the control group. Therefore, it appears that the destructive effect of ovariectomy on sheep bone metabolism is a reversible process and the standard bone parameters are reestablished within 6 months after surgery (SIGRIST et al., 2007). Another approach to reduce the bone volume of sheep was achieved through intracerebroventricularly leptin injection. This report elucidated that bone remodeling is also regulated by the central nervous system (POGODA et al., 2006).

2.3. Dog as animal models

Depending on the size and breed of dog, there may be some discrepancy in the

size and shape of canine bones in comparison to human bones. While adult human bone has a secondary osteonal structure, the canine bone is found to have a mixed microstructure of secondary osteonal bone and plexiform bone. Canine bone has a significantly higher mineral density than human bone. The rate of bone remodeling is different compared with humans (PEARCE et al., 2007). Dogs have already been used in studies modeling the human skeletal conditions like for example fracture healing, effects of immobilization, long-term effects of certain bone-active agents and allografts (TURNER, 2001). Dogs are like the human monogastric, but in their sexual cycle they are quite different. Female dogs ovulate twice a year and are therefore diestrus. So it appears that dogs have unchanged hormone levels during their long lasting periods of anestrus (TURNER, 2001). Despite some similarities between human and dog bone, a number of researchers remain uncertain of the potential of ovariectomy to induce significant bone loss in dogs. Results among laboratories have either differed or have been entirely encouraging (REINWALD & BURR, 2008). There is a report of beagles showing a 8-10% loss of bone density in the vertebrae in 8 to 12 months post ovariectomy (DREZNER & NESBITT, 1990). Ovariectomy in dogs leaded to either decreased or unchanged cancellous bone volume, whereas bone mineral density (BMD) was mostly unchanged. Additionally the fracture rate among ovariohysterectomized pet dogs, even in older age, is not comparable to those of postmenopausal women (EGERMANN et al., 2005). Although in the period of anestrus the levels of estrogen are extremely low, spontaneous fractures in dogs are almost unknown. Additionally the routinely removal of both ovaries and uterus appealingly does not lead to a major bone loss (TURNER, 2001).

2.4. Nonhuman primates as animal models

The nonhuman primate is the most closely related species and therefore the most physiologically similar to humans (SMITH et al., 2009). Old World monkeys such as baboons and macaques organ system, like the gastrointestinal tract, endocrine system and bone metabolism, closely resemble those of humans (NEWMAN et al., 1995). Females have a monthly cycle of 28 days and hormonal patterns including estrogen and progesterone levels similar to humans. Declining ovarian function and irregular menstrual cycles occur in macaques over 20 years and in baboons over 15 years of age (EGERMANN et al., 2005). However, spontaneous menopause does not occur in most nonhuman primates (NEWMAN et al., 1995).

The long life-span of nonhuman primates is also important in bone studies, but on the other hand also challenging since cynomolgus monkeys are typically at least 9 years, and rhesus monkeys at least 12 years old, reflecting the reported age that they reach peak bone mass (SMITH et al., 2009). Osteoporosis only occurs naturally in humans and nonhuman primates. In a study, rhesus monkeys showed a decline in bone density with advancing age and several aged individuals developed vertebral wedge fractures (CERRONI et al., 2000, 2003; EGERMANN et al., 2005). This report is the only one describing natural occurring osteoporosis and spontaneous fractures in animals. Ovariectomy in cynomolgus monkeys lead to a bone loss of -1.4 SD compared with healthy control animals (EGERMANN et al., 2005). In humans osteoporosis is defined as a BMD 2.5 SD or more under the mean value of healthy young adults. In other studies nonhuman primates showed a loss of BMD of 3% to 7% from the baseline value 16 to 18 months after ovariectomy. They lost bone mass after OVX, but the extent of the bone loss cannot be described as osteoporotic (SMITH et al., 2009). Additional strategies, like steroid medication or/and calcium low diet, are therefore necessary to induce significant bone loss comparable to human osteoporosis patients (EGERMANN et al., 2005).

2.5. Pig as animal models

Because of the well-characterized and striking anatomical similarities between pigs and humans, swine are used in biomedical research as relevant models for numerous human conditions and diseases, and have emerged as the preferred donor for xenotransplantation (VODICKA et al., 2005; SMITH & SWINDLE, 2006; LUNNEY, 2007; REINWALD & BURR, 2008). In both species bone metabolism is significantly affected by diet and nutrition. Humans and swine are both monogastric and true omnivores, so that the gross gastrointestinal physiological function of swine is more similar to humans than that of most other species (REINWALD & BURR, 2008). Domestic pig shows a rapid growth and attains body weights of 150 to 200 kg at maturity. Many authors describe pig as noisy and stubborn or even aggressive animal. Pigs are social and intelligent animals and, when handled and housed correctly, aggression problems are less likely encountered (SMITH & SWINDLE, 2006; REINWALD & BURR, 2008). The sexual cycle of the pig is quite similar to that of human and it is continuous throughout all seasons. In contrary to humans the pigs are quadrupeds and

therefore have different weight distributions. Their size permits the introduction of prosthetic implants and also it is possible to apply repetitive bone biopsies and to obtain large amounts of blood samples (NEWMAN et al., 1995). With regard to bone anatomy, morphology, healing and remodeling, the pig bone is considered to be closely representative of human bone. While having a denser trabecular network, the pig has a human like lamellar bone structure. Pigs have a similar rate of bone regeneration and cortical bone mineralization to humans (PEARCE et al., 2007). There is a close similarity of bone remodeling rates of pigs and humans and an overlap in Haversian canal diameters and secondary osteon dimensions among humans and pigs (REINWALD & BURR, 2008). Swine reach their peak bone mass at the age of 2.5 to 3 years, thereafter their bone mineral density begins to drop (TURNER, 2001). They are one of the few species where spontaneous vertebral fractures have been reported. These fractures occurred after pregnancy and during the lactation period, a disease that could be termed "porcine lactational osteoporosis" (SPENCER, 1979). Ovariectomy of sows did not significantly affect the variables of bone chemical and histological analyses (SCHOLZ-AHRENS et al., 1996). In Sinclair minipigs a (0.75%) calcium restricted diet in combination with ovariectomy resulted in a 7-10% reduction in spinal bone mineral density (BOYCE et al., 1995). Glucocorticosteroid-induced osteoporosis in adult Göttingen miniature pigs lead to bone mineral density depression of -10% from baseline. In the long term a slight further decline of 1% was observed (SCHOLZ-AHRENS et al., 2007).

2.6. Comparing different large animal models

Surely most similarities are found between old world monkeys and humans, but legal restrictions and housing conditions for the animals often create public distaste for their use. These ethical concerns and the relatively high risk of zoonotic disease transmission makes this model inconvenient (TURNER, 2001). In addition, housing and handling are demanding and therefore expensive, especially because the monkeys have to reach a certain age before they can be used (NEWMAN et al., 1995). It has to be taken into account that, in an economic climate, where financial resources for research are becoming increasingly limited, the choice of experimental animals must be scientifically and ethically justifiable as well as cost-effective (REINWALD & BURR, 2008). The second best model resembling the human physiological conditions, especially in the bone, is the

porcine large animal model (see table below) (PEARCE et al., 2007; REINWALD & BURR, 2008). Dogs or monkeys provoke emotional attachments and are individualized unlike farm animals as pigs or sheep (TURNER, 2001). Although osteoporosis can be induced in several animal models, spontaneous fractures without adequate trauma were only found in nonhuman primates. Ovariectomy alone seems to be adequate to reduce the mechanical properties of bone but does not reduce bone mineral density to levels comparable with those seen in osteoporotic human patients. Models using steroid medication combined with ovariectomy and calcium-wasting diet show the most severe bone loss (EGERMANN et al., 2005). Another limitation of osteoporosis animal models is that commonly female individuals were utilized for trials and the osteoporotic conditions in the senile male are overlooked. All of the animal models presented above have certain differences from humans and it is not yet proven that they accurately resemble the situation found in osteoporotic patients (TURNER, 2001). Today there is no truly satisfying large animal model for osteoporosis at hand (REINWALD & BURR, 2008). Future investigations must address the finding that the elimination of ovarian function does not always lead to osteoporosis and a high risk of fracture (EGERMANN et al., 2005).

Table 1: Summary of four key attributes in terms of similarity between animal and human bone (PEARCE et al., 2007)

	Canine	Sheep/Goat	Pig	Rabbit
Macrostructure	++	+++	++	+
Microstructure	++	+	++	+
Bone Composition	+++	++	+++	++
Bone Remodelling	++	++	+++	+

+ least similar, ++ moderately similar, +++ most similar

3. Relevance of RANKL in health and disease

3.1. Role of RANKL/RANK/OPG system in bone remodeling

3.1.1. RANKL/RANK/OPG signaling

The discovery of the RANKL/RANK/OPG system in the late 1990s greatly improved the understanding of bone remodeling and maintenance of the skeletal structure (BOYCE & XING, 2008; WRIGHT et al., 2009). Three key proteins are responsible for the regulation process of bone resorption: RANK (receptor-activator of nuclear factor kappa beta), its ligand RANKL (receptor-activator of nuclear factor kappa beta ligand) and a decoy receptor OPG (osteoprotegerin). This system is controlled by many osteotropic hormones (parathyroid hormone (PTH), 1,25dihydroxyvitaminD3 (Vitamin D3a), prostaglandin E2 (PG E2), estrogen, testosterone and prolactin) and cytokines (inflammatory cytokines e.g. interleukines: IL-1α, IL-1β, IL-6b, IL-11 and IL-17), tumor necrosis factor alpha and beta (TNFα and TNFβ) and transforming growth factor beta (TGF-β)) (WRIGHT et al., 2009). Osteoclasts' number and activity are closely dependent on the ratio of RANKL/OPG and every change in this ratio will have an effect on bone turnover (BOYCE & XING, 2008). The RANK/RANKL/OPG signaling system is essential for skeletal homeostasis and plays a major role in most animal models of bone diseases characterized by increased resorption (BOYCE & XING, 2008). RANK-RANKL signaling not only activates a variety of downstream signaling pathways required for osteoclast development, but also fine-tunes bone homeostasis both in normal physiology and disease via crosstalk with other signaling pathways. Interestingly all factors that inhibit or enhance bone resorption by osteoclasts also positively or negatively influence RANKL and OPG mRNA levels as well as protein levels (LEIBBRANDT & PENNINGER, 2008).

3.1.2. RANKL

RANKL is a member of the tumor necrosis factor (TNF) family, respectively its receptor RANK and its decoy receptor OPG belong to the TNF receptor family (KEARNS et al., 2008). RANKL exists as a homotrimeric protein and is typically membrane-bound on osteoblasts and activated T cells. There is also a soluble form which is secreted either through proteolytic cleavage of the membrane form or through alternative splicing. Most factors known to stimulate osteoclast formation and activity induce RANKL expression by osteoblastic stromal cells

(BOYCE & XING, 2008). Membrane bound RANKL ensures cell-cell contact allowing only microenvironmental function, whereas soluble RANKL can diffuse to the target cells leading to a systemic function. When more RANKL is released by osteoblasts, bone resorption will be increased through osteoclast differentiation, activation and survival (WRIGHT et al., 2009).

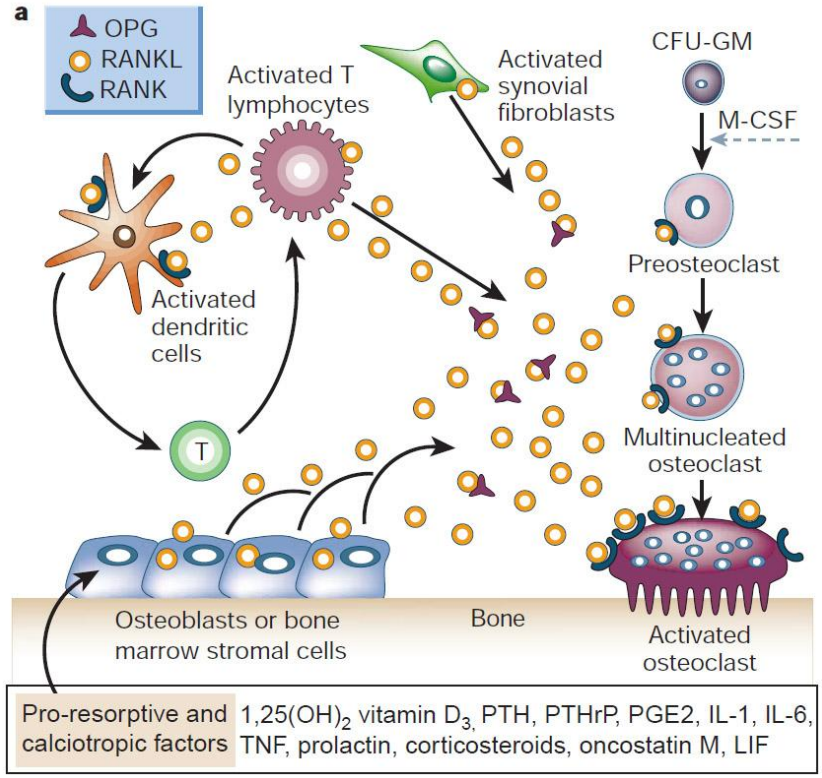
3.1.3. RANK

RANK is a type I transmembrane glycoprotein, expressed on the surface of osteoclast precursors, mature osteoclasts, dendritic cells, mammary gland epithelial cells and cancer cells. When RANKL binds to its receptor RANK a signaling cascade is initiated and in this activation process the following factors are involved: cytoplasmic adaptor protein TRAFs, followed by the downstream pathways including NF-kappaB, c-jun N-terminal kinase (JNK) or Src (proto-oncogenic tyrosine kinases) pathways. This leads to the expression of various genes, which facilitate the differentiation of monocytes into osteoclasts and also the activation of mature osteoclasts as well as their enhanced survival (WRIGHT et al., 2009).

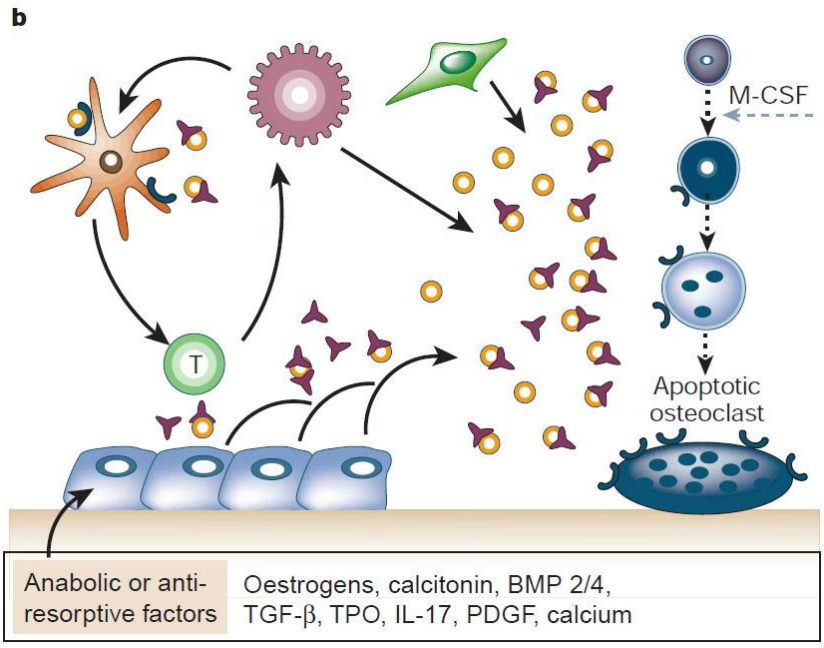
3.1.4. OPG

Osteoprotegerin was named so for its protective effects in the bone. OPG is a soluble glycoprotein secreted by osteoblasts and other mesenchymally derived cells. It is a factor without any transmembrane or cytoplasmic domain. OPG functions as a decoy receptor through binding with high affinity to RANKL, therefore the true receptor RANK will not be activated. Thus OPG inhibits osteoclast differentiation, activation and survival and therefore preserves bone substance (WRIGHT et al., 2009).

Figure 1: RANKL/RANK/OPG axis. RANKL expression is induced in osteoblasts and bone marrow stromal cells, and subsequently binds to its specific membrane-bound receptor RANK, that promotes osteoclast differentiation, activation and survival. OPG binds and neutralizes RANKL (BOYLE et al., 2003)



a) Pro-resorptive and calcitropic status



b) Anabolic and anti-osteoclastic status

3.2. RANKL/RANK/OPG axis in the state of disease

Estrogen deficiency in osteoporosis leads to an up regulation of RANKL, which increases bone turnover, whereas estrogen itself stimulates OPG production in osteoblasts and thus displays anti-resorptive effects (SIPOS et al., 2009). Duplications in the signal peptide of RANK have been linked to four families with familial expansile osteolysis or Paget disease of the bone, a rare autosomal dominant bone dysplasia characterized by focal areas of increased bone remodeling. Several mutations affecting the ligand binding domain of OPG have been found in patients suffering from juvenile Paget disease, an autosomal recessive osteopathy described by elevated bone remodeling, osteopenia, fractures and progressive skeletal deformity (LEIBBRANDT & PENNINGER, 2008). Serum RANKL levels are not clearly associated with bone mineral density, but with nontraumatic fractures. However, in one of the studies over half of the women (54.9%) had undetectable RANKL concentrations, and in another study 40% of samples were under the limit of detection (KEARNS et al., 2008). Levels of RANKL in the bone microenvironment may be more relevant. In a study, the cell surface concentration of RANKL was examined in connection with osteoporosis prevalence. Their findings suggest that up-regulation of RANKL on bone marrow cells is correlated with increased bone resorption induced by estrogen deficiency (KEARNS et al., 2008). During 28 days of continuous RANKL infusion, rats developed overall skeletal complications comparable to those in high bone turnover conditions, such as postmenopausal osteoporosis (YUAN et al., 2008). In the mouse three times injection of soluble RANKL leaded to a dose-dependent decrease of bone mineral density. It was assumed that bone loss easily could be controlled by varying the amount of injected sRANKL (TOMIMORI et al., 2009). The knowledge about the functionality of RANKL pathways leaded to new approaches for modern therapies, for example anti-RANKL monoclonal antibodies like Denusomab (Prolia®), which after the successful completion of phase III clinical trials are now released for the broadly treatment of osteoporosis (DOGGRELL, 2009; WRIGHT et al., 2009; UNIVERSIMED, 2010). Inhibition of RANKL function might be the most rational therapy to improve many osteopenic conditions and prevent bone destruction and cartilage damage in osteoporosis and arthritis (LEIBBRANDT & PENNINGER, 2008).

3.3. sRANKL transgenic mice

The group of Mizuno et al. generated two types of transgenic mice overexpressing soluble RANKL via DNA microinjection. One transgenic line CAG-promoter-sRANKL overexpressed sRANKL ubiquitously from an early developmental stage on, the other transgenic mouse line SG2-sRANKL overexpressed sRANKL only in the liver after birth. Unexpectedly, in the CAG-sRANKL line ubiquitous overexpression in the fetal stage resulted in a lethal phenotype. Also in the other line SG2-sRANKL some of the fetuses died at birth, but most of them grew up to adults and were fertile. As conclusion it is impossible to obtain living sRANKL transgenic progeny when the overexpression takes place uncontrolled during fetal development stage. In SG2-sRANKL mice at the age of 7-8 months the bone mineral density of femurs was significantly lower than in control mice. Additionally, the histological analyses revealed that trabecular bone mass was rapidly reduced with aging. Bone strength and stiffness were also markedly decreased. Bone resorption was increased by enhanced osteoclastogenesis and osteoclast activation (MIZUNO et al., 2002). These sRANKL overexpressing mice show a similar osteoporotic phenotype as OPG-deficient mice. The main difference between these both are that OPG-deficient mice have a low body weight and deformed skeletons, even before they are weaned. In contrast sRANKL transgenic mice have a normal body weight through their whole lifetime and there is no bone deformation in infants. Additionally, sRANKL transgenic mice exhibit a milder osteoporotic phenotype and lower serum ALP levels than OPG-deficient mice (BUCAY et al., 1998; MIZUNO et al., 1998; MIZUNO et al., 2002).

4. Gene regulation by a tetracycline inducible system

4.1. Inducible gene regulation

One powerful approach available to scientists is transgenic overexpression of genes of interest in animal models to study the role of these genes *in vivo* (SUN et al., 2007). But soon limitations of promoter-controlled transgene expression became obvious, because the constitutive system has no control over the timing of the expression. Constitutively active promoters mostly start transcription early in embryonic stage. If the transgene product is toxic to the embryo or fetus, this will result in failure to generate live progeny carrying the transgene. To address these

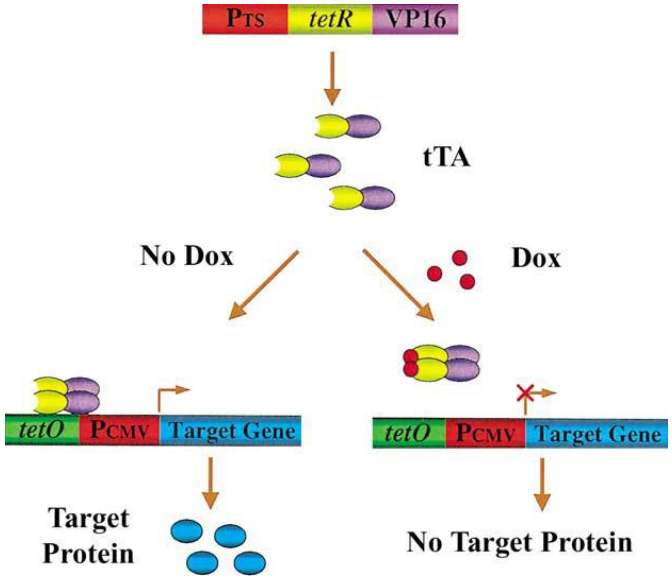
limitations different inducible transgenic modeling systems were established (ZHU et al., 2002). The most prominent and widely-accepted inducible systems so far are based on the tetracycline-controlled transcriptional regulator developed by Gossen and co-workers (GOSSEN & BUJARD, 1992; GOSSEN et al., 1995; STIEGER et al., 2009). To generate credible transgenic animal models to precisely mimic human disease states, it is critical to tightly regulate gene expression in the animals in a conditional manner. The ability to turn gene expression "on" or "off" in restricted cells or tissues at specific time points opens up a new level of research (SUN et al., 2007). Tetracycline (Tet) regulatable systems are based on the *E. coli* tetracycline resistance operon, which consists of the Tet repressor (TetR) protein and the Tet operator (TetO) DNA sequence (WISSMANN et al., 1986). In the absence of tetracycline or its derivate doxycycline (Dox), the TetR protein gets attached to the TetO DNA sequence, while in the presence of the drug, TetR changes its conformation to detaching from the DNA (ORTH et al., 1998). There are two basic variants of the tetracycline-inducible system: if transgene expression is allowed only in the absence of doxycycline, the system is called Tet-Off, whereas if transgene expression is allowed only in the presence of Dox, the system is called Tet-On (SUN et al., 2007; STIEGER et al., 2009).

4.2. Tet-Off (tTA)

The Tet-Off (tTA) system is suitable to maintain a long-time gene expression. To turn the system off, continuous administration of doxycycline (Dox) would be required (STIEGER et al., 2009). Especially when development-independent or adult-onset processes are under investigation and the target gene needs to be kept silent at embryonic and neonatal stage, long-term administration of Dox is required and may become a burden with undesired side effects (ZHU et al., 2002). In the absence of the regulating agent doxycycline, tTA binds the responsive elements tetracycline-resistance operon of E. coli transposon (TetO) and activates the minimum promoter from human cytomegalovirus (Pcmv) leading to transcription and expression of the downstream target gene. In the presence of Dox, tTA dissociates from TetO and terminates transcription of the target gene (ZHU et al., 2002). The activation of transgene expression may take several days, depending on the clearance kinetics of Dox from the system (ZHU et al., 2002; STIEGER et al., 2009).

These shortcomings limited the use of the Tet-Off system and leaded to the development of a new tetracycline depend system.

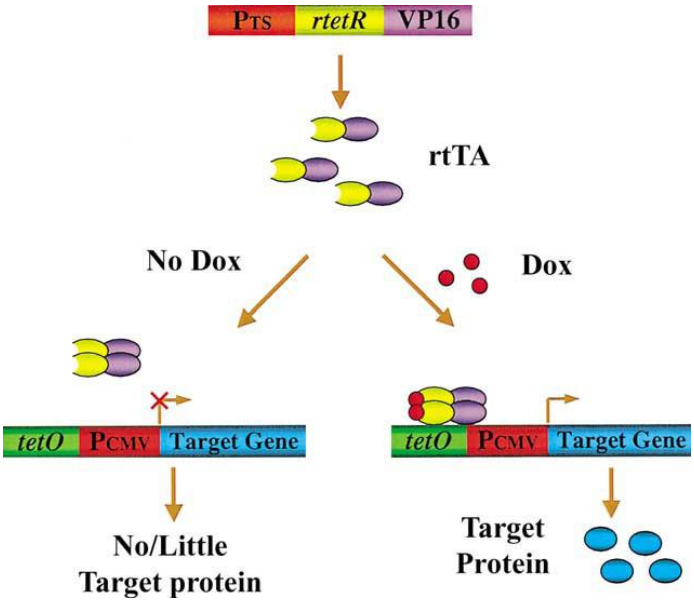
Figure 2: Tetracycline-controlled transcriptional activator (tTA) system: "Tet-Off" (ZHU et al., 2002)



4.3. Tet-On (rtTA)

The Tet-On (rtTA) system was created by mutations in the TetR domain of the transactivator tTA (Tet-Off), leading to a reverse behavior of the protein in interaction with the tetracycline response element (TRE) in the response to the presence or absence of Dox (GOSSEN et al., 1995). The main diversification in the Tet-On system is that only the presence of the inducer drug allows expression of a transgene. In the absence of Dox, rtTA does not bind to TetO and as a consequence no transcription of the target gene will take place. In the presence of Dox, rtTA binds to TetO and initiates the transcription of the target gene (ZHU et al., 2002). The activation of transgene expression was found to be more rapid compared to the Tet-Off system (GOSSEN et al., 1995; KISTNER et al., 1996; STIEGER et al., 2009). The rtTA system (Tet-On) has been used successfully in numerous transgenic mouse models with variety of transgenes targeted at various tissues and organs (KISTNER et al., 1996; ZHU et al., 2002; BACKMAN et al., 2009; RAO & MONKS, 2009; SONG et al., 2009).

Figure 3: Reverse tetracycline-controlled transcriptional activator (rtTA) system: "Tet-On" (ZHU et al., 2002)



4.4. Doxycycline

The inducer drug doxycycline, an analogue of tetracycline, is a well-documented antibiotic drug that has been used in the clinics for more than 30 years. Therefore it is considered as a save agent that can be used without major concern. The bioavailability of Dox after oral administration compared to intravenous administration is almost 100% and the serum half-life has been calculated to 14–22 h. The tissue penetration is excellent and includes the brain. Concentrations are the highest in liver, kidney and digestive tract, as it is eliminated primarily via urine and faeces (AGWUH & MACGOWAN, 2006; STIEGER et al., 2009). In Tet-inducible system transgenic mouse models doxycycline is administrated dissolved in the drinking water at ad libitum supply (KISTNER et al., 1996; RAO & MONKS, 2009). The Tet-based systems are steadily evolving towards an ideal inducible transgenic system, especially because they are coupled with a simple, well-understood, inexpensive, and easy-to-use inducing agent, doxycycline (ZHU et al., 2002).

5. Techniques of transgenesis in pigs

5.1. DNA Microinjection

Pronuclear DNA microinjection is seen as the injection of a gene construct directly into the pronucleus of zygotes. To hinder a mosaic integration and expression of the foreign gene, the DNA microinjection must take place at the one

cell stage (HOUDEBINE, 2005). This method was first established in the mouse (GORDON & RUDDLE, 1981) before being applied to various other mammalian species including the pig (BREM et al., 1985; HAMMER et al., 1985). Generally the efficiency of DNA microinjection is low. In the mouse the efficiency is 1-3% transgenic animals per microinjected embryos. In the pig the success of obtaining transgenic progeny is even lower, the same as in other animals like rabbits, rats and ruminants (HOUDEBINE, 2005). This inefficiency required microinjection and transfer of thousands of embryos to produce few transgenic offspring. In the end it is very costly to produce one single transgenic animal by DNA microinjection (ROBL et al., 2007). If integration of exogenous DNA occurs after embryonic cleavage begins, mosaic offspring can be obtained. For the species mouse the random integration of microinjected DNA has shown to bear a risk of insertional mutagenesis (RIJKERS et al., 1994). However, for pig, pathological side effects which could putatively be associated with insertional mutagenesis after DNA microinjection have not been reported so far (DEPPENMEIER et al., 2006). Nevertheless, expression levels of the transgene can differ due to position effects and variable numbers of integrated copies. An approach to reduce cost and labor of pronuclear microinjection would be the use of porcine embryos produced by in vitro fertilization (NAGASHIMA et al., 2003). Ovaries collected from slaughtered gilts are a source for oocytes, which undergo in vitro maturation (IVM) and in vitro fertilization (IVF). Resulting embryos would subsequently undergo DNA microinjection before being transferred to recipient sows. In vitro production systems yielding viable pig embryos and healthy piglets have been established (KIKUCHI et al., 2006), but still remain to be optimized (ALMINANA et al., 2008; GIL et al., 2008; KIKUCHI et al., 2008). Despite the overall low efficiency, most of the transgenic pig lines existing so far have been established by pronuclear microinjection technique. However other techniques of transgenesis have gained importance due to their higher efficiency and the potential to introduce targeted modification in the pig genome.

5.2. Sperm mediated gene transfer

Sperm cells are capable of binding and internalizing DNA molecules. This ability is used in sperm mediated gene transfer (SMGT) to transport exogenous DNA during fertilization into the embryo (LAVITRANO et al., 2006). SMGT is achieved by collection of sperm, co-incubation of sperm with exogenous DNA

and artificial insemination of gilts with DNA-loaded sperm. In 1989 the first transgenic mouse was produced by SMGT (LAVITRANO et al., 1989) and the first transgenic pig followed in 1997 (LAVITRANO et al., 1997). The big advantages of this method are its simplicity and the low costs. Unfortunately, the mechanism of DNA uptake by sperm during the co-incubation period is poorly characterized. A problem is the high variability of this method, which is showing in difficulties in reproducibility and low efficiency (ROBL et al., 2007). The crux of the matter seems to be the selection of the right sperm donor animals (LAVITRANO et al., 2003). It has been hypothesized that sperm from different boars differ in their ability to bind exogenous DNA which would explain the high variability of the SMGT method. In vitro incubation of radioactively labeled DNA with sperm showed that the DNA uptake of the sperm was highly correlated with sperm motility at the time of collection (WU et al., 2008b). Anyway it was shown that it is possible to produce piglets with a stable expression of the desired transgene (hDAF) and this founder animals passed the hDAF gene to their progeny (LAVITRANO et al., 2002; LAVITRANO et al., 2006). Other laboratories were also able to reach considerable transgene integration rates (WU et al., 2008b), but these piglets just showed transient transgene expression. New approaches to enhance efficiency and lower variability are linker base SGMT and intracytoplasmatic sperm injection (ICSI) mediated gene transfer. In Linker based SMGT, a monoclonal antibody is used which binds specifically to sperm cell surface by recognizing a specific antigen and DNA by its C-terminal end (HOUDEBINE, 2005). This is improving the uptake of exogenous DNA through receptor-mediated endocytosis. The transgenic sperm was transferred to sows through surgical oviduct insemination. The method has been reported to yield transgenic offspring (CHANG et al., 2002). As in SMGT, a central step of ICSI mediated gene transfer is the incubation of male germ cells with exogenous DNA. If the sperm membrane is disrupted, exogenous DNA could be more efficiently transferred into pig oocyte. First ICSI experiments using sperm that had been preincubated and then co-injected with exogenous DNA yielded in transgenic blastocysts which showed GFP (green fluorescent protein) expression (NAGASHIMA et al., 2003). With a new protocol of sperm pretreatment to disrupt the sperm membrane, it was also possible to obtain viable transgenic piglets (KUROME et al., 2007). Other groups succeeded in production of transgenic embryos via ICSI mediated gene transfer as well (WU et al., 2008a).

Clearly, more work is needed before SMGT is widely applied (ROBL et al., 2007).

5.3. Lentiviral gene transfer

The principle of lentiviral gene transfer is based on infection of porcine zygotes with retroviral vectors carrying transgenes. Lentiviruses belong to the family of retroviruses. Irrespective of cell cycle they can reach the host genome, because they can pass the nuclear membrane. So they are capable of infecting quiescent and embryonic cells (HOUDEBINE, 2005; ROBL et al., 2007), which reduces the formation of mosaics. Based on these advantages, lentiviral vectors are actually considered to be the most efficient method of viral transgenesis (ROBL et al., 2007). The generation of transgenic mice and rats using lentiviral gene transfer was first reported in 2002 (LOIS et al., 2002; PFEIFER et al., 2002). In the pig, lentiviral vectors based on the human immunodeficiency virus-1 (HIV-1) and on the equine infectious anemia virus (EIAV) were used for gene transfer. The recombinant lentiviruses were injected under the zona pellucida of zygotes, which were transferred to synchronized recipients later on. The overall efficiency of generating transgenic pigs by lentiviral gene transfer ranged at 13% (transgenic offspring per infected and transferred embryo) (HOFMANN et al., 2003; WHITELAW et al., 2004). A high proportion of transgenic G0 animals (94%) showed transgene expression, also over a long time (6 months) (PFEIFER et al., 2004). However, lentiviral vectors are limited to a DNA uptake only between 8-10 kb of foreign DNA, and they lead to varying gene expression in the transgenic pigs (HOUDEBINE, 2005; ROBL et al., 2007) and their progenies. The Hofmann group reported about low expression levels and hypermethylation in one third of G1 offspring, due to multiple integration sites in the founder animals, which segregated in the following generations (HOFMANN et al., 2006).

5.4. Somatic cell nuclear transfer (SCNT)

The generation of transgenic pigs by cloning is a multiple-step technology, including: transfection or transduction and selection of donor cells in cell culture; in vivo or in vitro maturation of oocytes; enucleation of the recipient oocytes; donor cell nucleus transfer; fusion of donor cell with recipient oocytes cytoplasm; artificial activation; in vitro culture of the reconstructed embryos and embryo transfer into synchronized recipient sows. The proof of concept of cloning was

confirmed in the sheep (SCHNIEKE et al., 1997; WILMUT et al., 1997), before being applied to the pig (BETTHAUSER et al., 2000; ONISHI et al., 2000; POLEJAEVA et al., 2000). Attractive characteristics of SCNT are the avoidance of mosaic phenotypes and the possibility of pre-selection of donor cells with regard to transgene expression or gender (LEE et al., 2005). SCNT exclusively enables gene targeting in pigs (PHELPS et al., 2003; RAMSOONDAR et al., 2003; TAKAHAGI et al., 2005) and assembling multiple genetic modification in a single pig line (ROBL et al., 2007). Sequential modifications cannot be done in a single step of cell culture, when primary cells with finite life spans are used. It requires serial cloning, where in each cloning step another genetic modification can take place (ROBL et al., 2007). Serial cloning has been reported up to three generations, with no negative impact on cloning efficiency and health of cloned offspring (CHO et al., 2007; KUROME et al., 2008). However, the efficiency of cloning is varying within relatively low values between 0.5% - 5.1% offspring per transferred SCNT embryos (DAI et al., 2002; KUROME et al., 2008). Considering the poor in vitro developmental competence of SCNT embryos, scientists prefer to transfer a high number of embryos (50-150) into each recipient after a short period of in vitro culture (1-8 cell stage) (VAJTA et al., 2007). Groups which perform SCNT on a regular and consistent basis tend to have better results, because routines are followed consistently and speedy completion of the NT-process results in minimal exposure of oocytes to detrimental conditions (KEEFER, 2008). Low efficiency in SCNT is based on the cumulative loss of cloned embryos and fetuses at every stage of development (KISHIGAMI et al., 2008). Increased mortality rates and health problems such as postnatal respiratory distress, contracture of the flexor tendon, enlarged tongue, cerebromeningitis, hemodynamic disorder and decreased birth weight have been reported in transgenic cloned piglets (LEE et al., 2003; PRATHER et al., 2004; PARK et al., 2005). Compared with cloned cattle and sheep, perinatal death and birth defects occur less frequently (CIBELLI et al., 2002; RUDENKO & MATHESON, 2007; KEEFER, 2008). As these abnormal phenotypes are usually not transmitted to offspring of the clones, they are apparently a result of epigenetic modification (PARK et al., 2002; KISHIGAMI et al., 2008). These dangers can be sidestepped by breeding the clones, as soon as they reach puberty (PEARSON, 2003; MIR et al., 2005). Despite of the low overall efficiency, SCNT is regarded as the most reliable approach to produce piglets with targeted genetic modifications (VAJTA

et al., 2007). Future research should focus on improving the health and production efficiency of somatic cell porcine clones (MATSUNARI & NAGASHIMA, 2009).

6. Examples of genetically modified pigs

6.1. Swine in biomedical research

Transgenic pigs are generated for biomedical research purposes, i.e. as animal model in medical research, as donor animal for xenotransplantation, as bioreactors for gene farming and as highly efficient productive livestock in agriculture. Among various possibilities, the established somatic cell nuclear transfer system with genetically engineered donor cells is an efficient and reliable approach to produce transgenic pigs (VAJTA et al., 2007). Various human disease models and donor pigs for xenotransplantation have been produced via SCNT.

6.2. Transgenic human disease models

6.2.1. Human heart disease model

Endothelial cell nitric oxide synthase (eNOS) over-expressing piglets were generated by nuclear transfer to study the role of nitric oxide metabolism in cardiac and skeletal muscle. The final goal was to generate large animal model for human heart disease (HAO et al., 2006). Nitric oxide (NO) is a messenger molecule, which modulates vascular function, structure and homeostasis. The enzyme endothelial cell nitric oxide synthase (eNOS) releases NO into the blood stream, where it plays an important role in the metabolism of cardiac and skeletal muscle. The created pigs carried the Tie2-eNOS transgene and also showed expression of the Tie2-eNOS in the endothelial cells of placental vasculature simultaneously to the endogenous eNOS (HAO et al., 2006).

6.2.2. Cystic fibrosis model

Cystic fibrosis (CF) is the most common lethal genetic disease in the Caucasian population (CARVALHO-OLIVEIRA et al., 2007), caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) (MATSUNARI & NAGASHIMA, 2009). CFTR knock-out mice failed to develop pulmonary and pancreatic symptoms, the leading causes of morbidity and mortality in CF patients (CARVALHO-OLIVEIRA et al., 2007; GUILBAULT et

al., 2007). Due to many anatomical and physiological similarities, pigs were considered as a more suitable animal model. CFTR knockout pigs developed meconium ileus, exocrine pancreatic destruction, and focal biliary cirrhosis, replicating abnormalities seen in newborn patients with CF (ROGERS et al., 2008). Additional the CF pigs showed few months after birth already typical lung symptoms like airway inflammation, mucus accumulation and infection (STOLTZ et al., 2010).

6.2.3. Alzheimer´s disease model

Disease-causing mutations for Alzheimer's disease (AD) were identified in the amyloid precursor protein gene (APP) and the presenilin 1 and presenilin 2 genes (PSEN1 and PSEN2). These mutations are associated with accumulation of the Aß peptide in the brain. Transgenic pigs were produced expressing the neuronal variant of the human amyloid precursor protein based on the Swedish APP mutation (KRAGH et al., 2009). APP695sw transgene expression, including protein, was observed in brain tissue and not in heart or liver tissues. Mutant protein accumulation will approximately take 1-2 years, and it's expected that after this time the transgenic pigs show the desired phenotype.

6.2.4. Diabetes mellitus type 3 model

Diabetes mellitus, one of the most serious chronic diseases in modern society (RATHMANN & GIANI, 2004), is characterized by high blood glucose levels and complications such as diabetic retinopathy, diabetic nephropathy and diabetic neuropathy. A model for maturity-onset diabetes of the young type 3 (MODY3) was developed by integrating a dominant-negative mutant hepatocyte nuclear factor 1alpha into pigs (UMEYAMA et al., 2009). These pigs manifested the pathophysiology of diabetes, such as high fasting blood glucose levels (>200mg/dl), abnormal formation of Langerhans islets and poor insulin secretion.

Table 2: Human disease models produced by SCNT

Objective of investigation	Gene	Reference	Technique
Human Nitric oxide metabolism in skeletal and cardiac muscle	Endothelial cell nitric oxide synthase (eNOS)	(HAO et al., 2006)	SCNT
Cystic fibrosis	Disruption of the CFTR gene	(ROGERS et al., 2008)	SCNT
Alzheimer's disease	dominant mutation APPsw	(KRAGH et al., 2009)	Handmade SCNT
Diabetes mellitus type 3	dominant-negative mutant hepatocyte nuclear factor 1alpha	(UMEYAMA et al., 2009)	transgenic-ICSI & SCNT

6.3. Xenotransplantation

Because of the world-wide shortage of human organs, researchers began with their studies on xenotransplantation (COZZI et al., 2009). Several genetically modified strains of pigs, representing different strategies against human rejection mechanisms, have already been generated by pronuclear DNA injection, sperm mediated gene transfer or SCNT (SACHS & GALLI, 2009). The rejection of a foreign organ can be classified in different stages: hyperacute rejection (HAR), acute humoral xenograft rejection (AHXR), immune cell-mediated rejection, and chronic rejection (KLYMIUK et al., 2010). Hyperacute rejection develops within 24 hours. It is mediated by the binding of preexisting xenoreactive antibodies to endothelial cells and activation of the complement system, which results in endothelial swelling and microvascular thrombosis (SCHUURMAN et al., 2003; SPRANGERS et al., 2008). The xenoantibodies are predominantly directed against α-1,3-Galactosyl (αGal) carbohydrate residues. These are expressed on endothelial cells of non-primate mammals, but are absent in humans, apes, and old world monkeys as these species lack the αGal-transferase gene to synthesize αGal. Because of cross-reactivity to surface antigens of the intestinal flora, natural anti-αGal antibodies are produced as soon as the gastrointestinal tract is encountered by microorganisms shortly after birth (SPRANGERS et al., 2008). In the year 2002 a breakthrough in pig genetic engineering was the birth of the first

homozygous Knockout of α-1,3-galactosyltransferase (αGalT) piglets (DAI et al., 2002; LAI et al., 2002; PHELPS et al., 2003; RAMSOONDAR et al., 2003; WATT et al., 2006; FUJIMURA et al., 2008), from that day on it was proven that gene targeting in the pig is possible (KLYMIUK et al., 2010). Development of αGal deficient pigs has reduced or eliminated the significance of αGal antigen in xenograft rejection. When αGal deficient pig organs are used for Pig-to-Primate Cardiac Xenotransplantation, xenograft rejection remains associated with antibody deposition, variable complement activation and microvascular thrombosis. Nevertheless organs from αGal knock out pigs are widely considered to be central for clinical xenotransplantation of solid organs (BYRNE et al., 2008).

7. Embryo transfer (ET) in the pig

7.1. Factors influencing ET success

The first birth of piglets after embryo transfer (ET) was reported in the year 1950 in Poltava, Ukraine (KVASNITSKI, 1950). Since then progress in reproductive biotechnologies has led to an increased use of embryo transfer in both swine research and swine production. The number of embryo transfers performed in pigs is low compared with that in cattle, despite the fact that bovine embryo transfer technology was developed at the same time (YOUNGS, 2001; VAJTA et al., 2007). This is due to the fact that rapid genetic improvement in pig easily can be achieved by selection programs without the use of embryo transfer. Another impediment to the widespread use of ET in the swine industry is that cryopreserving pig embryos is still no routine technology (HAZELEGER & KEMP, 2001; YOUNGS, 2001). There are various factors which might influence ET successes. One factor is synchronization of estrus cycle of recipient sow and development stage of transferred embryos. It is common to use hormones, first equine chorionic gonadotropin (eCG) followed by human chorionic gonadotropin (hCG), to induce estrus in gilts close to puberty. The eCG stimulates follicular development due to its FSH-like activity, and hCG causes ovulation because of its LH-like activity (YOUNGS, 2001). Accurate synchronization of the recipient gilt is crucial to the success of ET. A delay of 48 hours between donor and recipient estrus resulted in the degeneration of all embryos (GEISERT et al., 1991).

7.2. Pregnancy rates

First-estrus gilts are contraindicated for donating embryos, but they are quite acceptable as recipients. No difference in pregnancy rate (67.5% vs. 60%) or embryonic survival (69% vs. 75%) was reported in first-estrus versus third-estrus recipient gilts, respectively (ARCHIBONG et al., 1992). No evidence of uterine crowding adversely affecting litter size at Day 25 of gestation in females having 7 or fewer embryos present per uterine horn has been observed, suggesting that a minimum of 14 embryos should be transferred to each recipient (DZIUK, 1968). Pregnancy rates of 71% and 100% and embryo survival rates of 57% and 68% in gilts receiving 12 or 24 embryos, respectively, were reported (POPE et al., 1972). It was also reported that at least four viable embryos were necessary in pigs to maintain pregnancy in the early phases of embryo maternal communication (POLGE et al., 1966). Average pregnancy rates of 60% and embryonic survival rates (in pregnant recipients) of 60% are standard. So from all transferred embryos 35-40% survive and will result in living piglets. In all these experiments in vivo derived embryos were used and the success rate surely is lower, when dealing with manipulated embryos (YOUNGS, 2001).

7.3. Endoscopic embryo transfer

The surgical procedures of ET have been refined to a minimally invasive procedure, using endoscopy for transfer of embryos. Although they can be defined as surgical techniques, their advantage is that only a few small incisions are needed for insertion of instruments onto the abdominal cavity. Additionally endoscopy allows pre-examining the genital tract for reproductive abnormalities and successful ovulation without exposure and exteriorization of viscera during surgery (BESENFELDER et al., 1997; HAZELEGER & KEMP, 2001). Pregnancy rates up to 90 % were reported following routine application of endoscopic embryo transfer. The main disadvantage of this technique is that skilled personnel, anesthesia and surgical facilities are required (HAZELEGER & KEMP, 2001).

III. MATERIALS AND METHODS

1. Equipment and expendable items

1.1. In vitro works

Petri dishes:	35x10 mm, Becton Dickinson Labware
	50x9 mm, Becton Dickinson Labware
	60x15 mm, Nunc® Brand Products
Glassware:	Schott, Brand, Wertheim
Pipettes:	Eppendorf, Hamburg
Pipette filter tips:	Eppendorf, Hamburg
Neubauer counting chamber:	Brand, Wertheim
Centrifuge tubes:	15 ml, Greiner
	50 ml, Falcon®, Becton Dickinson Labware
Glass cover slips:	Marienfeld, Lauda-Koenigshofen
Reaction tubes:	0.5 ml / 1.5 ml / 2 ml, Eppendorf
Sterile benches:	Lamin Air®, HB 2472, Heraeus
	KR-130 BW, Kojair
Sterile filters:	Millipore Express®, 0.22µm, Millex® GP
Centrifuges:	Biofuge pico, Heraeus
	Rotanta 96, Hettich Zentrifugen
Incubator:	Type B 5060, Heraeus
	Model 500M, MMM Group
Microscopes:	Leitz Periplan, Leica
	Stermi SV6, Zeiss
	SMZ-10A, Nikon

Micromanipulator:	Eclipse TE 2000-U, Nikon
	Eclipse TE 300, Nikon
Warming plates:	HT 200, Minitube
Electro activation:	Multiporator, Eppendorf
Electro cell fusion:	Model LF 101, Nepa Gene
Cell transfection:	Nucleofector® II, Amaxa biosystems

1.2. Embryo transfer

Endoscopic instruments:	Karl Storz Endoskope
Ultrasonic device:	Echo camera, SSD-500, Aloka

2. Used media and stock solutions

2.1. Stock solutions

PBS (Phosphate-buffered saline)

8.00 g NaCl	(Sigma)
0.20 g KCl	(Sigma)
1.15 g Na_2HPO_4 x 2 H_2O	(Fluka, Neu-Ulm)
0.20 g KH_2PO_4	(Merck, Darmstadt)
0.10 g $CaCl_2$	(Sigma)
0.10 g $MgCl_2$ x 6 H_2O	(Sigma)

1.0 l Milli-Q water

sterile filtered, stored at room temperature

PBS− (Phosphate-buffered saline without calcium and magnesium)

8.00 g NaCl	
0.20 g KCl	
2.14 g Na_2HPO_4 x 7 H_2O	(Merck)
0.20 g KH_2PO_4	

1.0 l Milli-Q water

sterile filtered, stored at room temperature

PBS P/S (Phosphate-buffered saline with 2 % Penicillin/Streptomycin)

98 ml PBS

2 ml Pen/Strep stock solution

freshly prepared before use

Pen/Strep stock solution

0.65 g Penicillin (Seromed, Berlin)

1.33 g Streptomycin (Seromed)

100 ml Milli-Q water

(contains 100 U/ml penicillin and 100 µg/ml active streptomycin)

sterile filtered, stored at -20°C

2.2. Cell culture

Culture media for porcine fibroblasts and kidney cells:

DMEM (high glucose, without sodium pyruvate) (GIBCO)

1% non-essential amino acids (GIBCO)

1% sodium pyruvate (GIBCO)

0.1 mM Mercaptoethanol (7 µl/10 ml PBS 1%) (Sigma)

1% L-Glutamin 200 mM + 1% Pen/Strep (PAA)

10-15% FKS (GIBCO)

Serum Starvation media

DMEM (high glucose, without sodium pyruvate) (GIBCO)

1% non-essential amino acids (GIBCO)

1% sodium pyruvate (GIBCO)

1% L-Glutamin 200 mM + 1% Pen/Strep (PAA)

0.5 % FKS (GIBCO)

Trypsin/EDTA-solution for cell culture

Trypsin 0.25% (Difco)

EDTA 0.02% (Sigma)

PBS- (without calcium and magnesium)

sterile filtered, stored at -20°C

G418 selection Media

Porcine fibroblast culture medium (see above)

G418 (Geneticin) (GIBCO)

Porcine kidney cells: 1.2 mg/ml

Porcine fetal fibroblast cells: 0.6 mg/ml

2.3. Nuclear transfer

NCSU-23 Stock A

NaCl	6.355 g	(Sigma)
KCl	0.356 g	(Sigma)
$MgSO_4$ x 7 H_2O	0.293 g	(Sigma)
KH_2PO_4	0.162 g	(Sigma)
Milli-Q water	restocked up to 100 ml	

sterile filtered, stored at 4°C

NCSU-23 Stock B

$CaCl_2$ x 2 H_2O	0.5 g	(Sigma)
Milli-Q water	restocked up to 20 ml	

sterile filtered, stored at 4°C

Ready to use NSCU-23

$NaHCO_3$	0.421 g	(Sigma)
Glucose	0.2 g	(Sigma)
PenicillinG	0.013 g	(Sigma)
Streptomycin	0.010 g	(Sigma)
Taurine	0.175 g	(Sigma)
Stock A	20 ml	
Stock B	2 ml	
Milli-Q water	restocked up to 200 ml	

sterile filtered, stored at 4°C

Hepes-TaLP-PVP Stock A

NaCl	6.66 g	(Sigma)
KCl	0.24 g	(Sigma)
$MgCl_2$ x 6 H_2O	0.1 g	(Sigma)
NaH_2PO_4	0.042 g	(Sigma)
Na Lactate (60%)	1.85 ml	(Sigma)
Phenol red	2 ml	(Sigma)
Milli-Q water	restocked up to 100 ml	

sterile filtered, stored at 4°C

Hepes-TaLP-PVP Stock B

$CaCl_2$ x 2 H_2O	0.58 g	(Sigma)
Milli-Q water	restocked up to 20 ml	

sterile filtered, stored at 4°C

Hepes-TaLP-PVP Stock C

Hepes	2.4 g	(Sigma)
Milli-Q water	restocked up to 100 ml	
sterile filtered, stored at 4°C		

Ready to use Hepes-TaLP-PVP

Glucose	0.45 g	(Sigma)
Sorbitol	4 g	(Sigma)
PVP	1.5 g	(Sigma)
Streptomycin	0.025 g	(Sigma)
PenicillinG	0.033 g	(Sigma)
$NaHCO_3$	0.085 g	(Sigma)
Stock A	50 ml	
Stock B	5 ml	
Stock C	50 ml	
Milli-Q water	restocked up to 500 ml	
sterile filtered, stored at 4°C		

Porcine zygote medium

PZM-5	Institute for the functional Peptide, Yamagata, Japan

3. Establishment of transgenic cell lines

3.1. Cells

For transfection, porcine fetal fibroblasts (PFF) were obtained from 28 and 35 days old male wild type pig fetuses and tested in vitro for their SCNT development capacity. For recloning and additional transfection the following cell types were used: PFF, porcine ear fibroblasts (PEF), porcine kidney cells (PKC). As donation of other laboratories we received several different transgenic cell lines to perform somatic cell nuclear transfer and to produce transgenic offspring. These donated cell lines originated from mesenchymal stem cells (MSC) or PFF.

3.2. Transfection of cells

To establish a stable cell line expressing the desired construct plus antibiotic resistance, wild type fetal fibroblasts were electroporated by the Nucleofector® system. The foreign DNA was introduced into the cell by an electro current. Cells were harvested after passage three and counted in a Neubauer counting chamber. 5 µl DNA-solution (3.3 µg) containing the chosen gene construct and 100µl human dermal fibroblast Nucleofector® solution were mixed with 5 x 10^5 PFF cells. Then electroporation with the program U12 took place. After this treatment cells were pipetted with an Amaxa plastic pipette in 1 ml medium to a cell culture dish. The next day media was exchanged.

3.3. Mass cell selection

Three days after electroporation cells were confluent (around 90%) and were harvested. For selection of cells, they were seated with medium containing Geneticin (G418; 1.0 mg/ml) on a new cell culture dish. Every two days selection medium was changed. After one week of culture all cells had died, which did not integrate the antibiotic resistance gene; these cells were floating in the supernatant. Successfully transfected cells were able to survive selection and formed colonies. The cells were cultured until 90% confluence and then harvested and cryopreserved until SCNT was performed.

4. Vector design

4.1. Lentiviral vectors

The described lentiviral vectors (Figure 4) were constructed in the ExperiMed laboratory of Chirurgische Klinik und Poliklinik – Innenstadt LMU Munich by PD Dr. med Wolfgang Böcker. Head of Experimental Surgery and Regenerative Medicine (ExperiMed) laboratories is Prof. Dr. med. Matthias Schieker (www.experimed.de). Lentiviral transduction and selection of porcine fetal fibroblast cells were performed by Tamara Radic at ExperiMed laboratory. Transduced cells carrying the desired constructs were donated to our laboratory to produce transgenic offspring by SCNT.

Figure 4: Overview of lentiviral vectors

1) pLenti6-CMV-pRANKL (Bla+)

2) pLenti6-CMV-TetON (Bla+)

3) pLenti6-CMV-TetON-ΔBla (Bla-)

4) pLenti6-CMV-TetON-TRE$_{tight}$-pRANKL (Bla+)

5) CMV-TetOn-TRE$_{tight}$-pRANKL (Neo+)

These figures were a donation of PD Dr. med Wolfgang Böcker.

BSD: blasticidin-S deaminase (Basticidin resistance gene)
LTR: long terminal repeat
Pcmv: cytomegalovirus promoter
pRANKL: porcine receptor-activator of nuclear factor kappa beta ligand
pTRE tight: Tet-Advanced transactivator response element promoter (TARE)
RRE: Rev-responsive element
rtTA: reverse tetracycline-controlled transcriptional activator (Tet-On)
NEO: neomycin resistance

The lentiviral vectors starts and ends with long terminal repeats (LTR), which are responsible for the regulation of synthesis and processing viral RNA and other replicative functions. Another essential part of lentiviral vectors is the Rev-responsive element (RRE), because REV signaling mediates the export of the viral RNA out of the nucleus. The desired genes Tet-On and pRANKL were inserted to separate vectors (1–3) and also combined in one vector (4 and 5). These genes were controlled by a CMV promoter or the TRE tight promoter. For the later selection of cells with transgene integration, the antibiotic resistance gene for Blasticidin or Neomycin was introduced (except 3). In the last construct (5) all lentiviral elements, like the LTRs and the RRE, were cut out leaving only the genes of interest Pcmv-Tet-On and TRE$_{tight}$-pRANKL and additional the

Neomycin resistance gene. These assembly changes were applied to adapt the lentiviral vector to the Amaxa Nucleofector system. With the donated Tet-On-RANKL-Neo (5) vector porcine fetal fibroblast cells were transfected in our laboratory.

4.2. Conventional vectors

The vectors 6 and 7 were designed and constructed by Dr. rer. nat. Nikolai Klymiuk in our institute. The Tet-On and RANKL gene were integrated under the control of the CAG and the TARE (pTRE tight) promoter. Neomycin and blasticidin antibiotic resistance and the polyadenylation signal of the bGH gene was added. The poly adenosine tail contributes to the nuclear export, translation and stability of mRNA. The antibiotic resistance is needed for further selection of cells with stable integration of the desired genes. To avoid possible negative side effects of antibiotic resistance genes, loxP sites were introduced. With the enzyme Cre recombinase it is feasible to cut out specifically the loxP sites and the gene in between is deleted. Both gene constructs were used for cell transfection in our laboratory.

Figure 5: Overview of conventional vectors

6) CAG-rtTA: lox2 neo® – CAGpr – rtTA ORF – bGHpA

7) TARE-RANKL: TAREpr – RANKL ORF – bGHpA – lox2 bla®

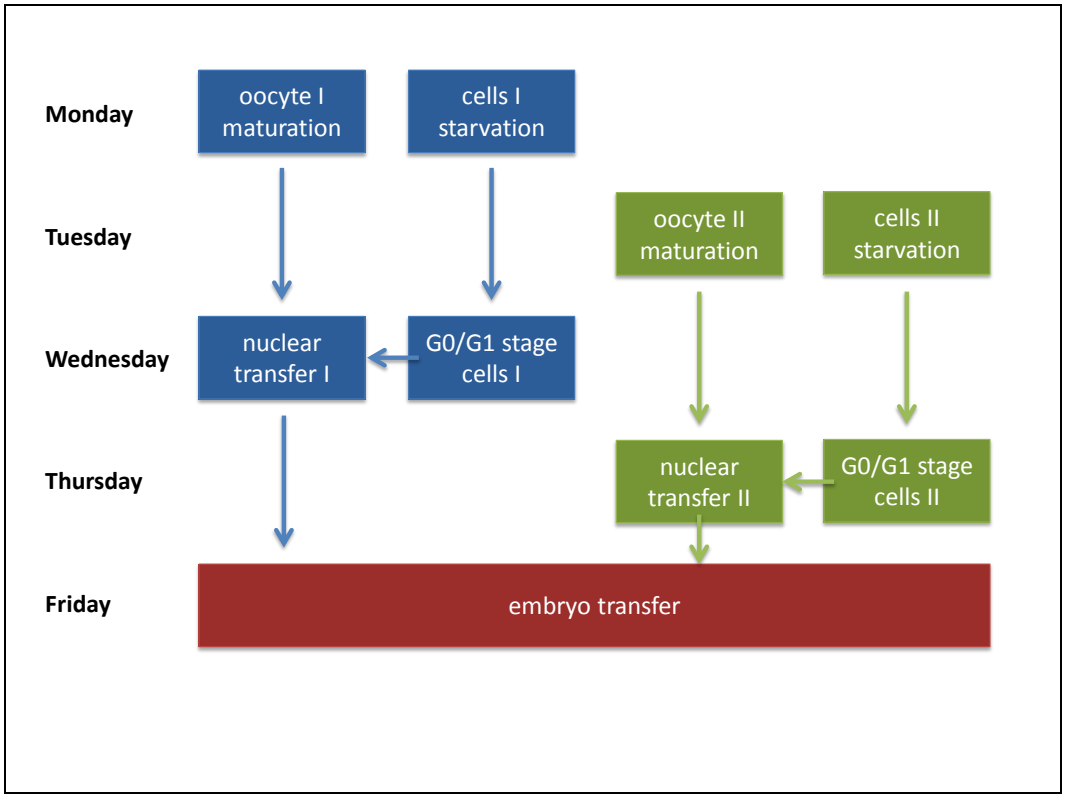
These figures were a donation of Dr. rer. nat. Nikolai Klymiuk.

bGH: bovine growth hormone gene
bla: blasticidin resistance
CAG: CMV early enhancer / chicken beta actin promoter
lox: locus of X-over P1 (lox P site)
neo: neomycin resistance
ORF: open reading frame
pA: (PolyA) polyadenylation signal
PR: promoter
RANKL: porcine receptor-activator of nuclear factor kappa beta ligand
rtTA: reverse tetracycline-controlled transcriptional activator (Tet-On)
TARE: Tet-Advanced transactivator response element promoter (pTRE tight)

5. Procedure of somatic cell nuclear transfer

Per week, two nuclear transfer experiments were scheduled, one performed on Wednesday and the other one on Thursday. Oocyte maturation and cell preparation must be exactly timed to fit in the procedure. All produced NT embryos were equally mixed and transferred to one or two recipients on Friday.

Figure 6: Regular work flow of NT and ET



5.1. In vitro maturation of oocytes

5.1.1. Ovary collection

Sixty to 90 ovaries per day were collected at a local abattoir and transported to the laboratory in phosphate buffered saline (PBS) containing 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulfate and 0.1% (w/v) polyvinylalcohol (PVA) in a warming box under the stable temperature of 36°C to 37°C degrees.

5.1.2. Oocyte collection

Cumulus-oocyte complexes (COCs) were collected by aspiration with a needle and syringe from ovarian antral follicles of 3.0-6.0 mm in diameter.

5.1.3. Selection of oocytes

In a first step all COCs which were visible were collected and washed in Tyrode´s

albumin lactate pyruvate (TALP) solution. Only COCs displaying over 3 layers of compacted cumulus cells with even cytoplasm were selected and used for further experiments. The remaining poor quality and altered COCs were discarded.

5.1.4. Oocyte maturation

120 to 180 first grade COCs per day were subsequently cultured in North Carolina State University medium-23 (NCSU-23) supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10% (v/v) porcine follicular fluid, 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulfate, 10 IU/ml equine chorionic gonadotropin (eCG; Intergonan, Intervet, Germany) and 10 IU/ml human chorionic gonadotropin (hCG; Ovogest, Intervet, Germany). The first 22 h they were cultured with the hormones eCG and hCG, and then for 20 h without these hormones in a humidified atmosphere of 5% CO2 and 95% air at 38.5 °C.

5.1.5. Denudation of matured oocytes

After 42 hours culture, the in vitro matured (IVM) oocytes with expanded cumulus cells were treated with 1 mg/ml hyaluronidase dissolved in TALP medium supplemented with 10 mM Hepes and 0.3% (w/v) polyvinylpyrrolidone (PVP) (Hepes-TALP-PVP) and were denuded of cumulus cells by gentle pipetting. Oocytes displaying evenly granulated ooplasm and extrusion of the first polar body were considered as fully matured and selected for the experiments.

5.2. Nuclear transfer in vivo experiment

Somatic cell nuclear transfer (SCNT) was performed using IVM oocytes as recipient cytoplasts.

5.2.1. Enucleation

Enucleation was performed using a chemically assisted method developed by Yin et al. (YIN et al., 2002). Oocytes were exposed to NCSU-23 medium supplemented with 0.1 µg/ml demecolcine, 0.05 mM sucrose and 4 mg/ml bovine serum albumin (BSA) for 0.5-1 h and subsequently enucleated by aspirating the first polar body and the adjacent cytoplasm using a beveled pipette (30 µm in diameter) in Hepes-TALP-PVP containing 0.1 µg/ml demecolcine, 5 µg/ml cytochalasin B (CB) and 10% FBS. Any protrusions observed on the surface of the oocytes were removed along with the polar body.

Figure 7: Enucleation

a) Penetration of the zona pelucida

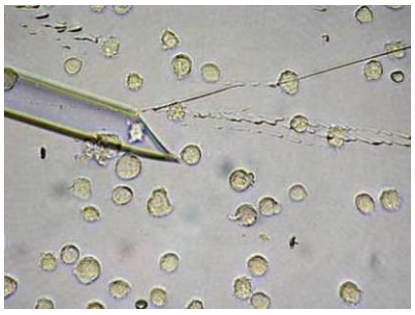
b) Soakage of the first polar body and the adjacent cytoplasm

c) Enucleated oocyte

5.2.2. Donor cell preparation

Cell cycle of donor cells was synchronized in the G0/G1 stage by serum starvation starting 48h before nuclear transfer. On the day of nuclear transfer, donor cells were detached from culture dish and singularized by trypsinization.

Figure 8: Appearance of donor cells just before injection



5.2.3. Donor cell injection

For cell insertion, round, small donor cells, with a smooth surface and a regular looking were selected. One single donor cell per enucleated oocyte was injected through the hole in the zona pelucida, originated by enucleation, into the perivitelline space.

Figure 9: Donor cell insertion

a) Introduction of the pipette through the enucleation hole

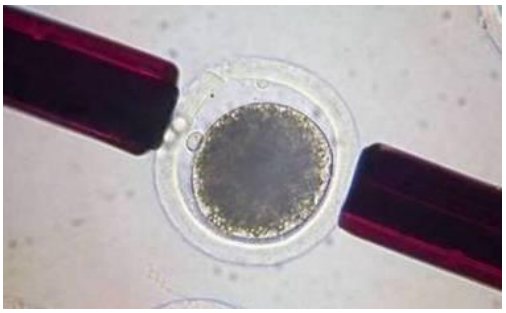
b) Injection of one donor cell

c) Reconstructed NT embryo

5.2.4. Fusion

Donor cell-oocyte complexes were placed in a droplet of 280 mM mannitol solution (pH 7.2) containing 0.15 mM $MgSO_4$, 0.01% (w/v) PVA, and 0.5 mM Hepes and held between 2 electrode needles. Membrane fusion was induced using an Electro cell fusion LF101 (NEPA GENE Co. Ltd.) by applying a single direct current (DC) pulse (200 V/mm, 20 µs ×1) and a pre- and post-pulse alternating current (AC) field of 5 V, 1 MHz for 5 s, respectively. Reconstructed embryos were cultured in NCSU-23 for 0.5-1h, followed by electrical activation.

Figure 10: Reconstructed NT embryo in between two electrodes of the fusion aperture



5.2.5. Activation

Reconstructed embryos were washed twice in an activation solution consisting of 0.3 M mannitol, 50 µM $CaCl_2$, 100 µM $MgSO_4$ and 0.01% PVA (300 mOsm), then placed between 2 wire electrodes (1 mm apart) of a fusion chamber slide and overlaid with activation solution. A single DC pulse of 150 V/mm was applied for 100 µs. To suppress extrusion of the pseudo-second polar body, activated oocytes were subsequently cultured in a medium containing 5 µg/ml cytochalasin B (CB) for 3 h.

5.2.6. In vitro culture of reconstructed embryos

The reconstructed embryos were stored for one or two days, until embryo transfer took place, in 20-µl droplets of porcine zygote medium (PZM-5) under paraffin oil in a plastic Petri dish maintained under a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C.

5.3. Nuclear transfer in vitro experiment

The procedures of nuclear transfer for in vitro experiments were basely the same as in an in vivo experiment, with one exception: the enucleation was confirmed by staining cytoplasts with 5µg/ml bisbenzimide (Hoechst 33342). Not completely

enucleated oocytes were rejected. Cleavage and blastocyst formation of reconstructed embryos were monitored during culture for 7 days. In vitro culture of embryos was performed in 20-µl droplets of porcine zygote medium (PZM-5) under paraffin oil in a plastic Petri dish maintained under a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C.

5.4. Embryo transfer

Six to seven months old prepuberal gilts (hybrids of "Schwäbisch-Hällisches Landschwein" and "Deutsche Landrasse") were used as recipients.

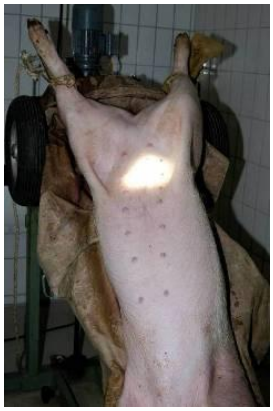
5.4.1. Estrus synchronization

Estrus synchronization was conducted by oral administration of altrenogest (Regumate, Serumwerk Bernburg, Germany) over a 15 day period, followed by intramuscular injection of 750 IU eCG, (Intergonan, Intervet, Germany) which has a follicle stimulating effect, 24 hours after last gestagen administration. Ovulation was induced 3 days later by intramuscular injection of 750 IU hCG (Ovogest, Intervet, Germany).

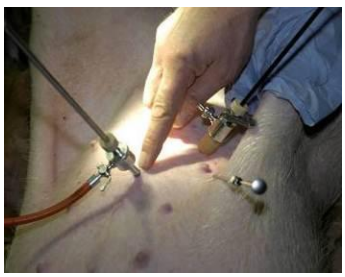
5.4.2. Endoscopic embryo transfer

Endoscopic embryo transfer was performed two days after Ovogest treatment. Recipients were anesthesized with a combination of 1.2 ml per 10 kg ketamine hydrochloride (Ursotamin, Serumwerk Bernburg, Germany) and 0.5 ml per 10 kg xylazine (Xylazin 2%, WDT, Germany) intravenously.

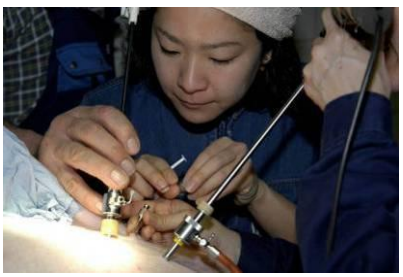
Figure 11: Endoscopic embryo transfer



a) Prepared recipient



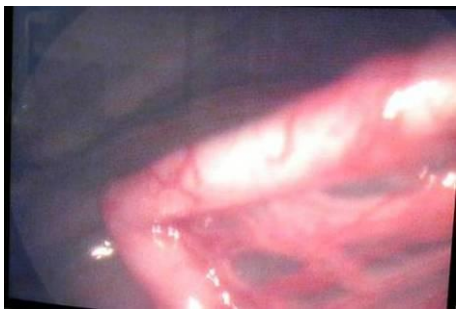
b) operation field with the endoscopic optics (left), grasper (middle) and trocar (right)



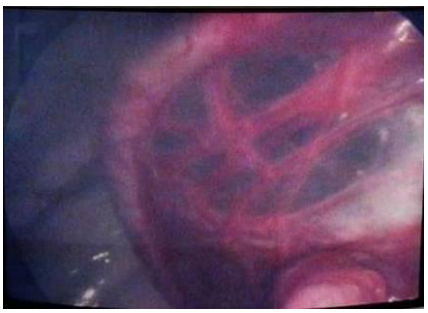
c) introduction of the embryo loaded catheter

Recipients were brought into 45° dorsal recumbency, and 53 to 147 embryos were transferred laparoscopically in to the right oviduct.

Figure 12: Introduction of the catheter into the oviduct



a) The right oviduct with inserted trocar



b) expansion of the right oviduct through the introduced catheter

6. Pregnancy control and birth

6.1. Pregnancy control

Pregnancy status was confirmed by ultrasound examination. These examinations were performed at least three times per recipient, the first check (TU1) always was scheduled for day 28, the second check (TU2) between day 50 and 70 and the third check (TU3) between day 90 and 110 after embryo transfer. Sows were classified as pregnant, when liquid filled chambered vesicles in the uterus were visible (TU1). At the later stages of pregnancy (TU2 + TU3) also fetuses were detected and evaluated.

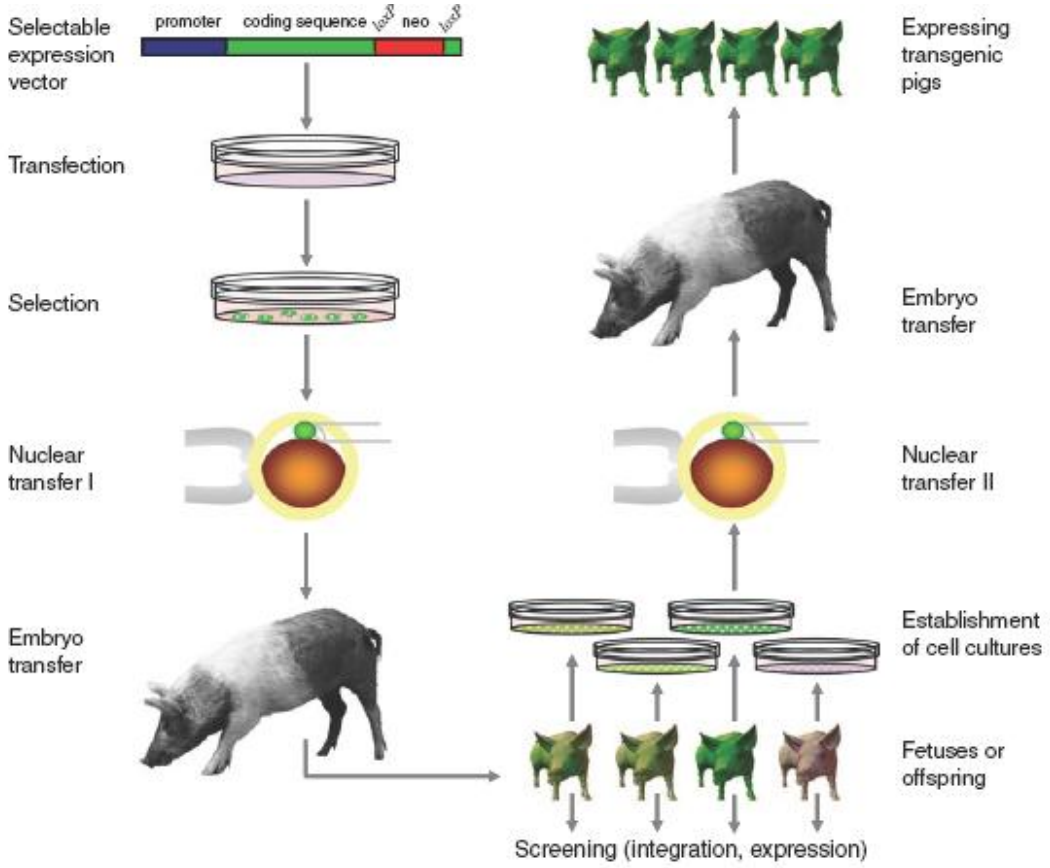
6.2. Induction of labor

If spontaneous birth did not occur until day 117, labor was hormonally induced by intra muscular application of 0.7 ml Cloprostenol (Estrumate, Intervet, Germany).

7. Overview: transgenic pig production via SCNT

The production schedule of transgenic SCNT pigs with mass cell selection instead of single cell clone selection provides the advantage of a shorter culture time and less stress of single culture of fibroblasts. The basic difference is that the screening step was done after SCNT and not before. Consequently, litters after the first round of SCNT represented different cell lines. After screening, a second round of nuclear and embryo transfer was necessary to obtain transgenic founder animals.

Figure 13: Efficient production of transgenic pigs via SCNT (AIGNER et al., 2010)



8. In vivo doxycycline stimulation

RANKL overexpression was in vivo stimulated by oral administration of Pulmodox® (Virbac Tierarzneimittel GmbH, Bad Oldesloe), with the active ingredient doxycyclinhyclat. Pigs were treated with doxycycline at a dosage of 40 mg/kg/day continuously over 6 days. Plasma blood samples were obtained on day 0 (before first doxycycline application), on day 3 and day 6 of doxycycline treatment. To distinguish the different RANKL plasma levels, ELISAs were performed by Tamara Radic at ExperiMed laboratory.

9. Statistical analysis

For statistical analysis the software of IBM SPSS Statistics, Version 18.0.0 was applied. The design of diagrams was optimized with Microsoft Excel 2010. Statistically verifiable relations were tested by bivariate correlations, for this purpose the correlation coefficients according to Pearson were calculated. The higher the value, the stronger is the correlation with a maximum of 1 and the common probability error of $\alpha^* = 0.05$ or $\alpha^{**} = 0.01$. Interrelationships were

pictured in cross-classified tables. Furthermore T-tests with unpaired random sampling were performed to test the significance level. Differences were considered significant at $P < 0.05$.

IV. RESULTS

1. Assessment of SCNT and ET

1.1. Overview of the years 2006 to 2009

In the year 2006 the technique of porcine somatic cell nuclear transfer was established in our laboratory. After the first birth of SCNT piglets obtained from wild-type porcine fetal fibroblast cells, SCNT procedures were continued with transgenic cells, either produced in our laboratory or obtained from other laboratories. The first transgenic SCNT piglets were born on January the 15th 2007. Until the end of 2009, 12880 NT embryos were produced and transferred to 145 recipients. Transfers resulted in 41 births with a total of 160 piglets, 13 terminated pregnancies with a total of 75 fetuses, 26 abortions and 65 non pregnant recipients. Most of embryo transfers were performed during 2008 and 2009, accordingly the majority of births occurred during 2008, 2009 and 2010. Over the years, number of cultured oocytes rose, but maturation rate (matured oocytes/total number of cultured oocytes) declined. Fusion rate (fused embryos/total number of reconstructed embryos) and cleavage rate (cleaved embryos counted at the day of ET/total number of cultured NT embryos) increased. The number of NT embryos transferred during ET ranged from 48 to 152, but the average number did not differ much over the years. Pregnancy rate was varying a lot compared over the years. Abortion rate declined whereas the years 2008 and 2009 were almost the same. Although the total number of born piglets was rising the average number of born piglets per recipient was almost stable, whereas the average number of still born piglets dropped in 2009. Overall cloning efficiency mounted and was highest at 2008. All these findings are tendencies, because there are too few cases to perform a statistical analysis.

Table 3: In vivo SCNT work of the years 2006 until 2009

Year	2006	2007	2008	2009	Total
Number of performed embryo transfers	11	13	53	68	145
Total number of cultured oocytes	1477	1933	13022	18006	34438
Average maturation rate in %	85	87	79	76	82
Average fusion rate in %	78	86	89	87	85
Average cleavage rate of NT embryos in %	78	80	88	90	84
Total number of NT embryos transferred	896	1121	4885	5978	12880
Average number of transferred NT embryos per recipient	81	86	92	88	87
Pregnancy rate in %	64	31	60	54	52
Abortion rate in %	57	50	28	30	41
Number of birth	2	1	19	19	41
Number of pregnancy stopped	1	1	4	7	13
Total number of piglets / fetuses	13	10	100	112	235
Average number of piglets / fetuses per recipient	4.33	5	5.26	4.31	4.73
Total number of stillborn or degenerated piglets / fetuses	4	2	20	15	41
Average number of stillborn or degenerated piglets / fetuses per recipient	1.33	1	1.05	0.58	0.99
Cloning efficiency in % (offspring per transferred NT embryos)	1.45	0.89	2.05	1.87	1.82

1.2. Outcome of in vivo SCNT procedure

Of all 169 transgenic SCNT pigs born until June 2010, 37% were healthy and showed a normal development, the remaining 63% were lost. These losses can be divided in different groups, like still born, early neonatal death, killed by mother and lethal disease. The still born piglets died at different stages of pregnancy, but mainly they reached the last trimester. Some of the stillborn piglets showed abnormalities, but the majority appeared to have a normal development until shortly before birth.

Table 4: Upgrowth of SCNT piglets

Condition of transgenic SCNT piglet	Overall number (%)	Cases and explanation
Healthy and normal development	63 (37)	- Transgenic SCNT pigs up to three years of age until now - Produced healthy and transgenic progeny
Still born	39 (23)	- Full grown and fully developed - Mummification - Abnormalities
Early neonatal death	52 (30)	- Severe underweight (< 900g) - Weakness, low viability - Abnormalities: Oversize tongue, Cleft palates, Atresia ani
Killed by mother	11 (7)	- Prolonged and laborious birth - Nervous sow
Lethal disease	4 (2)	- Malignant hyperthermia syndrome - Bacterial meningitis

Most piglets were lost due to early neonatal death of weak and not viable piglets, some additionally showed severe underweight. For them it is hard to enforce a claim to their siblings and get enough food, especially at the first days. Another occurrence were piglets with deformations like oversize tongue and cleft palate. For these piglets it is impossible to drink milk, because they cannot produce the vacuum pressure needed. Another abnormality which occurred at times was contracted tendons in the front legs, but almost all involved piglets survived with some assistance. A smaller group was killed by the mother sow. Gilts were used as recipients for embryo transfers and it is impossible to predict their future

mother qualities. Also most times labor had to be medically induced and in some cases birth was prolonged and painful. It has happened that between two piglets there was a 24 hour delay. Only few piglets died in the course of a disease.

1.3. Evaluation in vivo SCNT data

All descriptive statistical analyses shown in the following are sourced of the in vivo SCNT data from 19.06.2006 to 31.05.2010.

1.3.1. Impact of seasonal change

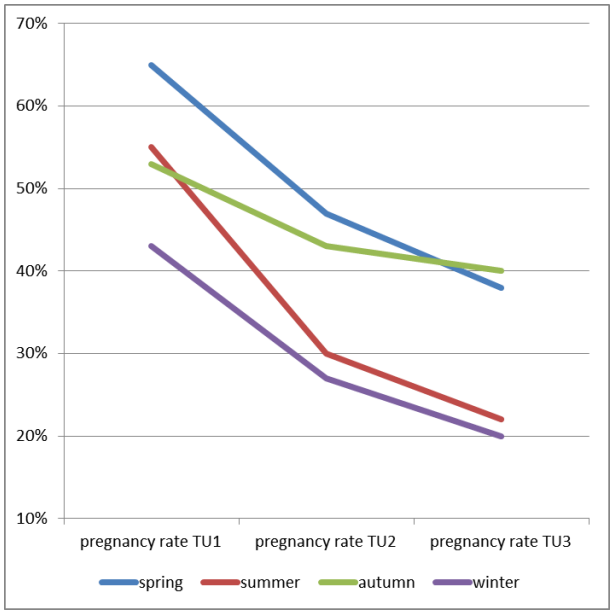
The in vivo SCNT experiments were assigned to the four seasons; the date of embryo transfer was used for the classification. The months March, April and May were classified as spring, June, July and August as summer, September, October and November as autumn and December, January and February as winter. When in the following seasons are mentioned, they always referred to the season in which the embryo transfer took place. During the seasons the maturation rate almost showed no variation at all. The pregnancy rate at the first check TU1 was best in spring (65%) and worst in winter (43%), summer (55%) and autumn (54%) had similar rates.

Table 5: Seasonal changes

	Spring	Summer	Autumn	Winter
Number of experiments	31	40	52	28
Maturation rate %	77	78	80	79
NT embryos transferred	2917	3504	4485	2378
Pregnant recipients (%)	20 (65)	22 (55)	28 (54)	12 (43)
Abortion (%)	7 (35)	11 (50)	5 (18)	4 (33)
Pregnancy stopped	2	3	5	3
Birth (%)	11 (55)	8 (36)	18 (64)	5 (42)
Piglets / Fetuses	49	53	103	38
Cloning efficiency %	1.68	1.51	2.30	1.60

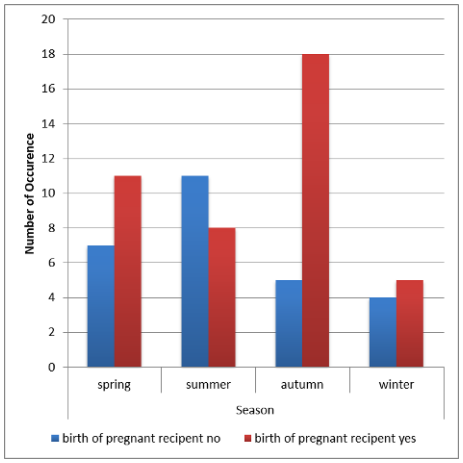
Most abortions occurred, when the embryo transfer was performed during summer (50%) and fewest after embryo transfers in autumn (18%). The abortion rate during spring (35%) and winter (33%) was almost the same. The highest percentage of pregnant recipients giving birth was found in autumn (64%), followed by spring (55%), winter (42%) and finally summer (36%) with the lowest percentage. The outcome of the SCNT in vivo experiments was that most piglets and fetuses were obtained from embryo transfers in autumn and fewest in winter, whereas spring and summer were almost equal in this respect. The calculated cloning efficiency (offspring per transferred NT embryo) revealed that clearly autumn was the best and the rest of season were almost alike, with the summer showing the worst performance of all.

Figure 14: Seasonal influence during course of pregnancy

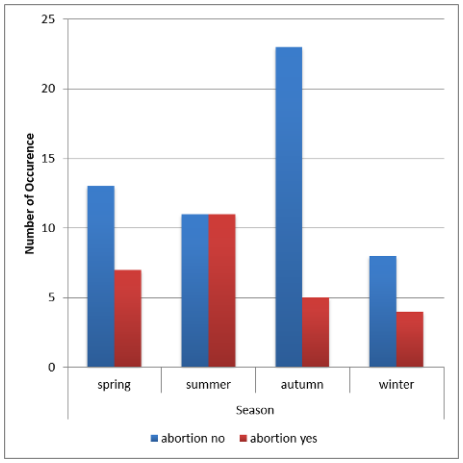


The influence of the seasons differed during the course of pregnancies. At the first pregnancy check (TU1), spring had the highest and winter the lowest pregnancy rate, autumn and summer were close together in between of them. During the ongoing gestastion it was reaveled that in all seasons pregnancies were lost, especially the pregnancy rate of summer dramatically dropped. This phenomenon was seen in the second pregnancy check (TU2) and even more clearly at the last pregnancy check (TU3). By the end of gestastion, the best results were obtained in spring and autumn, and the worst in winter and summer.

Figure 15: Seasonal influence on embryo transfer outcome



Pregnancies finished to term



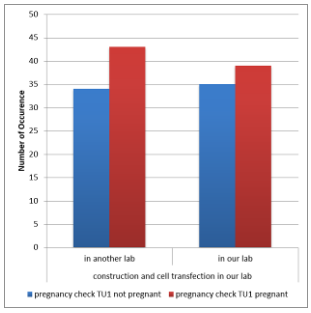
Abortion

Looking at the number of pregnancies finished to term at the differnt seasons, the autumn was outstanding. In autumn the range between birth and no birth was the highest, that means almost all sows wich became pregant during autumn also finished it succesfully. After the autumn, the spring had the second best birth rate. In winter the number of birth and no birth was almost equal. The only season where it was opposite down and more pregancies were lost than finished was the summer. During the seasons the number of abortion conducted almost reverse to the pregnancies finished to term. The main diffenerence is that stopped pregnancies for gaining fetuses, were counted as no abortion. It was revealed again that the autumn is the most eye-cathing season, with fewest abortions, then the spring and winter follows. In the summer the number of abortion and no abortion are equal, in all other seasons the number of no abortion is higher then the number of abortion.

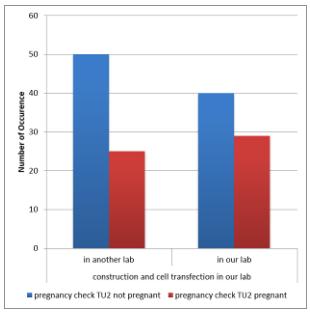
1.3.2. Effects of different SCNT donor cell treatment

Here it is distinguished between donor cells which were only treated in our laboratory and donor cells which were donated from several other laboratories. Cells from our laboratory were used in 74 in vivo experiments, other cells contributed to 77 experiments.

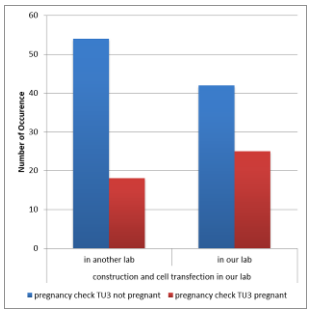
Figure 16: Performance of different donor cells during pregnancy



First pregnancy check TU1



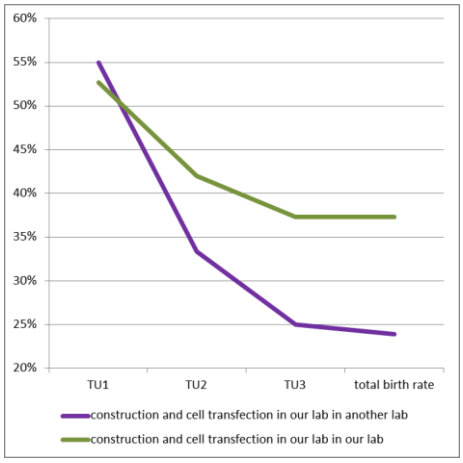
Second pregnancy check TU2



Last pregnancy check TU3

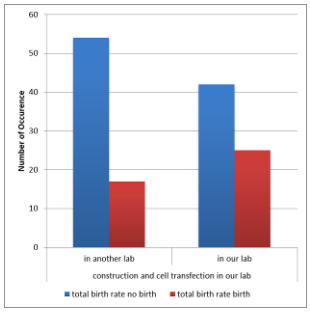
At the first pregnancy check TU1, both donor cell groups had very similar pregnancy rates. As the pregnancies went on the two groups began to alter. At the second check TU2 there were already more non pregnant recipients of donor cell of other laboratories, compared with our donor cells. This trend continued also at the last check TU3.

Figure 17: Comparison of different donor cells performance during the course of pregnancy until birth.

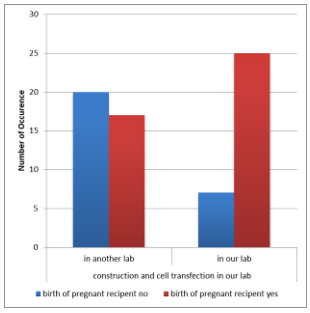


Pregnancy rates and total birth rates (pregnant recipients or occurance of birth divided by total number of recipients) were also calculated to elucidate the changes of donor cell perfomance in the course of pregnancy. Cells originated from other laboratories started with the highest pregnancy rate, but subsequently dropped dramatically. On the other hand cells treated only in our laboratory started second best, but showed a minor decline in following pregnancy rates. Finally our donor cells resulted in a noteable higher overall birth rate than donor cells of other laboratories.

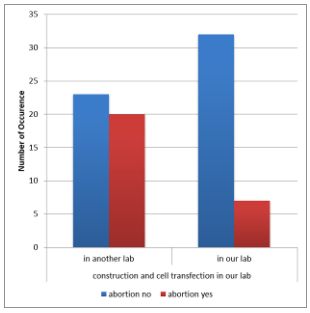
Figure 18: Birth and abortion rate in different donor cells



Total birth rate



Pregnancies finished to term



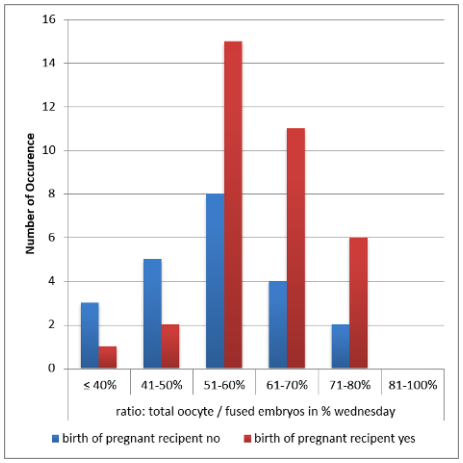
Abortion

The pregant recipient birth rate exposed a remarkable discrepancy between the donor cells obtained from other laboratories and donor cells orignated from our laboratory. Statistical analysis was performed and revealed a significant correlation and a significance level of 0.006 (T-test). Donor cells for SCNT procedure treated exclusively in our institue had a significantly higher chance, that pregancy results in birth. The abortion rate shows the same phenomenon just in reverse. When donor cells from our laboratory were used, there are significantly fewer abortions than cells from other source. Not only birth, but also stopped pregnancies with obtained fetuses were counted as no abortion.

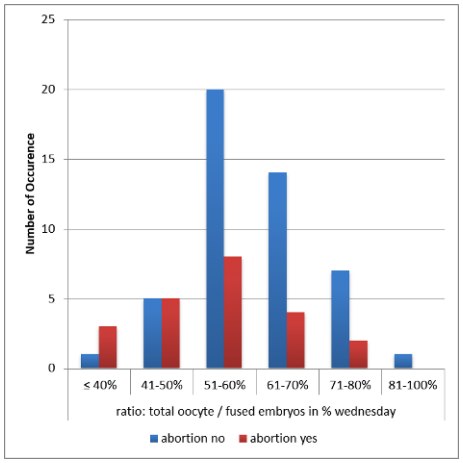
1.3.3. Different time periods of embryo culture

To examine the loss of oocytes from beginning of culture until fusion step of SCNT, the ratio of cultured oocytes to fused embryos was calculated. A ratio of 100% would mean no loss of oocytes.

Figure 19: Influence of oocytes loss during SCNT procedure on Wednesday



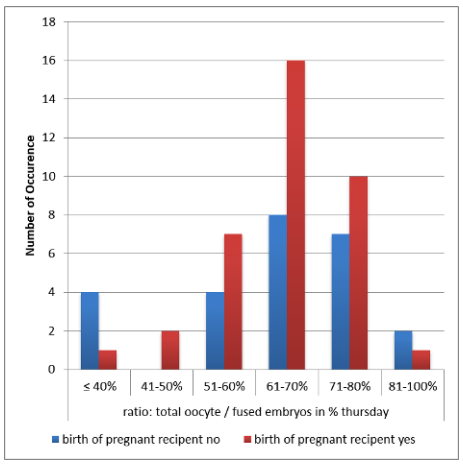
Pregnancies finished to term



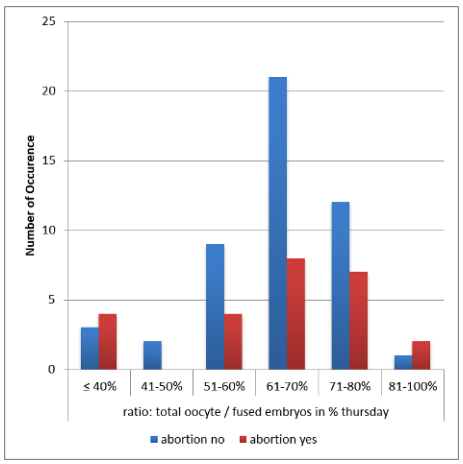
Abortion

On Wednesdays the ratio oocytes/fused embryos had a significant impact on the outcome of pregnant recipients. Most pregnancies persisted until the end and resulted in birth at a ratio better than 50% and under this value it was the other way around. A similar behavior in reverse was found in the abortion rate.

Figure 20: Influence of oocytes loss during SCNT procedure on Thursday



Pregnancies finished to term



Abortion

Thursday's ratio of oocytes/fused embryos showed neither connection to the birth of pregnant recipients nor to the abortion rate. The loss of oocytes during nuclear transfer process on Thursdays had no significant impact on the outcome like birth or abortion. Oocytes and NT embryos for experiments on Wednesday and Thursday were treated exactly the same way from in vitro maturation until fusion step of SCNT. The only difference occurred in diverging in vitro culture time of NT embryos. NT embryos produced on Wednesday underwent 2 days of culture and NT embryos of Thursday had only a one day in vitro culture. Subsequently NT embryos of both days were mixed and transferred together into recipient sows on Fridays.

Table 6: SCNT performed only on Wednesday or on Thursday

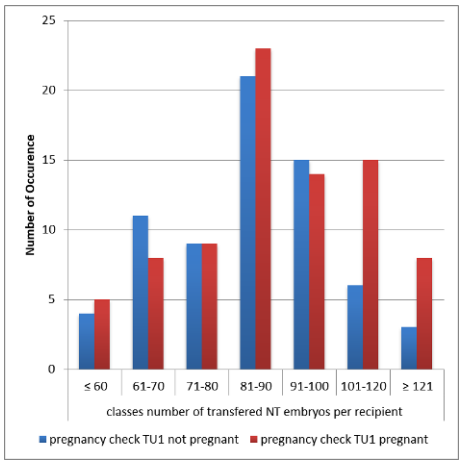
	Day two NT embryos transferred	Day one NT embryos transferred
number of experiments	12	15
cultured oocytes	1764	2193
maturation rate in %	79	78
number of transferred embryos	864	1284
pregnant recipient (%)	6 (50)	8 (53)
Birth (%)	2 (33)	4 (50)
pregnancy stopped	2 (33)	0
Abortion (%)	2 (33)	4 (50)
piglets/ fetuses	20	20
still born	1	1

Here are all in vivo SCNT experiments enlisted performed only on one day per week and therefore with different time periods of in vitro culture of embryos. Day one or day two embryos were transferred always on Fridays. In these cases the NT embryos could not be mixed, because only a single experiment was performed in these according weeks. Comparing both days ouctome it was visible that there are almost alike, total number of piglets and fetuses was even the same. Here it was elucidated, that SCNT embryos produced on different days both have a chance to develop to full term, although they are transferred in a different stage of development.

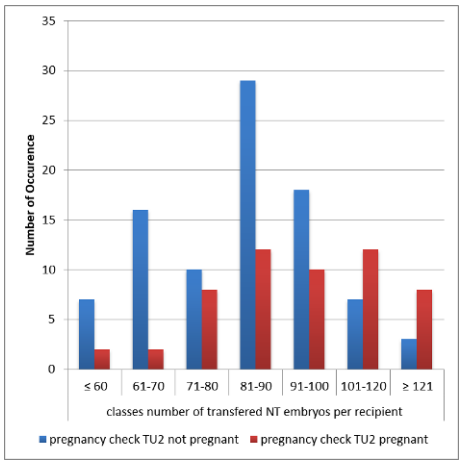
1.3.4. Number of transferred NT embryos per recipient

The number of transferred NT embryos ranged between 48 and 152, but in the main there were 81 to 90 NT embryos transferred per recipient. At the first examination of pregnancy status (TU1) no significant correlation between numbers of transferred embryos and occurrence of pregnancy could be found. Still there was a slight tendency, that recipients with over 100 transferred NT embryos had a better chance to become pregnant, whereas in recipients with under 100 transferred embryos the chance was almost equal.

Figure 21: Impact of number of transferred NT embryos on pregnancy (TU1+TU2)



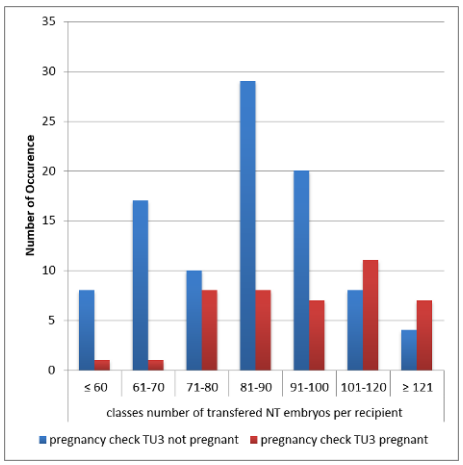
First pregnancy check TU1



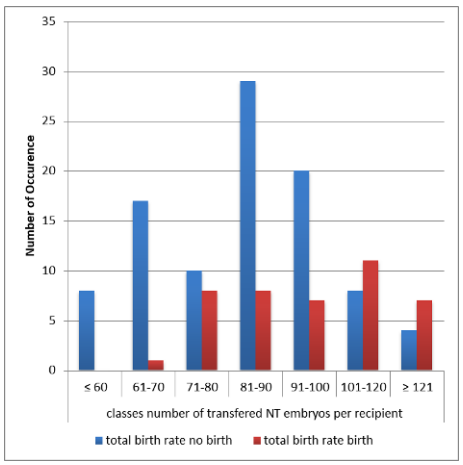
Second pregnancy check TU2

This tendency transformed during the course of pregnancy to a significant correlation. At the following pregnancy checks TU2 and TU3 it showed that when more than 100 embryos were transferred there was a significantly better chance of an enduring pregnancy. On the other hand, when under 70 embryos were transferred there was a significantly lower chance for a continuing pregnancy.

Figure 22: Number of transferred embryos and last pregnancy check and total birth rate



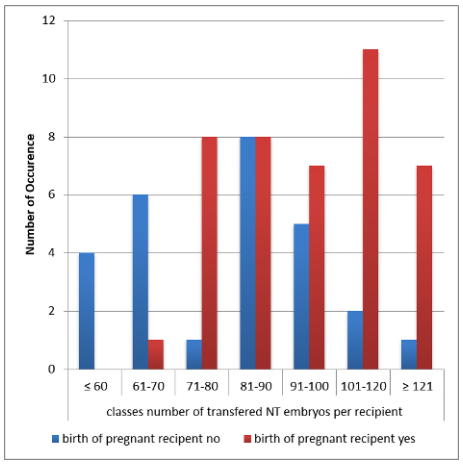
Last pregnancy check TU3



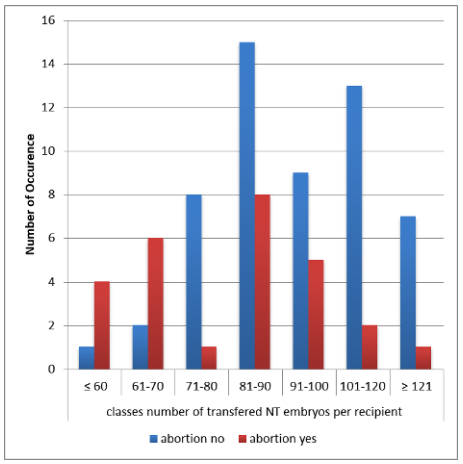
Total birth rate

The total birth rate behaved almost the same like the last pregnancy check TU3, there were only very few pregnancies lost in the last trimester.

Figure 23: Influence of the number of transferred NT embryos on birth of pregnant recipients and abortion



Pregnancies finished to term



Abortion

The trend visible during course of pregnancy also turned up in the birth of pregnant recipients and in the abortion rate and even clarified this trend. When over 100 NT embryos were transferred almost no pregnancies were lost and when under 70 NT embryos were transferred almost no pregnancy resulted into birth.

2. Production of cloned RANKL transgenic pig

2.1. In vitro SCNT embryo development competence of different cell lines

Before using new cell lines for in vivo SCNT experiments, they are tested for their in vitro development ability. At first wild-type porcine fetal fibroblast cell lines (PFF 06 and PFF 26) were tested, which served as basic cell lines for later transfection or transduction. The cell lines CMV RANKL, rtTA Bla+ and rtTA Bla- were transduced with lentiviral vectors at ExperiMed laboratory. All these cell lines showed the ability to generate blastocysts after SCNT.

Figure 24: CMV RANKL hatching blastocysts

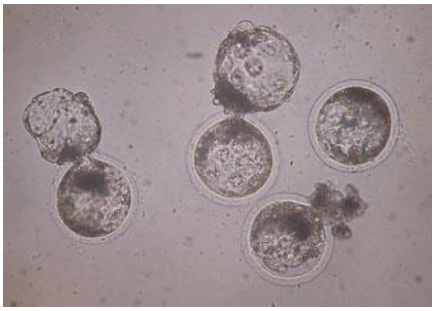


Table 7: In vitro test of wild type and lentiviral transduced cell lines

donor cell	Experiments	NT embryos	blastocysts (day 7)	blastocyst rate %
Wildtype cell lines				
PFF[1] 06	3	123	50	41
PFF[1] 26	1	30	4	13
Lentiviral transduced cell lines				
cmv[2] RANKL[3]	3	83	14	17
rtTA[4] Bla[5] +	2	46	2	4
rtTA[4] Bla[5] -	2	46	12	26

1) PFF- porcine fetal fibroblast
2) cmv- cytomegalovirus promoter
3) RANKL- receptor-activator of nuclear factor kappa beta ligand
4) rtTA- reverse tetracycline transcriptional activator (Tet-On)
5) Bla- Blasticidin

2.2. In vivo RANKL and Tet-On SCNT experiments

The different transgenic cell lines were established through two methods. In the beginning, the cells were modified via lentiviral transduction at the ExperiMed laboratory of Chirurgische Klinik und Poliklinik – Innenstadt LMU Munich (cell-lines: CMV-RANKL, Tet-On Bla+, Tet-On+RANKL+Bla). In another approach, cell lines were transfected by the Amaxa system in our laboratory (cell lines: Tet-On+RANKL+Neo, Tet-On+CAG promoter, 9894+TARE RANKL). For the production of the transgenic SCNT pigs 17 embryo transfers were performed. The pregnancy of 11 recipients and so a pregnancy rate of 64.71% was observed. These pregnancies resulted in one interruption with 4 obtained fetuses, 6 births with 25 born piglets and 4 abortions with an abortion rate of 36.36%. The overall cloning efficiency (offspring per transferred NT embryo) therefore accounted 1.65%.

Table 8: In vivo RANKL and Tet-On SCNT experiments

name of construct	experi-ment	NT embryos per recipient	Pregnant	pregnancy outcome	piglets / fetuses	stillborn
A) lentiviral transduction						
RANKL CMV-promotor	1	94	No			
RANKL CMV-promotor	2	53	Yes	Abortion		
RANKL CMV-promotor	3	75	No			
Tet-On Bla+	4	61	No			
Tet-On+RANKL+Bla	5	80	Yes	Abortion		
Tet-On+RANKL+Bla	6	80	No			
B) amaxa transfection						
Tet-On+RANKL+Neo	7	110	Yes	Stopped	4	1
Tet-On+RANKL+Neo	8	87	No			
Tet-On+RANKL+Neo	9	87	No			
Fetus 3 recloning	10	103	Yes	Birth	2	2
Fetus 3 recloning	11	82	Yes	Abortion		
Fetus 3 recloning	12	82	Yes	Abortion		
Tet-On + CAG pormoter	13	147	Yes	Birth	6	0
9894+TARE RANKL	14	110	Yes	Birth	5	2
9894+TARE RANKL	15	94	Yes	Birth	4	1
9894+TARE RANKL	16	83	Yes	Birth	5	3
9894+TARE RANKL	17	83	Yes	Birth	3	2

(A) Lentiviral transduction: Cells carrying the RANKL construct under control of the ubiquitous CMV promoter were used as donor cells for SCNT and were subsequently transferred to three recipients. One pregnancy could be detected but was lost before second ultrasound examination. Nuclear transfers of Tet-On and Tet-On-RANKL-Bla+ cells also leaded to one pregnancy, it was aborted as well. **(B) Amaxa transfection:** Donor cells transfected with a vector containing the Tet-On and the RANKL-TARE construct were used for 3 in vivo-experiments. One pregnancy could be established and was terminated on day 41 to obtain fetuses. Fetus 3 was recloned in 3 experiments, which lead to the birth of 2 stillborn Tet-On RANKL piglets. For a two-step strategy, the transactivator element Tet-On was introduced into a fetal fibroblast cell line. After SCNT, the reconstructed embryos were transferred into one recipient. Pregnancy resulted in six Tet-On transgenic piglets, which were sacrificed to obtain individual cell lines carrying the transactivator gene. Fetal cell lines were screened for expression of the Tet-On gene. After transfection with the RANKL construct containing a transactivator responsive element, a second round of SCNT was performed and embryos were transferred to 4 recipients. All of them became pregnant and finished to term, and altogether 17 piglets were born.

2.2.1. Recovery of Tet-On+RANKL+Neo fetuses

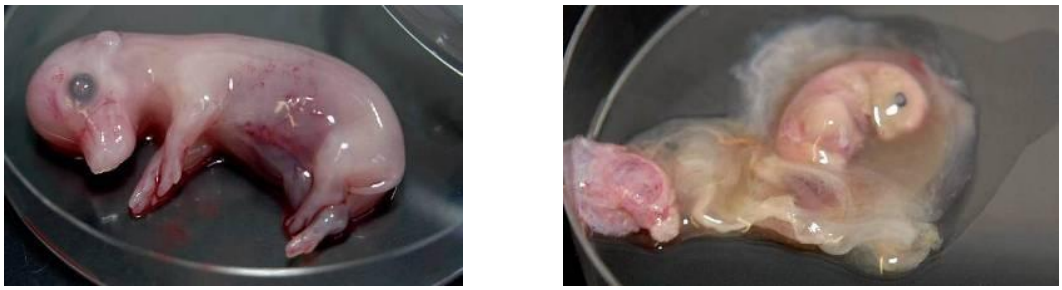
The pregnancy of recipient sow NT3 was terminated at day 41 after embryo transfer to gain the SCNT fetuses. Four fetuses could be obtained, of which three showed a normal development and one was already degenerated. Cell cultures were established from all fetuses, but only cells of Fetus 1, Fetus 2 and Fetus 3 were viable.

Table 9: Data of Tet-On+RANKL+Neo Fetuses

name of construct	Tet-On+RANKL+Neo
No. of experiment	7
Date of embryo transfer	20.06.2008
No. of recipient sow	NT 3
Date of birth	30.07.2008
No. of piglets / fetuses	4
Degenerated	1

Genotyping elucidated that Fetus 2 and Fetus 3 were carrying the Tet-On and RANKL gene and Fetus 1 did not. In the fetuses number 2 and 3 mRNA and protein of Tet-On could be detected. Expression of RANKL was only visible in Fetus 3, but this expression did not alter under in vitro doxycycline stimulation. These analyses were all performed by Tamara Radic at ExperiMed laboratory.

Figure 25: Tet-On+RANKL+Neo fetuses



a) Fetus 3: normal developed b) Fetus 4

2.2.2. Recloning of Fetus 3

The piglets, number 9878 and number 9879, were both born dead after a prolonged time of labor. Because these piglets probably had been already dead some time before labor started it was impossible to perform a complete analysis.

Table 10: Data of piglets recloned from Fetus 3

Name of construct	Fetus 3 recloning
No. of experiment	10
Date of embryo transfer	13.03.2009
No. of recipient sow	NT 44
Date of birth	08.07.2009
No. of piglets / fetuses	2
Stillborn	2

From the established cell lines of each piglet it could be elucidated that both were expressing Tet-On and RANKL at the mRNA level. The RANKL expression of these cell lines could not be stimulated by in vitro doxycycline administration. These in vitro tests were accomplished by Tamara Radic at ExperiMed laboratory.

2.2.3. Birth of Tet-On+CAG piglets

Out of the Tet-On + CAG transgenic cell line after SCNT procedure and embryo transfer 6 living piglets were born, of which each was a donator for a porcine kidney cell line. Two piglets died within 2 days after birth and a third piglet died 2 weeks later. The remaining 3 piglets were sacrifized to obtain samples for the analysis and the cell culture.

Table 11: Data of Tet-On+CAG piglets

Name of construct	Tet-On + CAG promoter
No. of experiment	13
Date of embryo transfer	27.03.2009
No. of recipient sow	NT 45
Date of birth	21.07.2009
No. of piglets / fetuses	6
Stillborn	0
Early neonatal death	3
Normal development	3

Only from piglet number 9893 we could not gain any living cells. The following analysis steps revealed that all piglets were carrying the Tet-On gene. All individual cell lines were transfected with the TARE RANKL gene and screened for best induction after in vitro doxycylin treatment. It appeared that the cell line of piglet 9894 transfected with TARE RANKL showed the best induction after doxycylcin administration and additionally this cell line showed almost no basal expression of RANKL. All mentioned screening and analysis steps were performed by Tamara Radic at ExperiMed laboratory.

Figure 26: Piglets of NT45 (piglets 9891 – 9895)

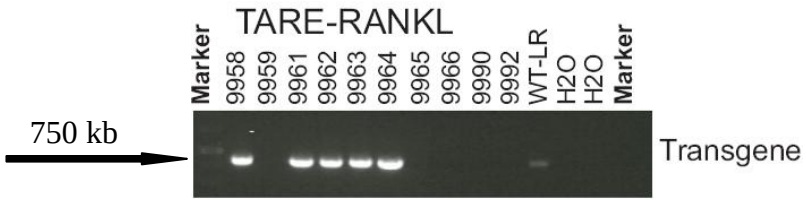
2.2.4. Birth of Tet-On 9894 + TARE RANKL piglets (two step strategy)

The kidney cell line of the Tet-On transgenic piglet 9894+TARE RANKL was chosen for further experiments. SCNT embryos reconstructed from this cell line were transferred to 4 recipients, which all finished to term. The total outcome was 17 born piglets of which 8 were still born. Unfortunately, the majority of these piglets were lost during the first days after birth. Only two survived and showed a normal development. Genotyping revealed that one of these two piglets carried the TARE RANKL gene (piglet 9961). Most of the other piglets did not reach the end of the first week, but one piglet also died after 24 days. These piglets displayed a low viability and some of them had severe underweight. Additional the weakness of piglets leaded to failure of milk production of recipient sow NT81 and NT84.

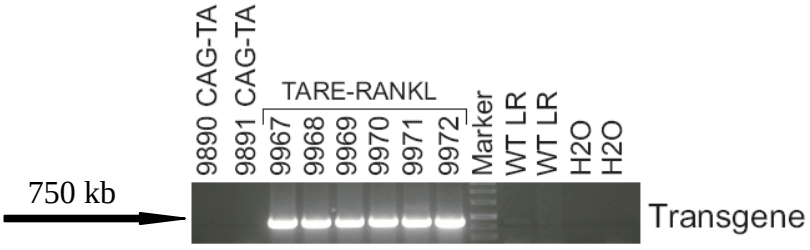
Table 12: Data of 9894+TARE RANKL piglets

No. of experiment	No. 14	No.15	No. 16	No. 17
Date of embryo transfer	30.10.2009	06.11.2009	13.11.2009	13.11.2009
No. of recipient sow	NT 79	NT 81	NT 83	NT 84
Date of birth	23.02.2010	02.03.2010	09.03.2010	08.03.2010
No. of piglets / fetuses	5	4	5	3
Stillborn	2	1	3	2
Killed by mother	0	1	0	0
Early neonatal death	1	2	2	1
Normal development	2	0	0	0

Figure 27: Genotyping of 9894+TARE RANKL piglets 9958-9972



At genotyping the following piglets were tested positive for TARE RANKL; litter of recipient 79: 9958, 9961 and 9962; litter of recipient 81: 9963 and 9964. No signal was found in the piglets 9959, 9965 and 9966.



Genotyping PCR revealed that the piglets (9967- 9972) of recipient 83 and 84 were carrying the TARE RANKL gene. These figures were a donation of Dr.rer.nat. Nikolai Klymiuk

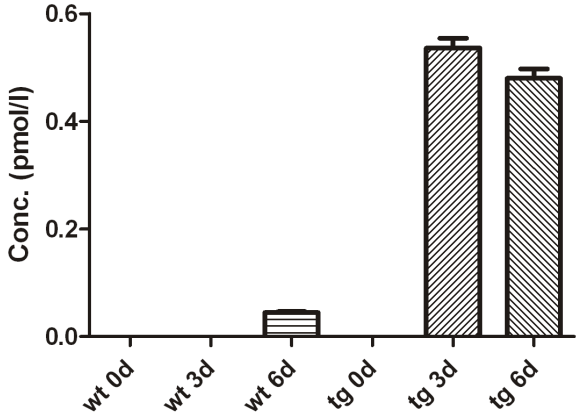
CAG: CMV early enhancer / chicken beta actin promoter
WT LR: wild-type landrace pig
RANKL: receptor-activator of nuclear factor kappa beta ligand
TARE: Tet-advanced transactivator response element promoter (TRE tight)
TA: reverse tetracycline-controlled transcriptional activator (Tet-On)
H2O: water control

Only in recipient sow NT79 piglets were strong and developed in a regular schedule. One reason could be that in this sow no interference during labor was necessary and she displayed right away good mother qualities. All other recipients (NT81, NT83 and NT84) had a prolonged time of labor and piglets could be only developed through manual help. Recipient sow NT81 killed one of its piglets by biting it.

Figure 28: Piglets of NT79 (piglets 9959 and 9961)

After weaning RANKL overexpression was stimulated by oral doxycycline administration over 6 days. The transgenic pig number 9961 carrying the Tet-On and the TARE-RANKL gene displayed an induction of RANKL expression at day 3 and day 6 of doxycycline treatment. The wild-type control which does not carry the TARE-RANKL gene showed only a basal expression of endogenous RANKL. The TARE-RANKL transgenic pig had a fivefold higher RANKL blood plasma level after doxycycline stimulation than the age matched wild-type control.

Figure 29: Doxycycline-inducible expression of sRANKL



wt: wild-type pig number 9959
tg: transgenic pig number 9961

Conc.: concentration in the blood plasma
d: day

This figure was a donation of Tamara Radic

V. DISCUSSION

1. SCNT over the years 2006 – 2009

In the years 2006 and 2007 we were occupied by establishing and improving the routine schedule of in vivo SCNT experiments and embryo transfers. During these two years only few experiments were performed and the outcome was only moderate. But starting with 2008 the number of cases multiplied by 5 times and SCNT was performed on a regular and professional basis. The effect showed in the number of born piglets and obtained fetuses, additionally the overall cloning efficiency was rising, too. The handling of SCNT embryos during micromanipulation and in vitro culture slowly improved. This enhancement became visible through the continuously elevating fusion and cleavage rate of embryos. Similar observations were noted by C.L. Keefer; disposing the thesis that better results were obtained of groups which conduct somatic cell nuclear transfer on a regular and consistent basis (KEEFER, 2008). Over the years 2006 until 2009 our average cloning efficiency varied between 0.89% and 2.05%, and was thus in the standard range among swine somatic cell nuclear transfer practice of 0.5% – 5.1% offspring per transferred SCNT embryos (DAI et al., 2002; KUROME et al., 2008; PETERSEN et al., 2008).

2. Outcome of transgenic porcine SCNT embryo transfers

There was not only a hazard of losing SCNT fetuses during pregnancies, but also a great deal of SCNT piglets died shortly after birth. These cumulative losses during all stages of development were one major reason for the low overall cloning efficiency; this phenomenon was found also in other groups (PARK et al., 2005; KISHIGAMI et al., 2008). For example Park et al. reported in detail about 40 somatic cell cloned piglets, out of which 5 (12.5%) were stillborn, 22 (55%) died suddenly during the first week of life and 12 (30%) were healthy (PARK et al., 2005). Our outcome of SCNT piglets were in total 169 born piglets, of which 39 (23%) were stillborn, 52 (30%) were lost in an early neonatal stage and 63 (37%) were healthy and showed a normal development. These results were comparable to the findings of Park et al. implementing that our high number of losses among SCNT piglets is due to the cloning procedure. It was often discussed

that the emergence of high numbers of abnormalities and the elevated mortality rate of SCNT embryos and piglets is due to reprogramming failures and epigenetic imprinting of the adult donor cells (JAENISCH et al., 2005). In this context there were reports of altered gene expression of SCNT offspring and extra embryonic tissue (CHAE et al., 2009; TIAN et al., 2009). But the exact mechanism of nuclear remodeling and reprogramming after SCNT still needs to be clarified (ZHAO et al., 2010). As long as this issue is not fully decoded, one has to resign to the fact that somatic cell cloning has some detrimental side effects visible in low overall efficiency and a high mortality rate in all developmental stages. Nevertheless, for the generation of transgenic pigs this is so far the most efficient and reliable technique (VAJTA et al., 2007).

3. Statistical analysis

3.1. Experimental setup

In our in vivo SCNT trials the main focus was laid on the production of transgenic piglets. Therefore we conducted experiments also under unfavorable conditions. Some recipient sows reacted poorly to hormonal synchronization and in the absence of a practical alternative these recipients were used anyway. In some weeks, oocyte in vitro maturation rate was noticeably depressed, additionally the amount and appearance of donor cells varied in each batch. The resulting NT embryos showed not only diversity in number but also in quality. Nevertheless, all reconstructed NT embryos were transferred, so that even a minimal chance of success is not wasted. This however caused a high level of variation among our results.

3.2. Seasons

Although pigs are known for their seasonally varying fertility, we could not find a significant correlation between different seasons and our nuclear transfer and embryo transfer data. There was only a tendency for a ranking of seasons visible, outstanding the autumn was best performing, followed by spring and winter and finally the summer was worst. The group Koo et al. also compared pregnancy rate and delivery rate of their porcine SCNT in vivo experiments throughout the different seasons. The mentioned seasons always referred to the date of embryo transfer. They recorded that the highest pregnancy rate was in the autumn group, although in the delivery rate spring was significantly higher than autumn. Their

winter group showed the worst performance in both pregnancy and delivery rate (KOO et al., 2009). The findings in our in vivo experiment were slightly divergent. The highest pregnancy rate had our spring group and the lowest our winter group. The fewest losses during pregnancy were recorded when embryo transfer was done in autumn and the highest losses came from the summer group. This high abortion rate of our summer group could be caused by seasonal infertility which is also found in conventional domestic pigs. There are reports of increase in the number of sows returning to estrus in the summer-autumn period and autumn abortion syndrome in domestic pig production (BERTOLDO et al., 2009). That means pigs inseminated during the summer months have a higher abortion rate later on, which confirmed our findings. So the best overall cloning efficiency was recorded in the autumn group. The group of Koo et al. assigned the spring as the season which showed best results after embryo transfer. Also there were no piglets born in their winter group (KOO et al., 2009). In our case we recorded birth of piglets from embryo transfers during the winter months, although the pregnancy rate was quite low. This could be due to the different embryo transfer procedures. Our method of embryo transfer was with endoscopic devices and they did a surgery with relocation of the uterine tract outside of abdominal cavity. Additionally they had no heatable environment at the pig stables to carry out their surgery (KOO et al., 2009), in contrast in our faculty we had a room which could be heated during winter time. The sudden drop of temperature surely has a detrimental effect on porcine SCNT embryos. Another finding was that the in vitro maturation rate of oocytes from slaughtered prepubertal sows almost did not differ according to the seasons, meaning that the seasons had a bigger influence on the recipient sows than on the in vitro matured oocytes gained at the abattoir. Unfortunately the variability was so high and the number of cases was too low that no significant correlations could be found. Probably there will be a significant correlation if more cases are available in the next years. One reason for the low variation of fertility throughout the year is that pigs were housed in standardized climate and nutrimental conditions.

3.3. Different treatment of donor cells

Origin and history of cells for nuclear transfer had an impact on the duration of pregnancies and therefore viability of the SCNT fetuses. Cells which were only treated in our laboratory had an in vitro culture period as short as possible to

prevent side effects of the artificial living conditions. This was achieved by applying mass selection after transfection and no single clone selection. Especially fibroblast cells hardly tolerate solitary culture conditions and when accompanied by the selection pressure they can bear defects afterwards. In contrast, the mass cell selection procedure is less harmful to the cells. This resulted in a better donor cell quality and improvement of in vivo SCNT outcome. The resulting piglets of one litter represented different cell clones with different integration and expression patterns. Thus, it was possible to establish several transgenic pig founder lines of one single in vivo SCNT experiment. The main disadvantage of mass cell selection is that cells not carrying the desired genes could slip through the selection step and result to nontransgenic SCNT piglets. In fact the percentage of non-transgenic SCNT piglets derived from mass cell selection was quite low, indicating that selection for two passages is sufficient to have almost all cells stable transfected. Cells donated from other laboratories underwent different culture conditions and handling by various people. Additionally all cells of other origin needed transportation either frozen in liquid nitrogen or fresh in a culture bottle. All these factors could influence the cell quality and it turned out that there was a significantly different abortion rate of SCNT embryos generated with cells produced in our lab vs. cells provided by collaboration partners. The developmental competence test of porcine wild-type cell lines revealed that even cells treated exactly in the same manner and with no genetic modification showed a different performance. That means each cell line has its individual capacity to successfully serve as donor cell for the nuclear transfer procedure. But nevertheless the donor cell treatment and therefore cell quality at the time point of nuclear transfer had a tremendous impact on the outcome of the in vivo SCNT experiments. One reason for these diverging performances could be found in different epigenetic imprinting or other changes in the donor nuclei. To form a viable embryo the donor cell has to accomplish various gene expression changes involving epigenetic modification and chromatin remodeling. Every malfunction during these events can have detrimental effects on the SCNT embryos and piglets (MAGNANI et al., 2008; ZHAO et al., 2010). A long time of in vitro culture and improper handling of donor cell could lead to alterations in the nucleus, which have a negative impact on the development capacity of SCNT embryos.

3.4. Different time span of in vitro culture of SCNT embryos

In our schedule of nuclear transfer and embryo transfer regularly two experiments per week on Wednesday and Thursday are performed and from both experiments embryos are cultured until Friday. NT embryos of both experiments were mixed in equal shares and transferred together into one or two recipients. That means that NT embryos from Wednesday have a one day longer time span of in vitro culture before embryo transfer. The disadvantage of our routinely sequence of operations is that after mixing the NT embryos before embryo transfer it is impossible to distinguish between NT embryos of Wednesday or Thursday. So there was no final proof which NT embryos (Wednesday or Thursday) maintained through gestation and resulted in piglets. When NT embryos were not mixed, because only one experiment per week was performed, there was no difference in pregnancy or abortion rate. Although NT embryos underwent either one or two days of in vitro culture before they were transferred on Fridays, in vivo experiments on Wednesdays and Thursdays had a very similar outcome. In other groups a 24 h delay of estrus synchronization status and development stage of SCNT embryo resulted in a significant alteration in pregnancy and delivery rates. It was postulated that an asynchrony between the reconstructed embryos and the recipients (pre-ovulation status) had a positive influence on the cloning efficiency in pigs. The authors reasoned this phenomenon by an improvement of the uterine environment for embryos that were delayed in development compared with their in vivo produced counterparts (PETERSEN et al., 2008; KOO et al., 2009). Interestingly when in vivo derived embryos were transferred already a recipient asynchrony of 48 hours resulted in the degeneration of all embryos (GEISERT et al., 1991).

3.5. Number of transferred SCNT embryos

Although it occurred that a recipient gave birth to 3 piglets after transfer of only 62 NT embryos, the chances are significantly higher as more embryos are transferred per recipient. The best chances of a successful pregnancy outcome were achieved with over 100 NT embryos per recipient and the worst chance with fewer than 70 NT embryos transferred. This highlights that during pregnancy most NT embryos are lost and only around 1- 5% of the embryos transferred survive until birth. Therefore this high number of embryos per recipient seems to be necessary unless the developmental capacity of SCNT embryos improves

greatly. Additionally, in the pig at least four viable conspectuses are needed to ensure the onset of early pregnancy (POLGE et al., 1966). On account of this it is still gold standard to transfer high numbers of porcine NT embryos (50-150 or even more) into each recipient (VAJTA et al., 2007; PETERSEN et al., 2008). Interestingly, in our experiments the number of transferred embryos was not so important to establish a pregnancy. At the first pregnancy check there was no significant relation between occurrence of pregnancy and number of transferred NT embryos. But embryo number had a significant influence on later positive pregnancy controls. An explanation for this phenomenon could be that at the first pregnancy check also parthenogenetic embryos could be detected. It was reported that they can develop to at least the limb-bud stage at day 29 and then they subsequently degenerate (KURE-BAYASHI et al., 2000). In our SCNT procedure we conducted blind enucleation to avoid the unnecessary and harmful UV light detection step. So it is possible that some of the transferred NT embryos had a remaining nucleus of the oocytes and underwent parthenogenesis. Signaling of parthenotes is adequate to maintain early pregnancy until around day 29 (KAWARASAKI et al., 2009). But just as well the incidence of lost pregnancies between first and second pregnancy check could be also caused by the low developmental capacity of SCNT embryos.

4. Production of transgenic Tet-On and RANKL pigs

First attempts to generate piglets, carrying the RANKL construct under control of the constitutively active CMV promoter, resulted in a single pregnancy, which was aborted later on. Transgenic mice with constitutive and ubiquitous overexpression of sRANKL all died few days before birth. When the overexpression took place exclusively postnatal, most transgenic mice grew up to be adult and became fertile. It was postulated that sRANKL overexpression during late fetal stage had lethal consequences (MIZUNO et al., 2002). In our case we obtained no piglets with constitutive and ubiquitous RANKL overexpression, yet we only conducted three embryo transfers. The descried abortion could be accounted as a common event, which we observed in a similar rate in other in vivo SCNT trials. Because it was impossible to exclude a harmful effect of RANKL overexpression during fetal development, we additionally integrated a controllable gene expression system (Tet-On). This should enable a switch on of RANKL expression at any desired time point (SUN et al., 2007; STIEGER et al.,

2009). During the further approach it was impossible to obtain any offspring out of lentiviral transduced donor cells, although they supported in vitro formation of blastocysts. In our studies it appeared that cell lines, which had been transfected in our own laboratory, displayed a better performance as donor cells for in vivo SCNT experiments. So a change of strategy took place and subsequent donor cells were all only treated in our laboratory. The results of the production of a double transgenic pig in only one step of transfection and SCNT resulted in 4 fetuses and 2 stillborn piglets after recloning of a transgenic fetus. Unfortunately hardly any doxycycline in vitro induction was detectable in this Tet-On+RANKL+Neo double transgenic fetuses and the recloned piglets. Therefore a new approach was applied in which the Tet-On and RANKL genes were introduced step by step. In the first round of transfection and SCNT Tet-On transgenic piglets were created. Screenings for the highest level of Tet-On expression and the best inducibility of a subsequently transfected TARE-RANKL expression construct were performed under in vitro cell culture conditions. Best performance showed the cell line derived from the Tet-On piglet number 9894 and transfected with TARE RANKL. Subsequently this cell line was the donor for the next SCNTs and resulted in the birth of double transgenic piglets. Until today there is no published report about a transgenic pig carrying the Tet-On inducible gene expression system. In mice several transgenic mouse models with controllable gene expression via doxycycline administration were already established (KISTNER et al., 1996; ZHU et al., 2002; BACKMAN et al., 2009; RAO & MONKS, 2009; SONG et al., 2009). So we produced the first piglets which exhibited functional Tet-On and also showed inducible expression of the desired gene. Future examinations will reveal what impact long time overexpression of RANKL has on piglet number 9961 originated of Tet-On 9894+TARE RANKL cell line. This piglet may develop an osteoporotic phenotype comparable to the human status of disease. In 7-8 months old mice overexpression of sRANKL resulted in a significant deprivation of the femur bone mineral density, due to an enhanced osteoclastogenesis (MIZUNO et al., 2002).

5. Conclusion

In the continuing working processes our findings are integrated if possible. The aim is to transfer at least 100 NT embryos per recipient. We changed embryo transfer regime and recipient sow synchronization. Now NT embryos are

transferred on the same day as nuclear transfer takes place, meaning directly on Wednesdays and Thursdays. Estrus synchronization is shifted so that sows are in a pre-ovulation stage at embryo transfer, corresponding to research results of other groups (PETERSEN et al., 2008; KOO et al., 2009). Other laboratories who want to donate cells for nuclear transfer procedures, we offer a list of recommendations for treatment and handling of donor cells.

After the successful in vivo induction of RANKL expression of the Tet-On 9894+ TARE RANKL pig, it will serve as founder for a transgenic pig line by recloning and natural mating. If an osreoporotic phenotype can be induced, this novel model will serve for fracture studies in collaboration with surgeons to elucidate the influence of the osteoporosis disease state on the healing processes.

VI. SUMMARY

Inducible expression of RANKL in transgenic pigs under the control of the Tet-On system

Because of the tremendous need for transgenic large animal models for human diseases, the process of SCNT is a crucial step in transgenic pig production. In our study, we evaluated the particular steps during the production for their impact on the efficiency of cloning transgenic pigs. For this purpose, statistical analysis was performed for all SCNT data from the years 2006 until June 2010. The RANKL transgenic osteoporosis model was chosen for an example for the production steps needed to finally achieve a disease model, to elucidate pitfalls and chances of SCNT procedure. In total 151 in vivo SCNT experiments using different transgenic cell lines were carried out, resulting in 243 piglets and fetuses. Statistical analysis revealed that donor cells treated exclusively in our laboratory had a significant better birth rate than donor cell originated of other laboratories. Furthermore, there was a significant relation between number of transferred NT embryos and later pregnancy checks, birth rate and abortion rate. The more NT embryos were transferred, the more pregnancies finished to terms. It was also elucidated that in our studies a different in vitro culture time of 24 or 48 hours had no significant impact on the outcome like pregnancy or birth rate. Seasonal changes during the years had no significant influence on pregnancy rate, birth or abortion. But there was a strong tendency that autumn showed best performance of all seasons, and most pregnancies were lost after embryo transfers during the summer. All these findings will be integrated in future in vivo SCNT experiments and embryo transfers. For the production of a transgenic osteoporosis model 17 in vivo experiments took place so far, with an outcome of 4 fetuses and 25 piglets. For gaining a controllable expression of RANKL, it was necessary to establish double transgenic pigs to sidestep harmful effects of RANKL overexpression during the fetal development. First attempts to integrate both genes, tetracycline controlled transactivator (Tet-On) and RANKL, in a single step of cell transfection and SCNT, had no satisfying result. We obtained 4 fetuses and stillborn recloned piglets carrying both genes, but they showed only expression of Tet-On and it was impossible to induce RANKL overexpression. Therefore the strategy was changed in favor to two rounds of transfection and nuclear transfer.

First Tet-On transgenic piglets were established and screened for integration and expression. Piglet 9894 showed the best expression and severed as donor for next cell transfection step. These Tet-On + TARE RANKL cells were in vitro tested for their inducibility. Thereafter SCNT and embryo transfer of the best candidate were performed and they resulted in 4 pregnancies which all finished to term. One double transgenic piglet could be raised and will be kept until adulthood to establish a line of Tet-On +TARE RANKL transgenic pigs. Importantly, this founder animal showed inducible RANKL overexpression. Other constructs might be based on the existing Tet-On cell line in the future, offering an inducible system for a broad variety of different transgenes. Thus a functional Tet-On system in the pig is reported for the first time.

VII. ZUSAMMENFASSUNG

Induzierbare Expression von RANKL in transgenen Schweinen unter der Kontrolle des Tet-On Systems

Da es einen enormen Bedarf an transgenen Großtieren als Modelltiere für humane Erkrankungen gibt, wie zum Beispiel für Osteoporose, wurde die Generierung von transgenen Schweinen mittels somatischen Kerntransfers genauer untersucht. Dabei war das Ziel herauszufinden, welchen Einfluss die einzelnen Arbeitsschritte auf die Produktionseffizienz haben. Aus diesem Grund wurde eine statistische Analyse aller in vivo Kerntransfers von Anfang 2006 bis zum Juni 2010 durchgeführt. An dem Beispiel eines RANKL transgenen Osteoporose-Models wurden alle nötigen Produktionsschritte dargestellt und die Schwierigkeiten und Vorteile des somatischen Kerntransfers beschrieben. Die insgesamt 151 in vivo Experimente, wobei unterschiedliche transgene Zelllinien genutzt wurden, resultierten in 243 Ferkeln und Feten. Statistische Analysen zeigten, dass Spenderzellen, die ausschließlich in unserem Labor behandelt wurden, nach Kerntransfer zu einer signifikant höheren Geburtsrate der trächtigen Empfänger führten als Spenderzellen aus anderen Laboren. Weiterhin ergab sich ein Zusammenhang zwischen der Anzahl übertragener Kerntransfer-Embryonen und der späteren Trächtigkeitsrate, Geburtsrate und der Abortrate. Je mehr Embryonen übertragen wurden, desto mehr Trächtigkeiten wurden erfolgreich beendet. Es wurde auch sichtbar, dass in unseren Versuchen eine unterschiedliche in vitro-Kulturdauer der Kerntransfer-Embryonen von 24 bzw. 48 Stunden keinen signifikanten Unterschied in der Trächtigkeits- oder Geburtsrate verursachte. Auch für die verschiedenen Jahreszeiten konnte kein signifikanter Einfluss auf Trächtigkeit oder Geburt nachgewiesen werden. Es zeigte sich aber die Tendenz, dass im Herbst die besten Bedingungen für einen positiven Verlauf der Trächtigkeit herrschen und nach Embryotransfers im Sommer die höchste Abortrate auftritt. All diese Untersuchungsergebnisse werden zukünftig in unseren Arbeitsalltag integriert und der Kerntransfer und Embryotransferablauf optimiert. Zur Erstellung eines transgenen Osteoporosemodells wurden 17 Embryotransfers durchgeführt, die in 4 gewonnenen Feten und 25 geborenen Ferkeln resultierten. Um eine regulierbare RANKL Expression zu erhalten war es notwendig, doppelt transgene Schweine zu erstellen, so dass negative Nebeneffekte der RANKL

Überexpression während der Fetalentwicklung vermieden wurden. Die ersten Versuche, Tetrazyklin Transaktivator (Tet-On) und RANKL in einem einzigen Schritt der Zelltransfektion und des in vivo Kerntransfers zu integrieren, führte zu wenig befriedigenden Ergebnissen. Es wurden 4 doppelt transgene Feten und 2 totgeborene Ferkel gewonnen, doch es konnte nur die Expression von Tet-On nachgewiesen werden, da die RANKL Expression nicht induzierbar war. Deswegen wurde die Strategie zu Gunsten von zwei Einzelschritten der Zelltransfektion und in vivo Kerntransfers gewechselt. Zuerst wurden Tet-On transgene Ferkel erstellt und auf Integration und Expression hin untersucht. Das Ferkel 8994 zeigte die beste Expression und seine Zellen wurden für den nächsten Zelltransfektionsschritt verwendet. Die daraus resultierenden Tet-On + TARE RANKL-Zellen wurden in vitro auf ihre Induzierbarkeit getestet. Als Spenderzellen für weitere in vivo Kerntransfers diente der beste Kandidat aus diesen Tests. Alle 4 Embryotransfers resultierten in Trächtigkeiten, die alle auch ausgetragen wurden. Ein doppelt transgenes Ferkel konnte aufgezogen werden, das zum einen nach Erreichen der Geschlechtsreife als Gründer einer transgenen Schweinelinie dienen wird, und im in vivo-Test eine induzierbare Expression von RANKL zeigte. Die regulierbaren Tet-On Zelllinien können auch für weitere zukünftige Konstrukte Verwendung finden, was die Möglichkeit mannigfaltiger genetischer Manipulation durch ein induzierbares System eröffnet. Hiermit wird das erste Mal von einem funktionalen und kontrollierbaren Tet-On System im Schwein berichtet.

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IX. LIST OF FIGURES

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XI. ACKNOWLEDGEMENT

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