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Regulated gene over-expression of Receptor Activator of NF-ĸB Ligand (RANKL) in a porcine animal model

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1. INTRODUCTION

Bone-related diseases, such as osteoporosis, affect hundreds of millions of people worldwide and pose a tremendous burden to health care [1]. Osteoporosis, characterized by enhanced bone resorption, leads to reduced bone mass and increased risk of fractures. Furthermore, it is a disease of rapidly increasing prevalence and high morbidity. Thus, increased awareness of this condition on the part of the medical community and the public has led to an increase in screening measures to make the diagnosis and in improvements in its treatment. Therefore, newer therapeutic approaches based on a better understanding of the pathophysiology of osteoporosis are needed [1].

Bone is constantly being remodeled. This is the natural, healthy state of continuous uptake of old bone (resorption) followed by the deposit of new bone (formation). The cells that form new bone are called osteoblasts and the cells responsible for resorption of old bone are called osteoclasts. Osteoporosis occurs as a result of a mismatch between osteoclasts and osteoblasts activities. The result is a thinning of the bone with an accompanying loss in bone strength and a greater risk of fracture. Although many acquired or environmental factors are known to affect the adult bone mineral density [2], genetic factors are also known to play a major role [3]. Understanding the mechanisms that control the remodelling process and its regulation will clarify not only the local control of the function of bone cells but also the pathophysiology of accelerated bone loss in osteoporosis.

One of the most significant advances in bone biology in the past decade has been the identification of receptor activator of nuclear factor-kappa B (RANK) and its ligand (RANKL) as key regulators of bone remodelling. RANKL, a member of the tumor necrosis factor superfamily, is secreted by osteoblasts in response to a variety of hormonal and cytokine signals known to be important for bone metabolism and is the principal regulator of the differentiation and activity of osteoclasts. RANKL signalling is mediated by its receptor RANK on osteoclasts; the binding of RANKL to RANK is inhibited by sequestration to a soluble decoy receptor, osteoprotegerin (OPG). OPG is co-secreted by osteoblasts and serves to modulate the effective levels of RANKL. As expected, animal models confirm that RANKL-excess shifts that balance of bone metabolism in the direction of catabolism and causes osteoporosis. Conversely that absence of RANKL increases bone density. Therefore, RANKL inhibition offers the therapeutic possibility to treat osteoporosis.

The successful development of transgenic animal models for human diseases has led to remarkable impacts on the diagnosis, treatment and intervention in human diseases. Furthermore, they have helped to clarify our understanding of disease pathophysiology.

Unfortunately, many mouse disease models often do not reflect the relevant human conditions sufficiently. Pigs however show similarities in size, physiology, organ development and course of disease. Therefore, they represent an important and ideal mammalian model for human biology research.

Taken together, the main aim of this doctoral thesis is: Establishment of an osteoporotic large animal model by regulated over-expression of extracellular domain of porcine Receptor Activator of NF-KB Ligand (sRANKL). In order to accomplish the main aim, the following project goals were defined:

1. Cloning and stable over-expression of the porcine soluble RANKL protein in swine cells.
2. Characterization of osteoclast formation in porcine system.
3. Investigation of the effects of porcine sRANKL over-expression on osteoclast formation.
4. Establishment of an inducible Tet-On-psRANKL system *in vitro*.
5. Generation of transgenic pigs over-expressing porcine sRANKL upon induction.
6. Characterization of transgenic piglets and re-cloning of the best expressing one.
7. *In vivo* induction of psRANKL expression to study osteoporotic markers.

This study is a part of the DFG research group "Mechanisms of fracture healing and bone regeneration in osteoporosis" (DFG 793/project 2). It established the first transgenic large animal model for osteoporosis, which can be used to study fracture healing.

1.1 OSTEOPOROSIS

1.1.1 Definition and risk factors

Osteoporosis is a chronic bone disorder characterized by reduced bone mass and alteration of bone architecture (Figure 1.1), resulting in increased bone fragility and fracture risk [4]. Although there are no current practical methods to assess overall bone strength, bone mineral density (BMD) correlates closely with skeletal load bearing capacity and fracture risk [5]. The World Health Organization (WHO), therefore, defines osteoporosis in humans as a BMD based on the T-score. T-score is the difference between the patient's BMD and the mean BMD of a young adult reference population. Based on T-score, four different variations exist: healthy (a T-score of –1.0 or higher), osteopenia (a T-score of less than –1.0 but higher than –2.5), osteoporosis (a T-score of –2.5 or less) and severe osteoporosis (a T-score of –2.5 or less with a fragility fracture) [6].

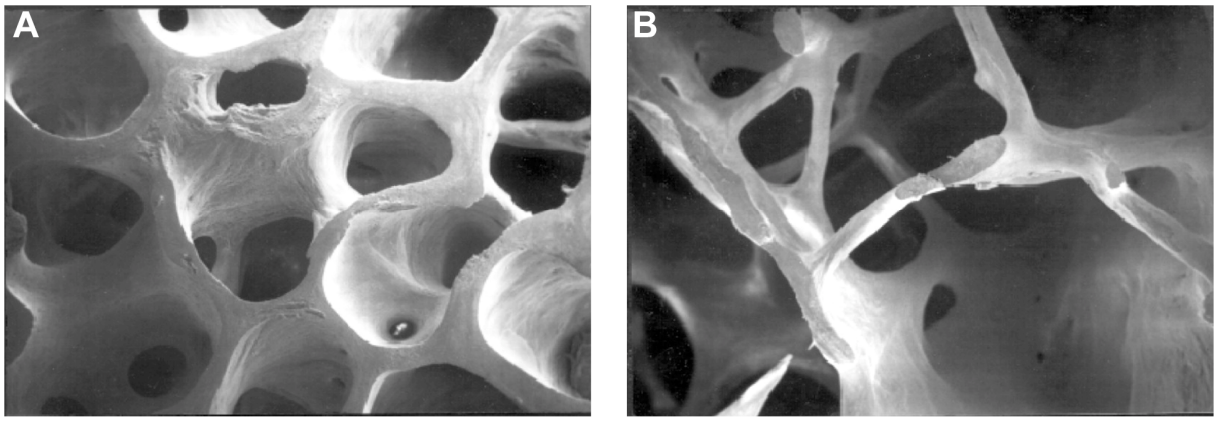

Figure 1.1.: Comparison of bone architecture from healthy (A) and osteoporotic patient (B). From [4]

Although age is a common cause associated with bone fracture, there are several other important secondary causes of osteoporosis. Other pathogenetic factors include reduced physical activity, vitamin D insufficiency, genetic factors, sex hormone status, nutrition, smoking, alcohol excess, low weight, low muscle mass, corticosteroid use, Caucasian or Asian race, family history of fragility fracture, heparin, hypogonadism, thyrotoxicosis, deficiency of calcium, gender, geographical region, bone quality, primary or secondary amenorrhea, caffeine intake and rheumatoid arthritis [7-14].

1.1.2 Forms of osteoporosis

Osteoporosis can be classified as primary or secondary. The primary osteoporosis is a multifactorial illness including genetic alterations, hormonal changes and a sedentary lifestyle and it is mainly seen in elderly people [15]. Postmenopausal osteoporosis, the most common primary form of osteoporosis, is often observed in trauma units dealing with fracture treatment. Secondary forms of osteoporosis are predominantly medication/drug-induced forms, such as glucocorticoid-induced osteoporosis. Secondary osteoporosis can be observed in both young and old people [16].

1.1.3 Clinical aspects of osteoporosis

The bone strength primary reflects the integration of bone density and quality. Bone density is expressed as grams of minerals per area or volume. While bone quality is defined as the architecture, turnover, damage accumulation and mineralization of the bone [17]. When a failure-inducing force, such as trauma, is applied to an osteoporotic bone, a fracture occurs, thus osteoporosis is a significant risk factor for fractures. These fractures are a major cause of morbidity and mortality in the elderly people. Screening for individual fracture risk is still not reliable in usual clinical settings. The following factors have to be taken into account in order

to estimate the progression of the disease: bone architecture and geometry, mineralization, microdamage accumulation and properties of the collagen and mineral matrix [18]. Furthermore, there is a clear relationship between aging and the incidence of osteoporosis. Women are also affected more often than man, due to the postmenopausal estrogen deficiency [16,19].

1.1.4 Osteoporosis treatment

Treatment for osteoporosis requires a multidisciplinary approach encompassing patient information, lifestyle advice, physical measures, pharmacological therapy and in some cases interventional options. The choice of treatment is influenced by factors such as bone mineral density, stage of disease progression, nature and site of fracture, patient age, underlying co-morbidities and side effects. The goal of treatment of osteoporosis is the prevention of bone fractures by reducing bone loss or by increasing bone density and strength [20].

Currently, the most effective medications for osteoporosis that are approved by the Food and Drug Administration (FDA) are antiresorptive agents bisphosphonates, which decrease the removal of calcium from bones and inhibit resorption [21]. Bisphosphonates such as alendronate (Fosamax®), risedronate (Actonel®), raloxifene (Evista®), ibandronate (Bonviva®) and the recently approved zoledronate (Zometa®) are the examples of antiresorptive agents [22]. Furthermore, a newly approved medication by FDA for use in postmenopausal women with risk of osteoporosis is an inhibitory antibody against RANKL, Denosumab (Prolia®) which increases bone mineral density, decreases bone resorption and prevents fractures [23,24]. Calcium and vitamin D supplements should be co-prescribed with other treatments for osteoporosis. A daily intake of elemental calcium (1 200 mg per day) and vitamin D3 (800–1 000 IU per day) are considered to be necessary for prevention of osteoporosis [25].

1.1.5 Biochemical markers of osteoporosis

The field of bone turnover markers has developed considerably in the past decade. Biochemical monitoring of bone metabolism depends upon measurement of enzymes and proteins released during bone formation and resorption. Various biochemical markers are now available, although these markers are not recommended for use in diagnosis of osteoporosis, they appear to be useful for the individual monitoring of osteoporotic patients [26]. A summary of bone formation markers is as follows: serum total alkaline phosphatases, serum bone–specific alkaline phosphatases, serum osteocalcin and serum type 1 procollagen C-

terminal/N-terminal (C1NP or P1NP) and a summary of bone resorption markers is the following: urinary hydroxyproline, urinary total pyridinoline (PYD), urinary free deoxypyridinoline (DPD), urinary collagen type 1 cross-linked N-telopeptide (NTX), urinary or serum collagen type 1 cross-linked C-telopeptide (CTX), bone sialoprotein (BSP) and tartrate-resistant acid phosphatase 5b (TRACP5b) [27].

Diagnosis of osteoporosis is not based on evaluation of bone markers, rather the BMD assessment is still the criterion standard for evaluation and diagnosis [28]. The level of bone mass can be assessed with adequate precision by measuring BMD using dual-energy x-ray absorptiometry (DEXA) [29]. However, this measurement does not capture all risk factors for fracture. BMD assessment with DEXA is a surrogate marker of treatment efficacy that has been widely used in clinical trials. However, mean values for markers of bone turnover are higher in osteoporosis patients than in the matched controls [30].

Such markers can also be useful in selected cases to improve the assessment of individual fracture risk when BMD measurement by itself does not provide a clear answer. The combined use of BMD measurement and bone markers is likely to improve the assessment of the risk of fractures in bone disorders [27,31,32].

1.1.6 The health economics of osteoporosis

Fractures due to osteoporosis and osteoporosis itself do not only have a major impact on peoples' health and quality of life, but they also place an enormous economic burden on healthcare systems in Europe and worldwide. As the population demographics change, an increased number of osteoporotic fractures are expected. A growth that may in some societies be compounded by a rise in age-specific incidence. Thus, osteoporosis potentially could increase to epidemic proportions within 50 years. Driven by this, there is a marked increase in research into the pathogenesis of bone loss and a heightened interest in developing new therapeutic agents to prevent and treat the disease [33].

According to the World Health Organization report from 1994, about 30% of postmenopausal women are estimated to have osteoporosis [6]. The prevalence rate of osteoporosis ranges between 13% and 30% for the US and the UK [33-35]. The prevalence in Germany is estimated to be between 4 million and 7 million people [36-38]. One out of three postmenopausal women and one out of five men over the age of 50 years will experience osteoporotic fractures [39]. The report of the European Commission from 1998 estimates an increase in the incidence of hip fractures in Germany until the year 2040, from 117 000 in 2000 to 240 000 in 2040 [40]. As the median age of the German population increases, the

costs associated with osteoporotic fractures are also likely to increase. Thus, the osteoporosis is responsible for millions of fractures annually and total medical care costs in Europe, including hospitalization and rehabilitation. These were 76,7 billion € in 2005 [41].

Due to its social and economic implications, osteoporosis is a major public health problem. Osteoporosis-related fractures account for more hospital days per year than diabetes, myocardial infarction or breast cancer [39,42,43]. Therefore, it is essential to identify individuals at high risk and take preventive measures in order to reduce the costs associated with osteoporosis. Furthermore, the osteoporosis issue demonstrates the need for effective prevention and treatment strategies [39].

1.2 BONE REMODELLING

During the normal homeostasis of bone, formation and resorption are balanced so when old bone is resorbed by osteoclasts, a similar amount of new bone is formed by osteoblasts. The purpose of remodelling is to regulate calcium homeostasis, repair micro-damaged bones and to shape and sculpture the skeleton during growth. During osteoporosis there is an abnormality in the remodelling process of bone in which bone resorption exceeds bone formation, thus leading to a net loss of bone.

Bone remodelling occurs in focal and particular units called basic multicellular units (BMU) where osteoblasts and osteoclasts are coupled together *via* paracrine cell signalling [44]. Their action is controlled by a number of chemical factors, which either promote or inhibit the activity of the bone remodelling cells. This process consists of five phases: (1) initiation: preosteoclasts are stimulated and differentiate under the influence of cytokines and growth factors into mature active osteoclasts; (2) resorption: osteoclasts digest the mineral matrix; (3) reversal phase: end of resorption; (4) formation: osteoblasts synthesize a new bone matrix; and (5) quiescence: osteoblasts become resting bone lining cells on the newly formed bone surface (Figure 1.2).

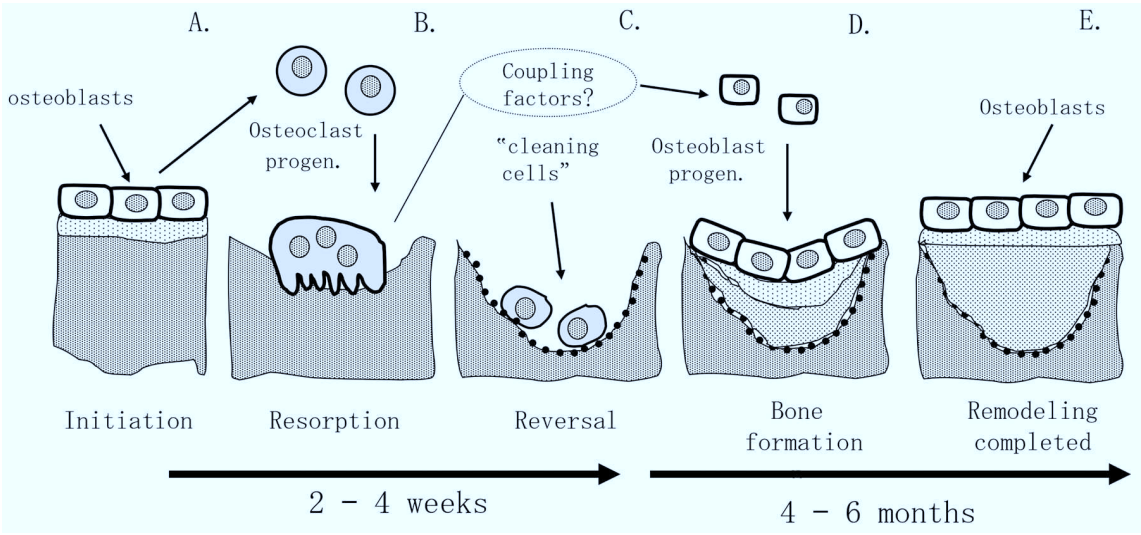

Figure 1.2.: Remodelling of bone in a bone multicellular unit starts with osteoblastic activation of osteoclast differentiation, fusion and activation (A, B). When resorption lacunae are formed, the osteoclast leaves the area, and mononucleated cells of uncertain origin appear and "clean up" the organic matrix remnants left by the osteoclast, also possibly forming the cementum line (dotted line) at the bottom of the lacunae (C). During the resorption process, coupling factors are released from the bone extracellular matrix and these growth factors contribute to the recruitment and activation of osteoblasts to the resorption lacunae (D). The osteoblasts will then fill the lacunae with new bone and when the same amount of bone is formed as that being resorbed, the remodelling process is finished and the mineralized extracellular matrix will be covered by osteoid and a one-cell layer of osteoblasts (E). From [4].

1.2.1 Cells involved in bone remodelling

As mention above, the cells responsible for bone remodelling are osteoblasts, which secrete new bone and osteoclasts, which break bone down (Figure 1.3). The structure of bones requires close cooperation between these two cell types.

Osteoblasts are mononuclear cells of mesenchymal origin involved in the bone formation. They produce and secrete the major part of the organic bone matrix in a well-regulated process to form mineralized bone [45]. Osteoblasts are postproliferative and cuboidal cells lining the bone matrix at sites of active bone production. Osteoblasts can also be recognized by their ability to synthesize a number of phenotype-specific bone matrix macromolecules such as osteocalcin, bone sialoprotein, osteopontin, alkaline phosphatase, proteoglycans, certain hormone receptors, cytokines and growth factors [46].

Osteoclasts, first named by Kölliker [47], are multinucleated cells from hematopoietic origin; more specifically from cells in the monocytes-macrophage linage [48,49]. Osteoclasts are the only cells known to be responsible for bone resorption. They are localized on bone surfaces and have a phagocytic-like mechanism similar to circulating macrophages [50].

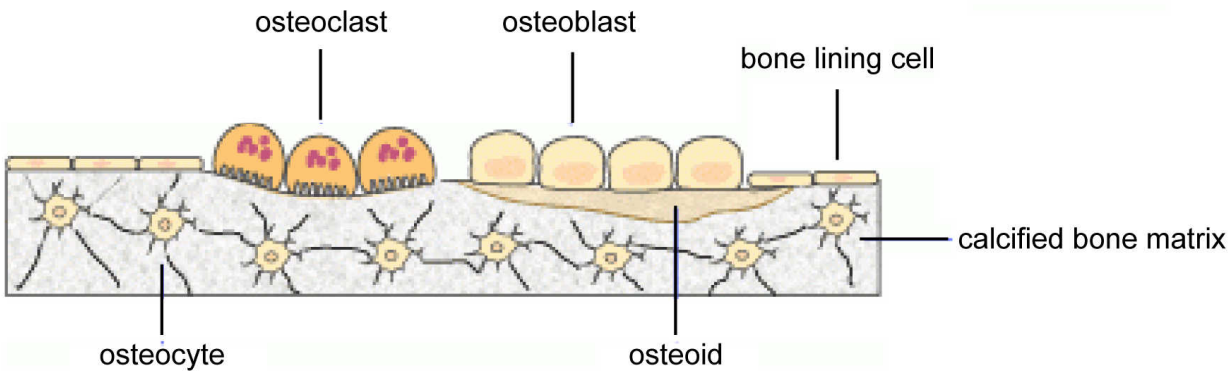

Figure 1.3.: Bone remodelling cells (osteoblasts and osteoclast), support cells (osteocytes and bone lining cells), nonmineral matrix of collagen, noncollagenous proteins (osteoid) and inorganic mineral salts deposited within the matrix. From www.iofbonehealth.org.

1.2.2 Cellular mechanism of bone resorption

Morphology of the osteoclast

An osteoclast is a large cell that is 40 mm in diameter. They contain 15-20 oval closely packed nuclei and are found in pits on the bone surface, which are called Howship´s lacunae. Osteoclasts are characterized by a cytoplasm with a homogeneous foamy appearance, which is due to a high concentration of vesicles and vacuoles (Figure 1.4). These vacuoles are lysosomes filled with acid phosphatase [51]. At a site of active bone resorption, the osteoclast forms a specialized cell membrane, the "ruffled border (RB)," that touches the surface of the bone tissue [52].

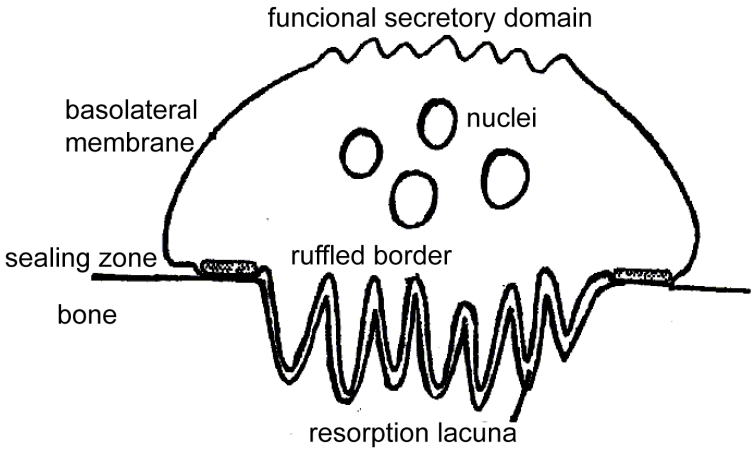

Figure 1.4.: Schematic illustration of a bone resorbing osteoclast. Modified from [52].

Osteoclast formation

Osteoclast differentiation has various characteristic features such as multinucleation, induced by the cell fusion of mononuclear osteoclasts; synthesis of the vacuolar proton pump and acids to dissolve the bone mineral; formation of ruffled borders, to secret protons and acids; formation of a sealing zone, to prevent proton and acid leakage [53].

The differentiation of osteoclast precursors into mature multinucleated osteoclasts requires different factors including hormonal and local stimuli [54]. As first noted in 1990, osteoblastic or bone marrow stromal cells are also required for osteoclast differentiation [55]. These accessory cells express the two molecules that are essential and sufficient to promote osteoclastogenesis: the macrophage colony-stimulating factor (M-CSF) [55-57] and the receptor for activation of nuclear factor kappa B ligand (RANKL) (also known as OPGL, ODF and TRANCE) [48,55,58-63].

By a process that requires cell-to-cell contact, RANKL will activate its cognate receptor RANK, on osteoclast progenitor cells. Together with the activation of the receptor c-Fms by M-CSF, which is imperative for macrophage maturation, this will lead to an expansion of the osteoclast progenitor pool, an increased survival of these cells and the initiation of a differentiation program which terminates in fusion of the mononucleated progenitor cells and the development of the osteoclasts. Finally, these osteoclasts become activated to bone-resorbing osteoclasts. Osteoblasts also secrete a protein that strongly inhibits osteoclast formation called osteoprotegerin (OPG). It acts as a decoy receptor for the RANKL, thus inhibiting the true connection between osteoclasts and osteoblasts via RANK and RANKL (Figure 1.5). It is the balance between the expression of RANKL and OPG which dictates the quantity of bone resorbtion [64]. Moreover, complete osteoclastogenesis can now be achieved *in vitro* with pure populations of macrophages, which are exposed only to M-CSF and RANKL [58].

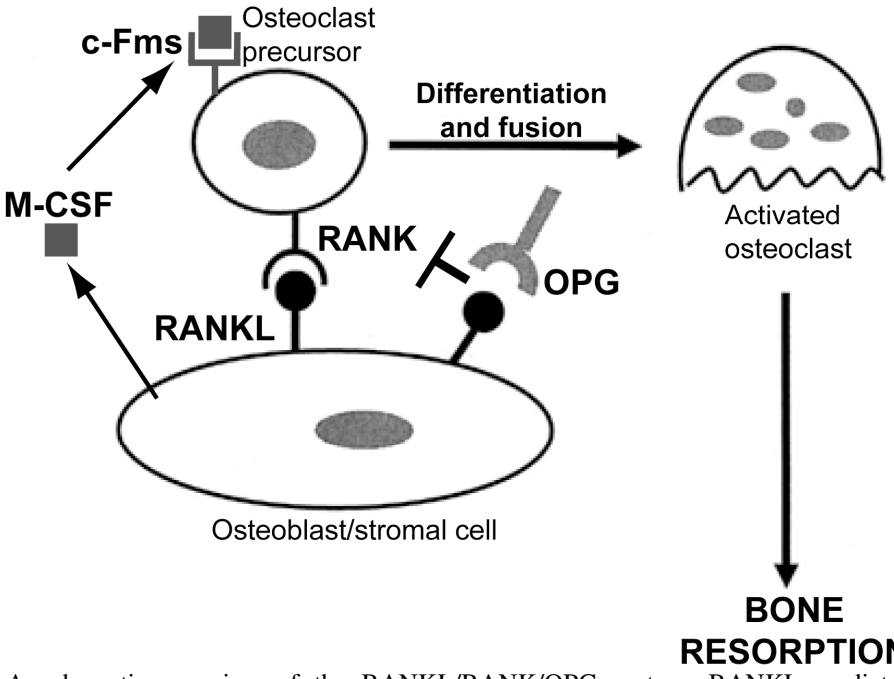

Figure 1.5.: A schematic overview of the RANKL/RANK/OPG system. RANKL mediates a signal for osteoclast formation through RANK expressed on osteoclast progenitors. OPG counteracts this effect by competing for and neutralizing RANKL. Modified from [62].

Osteoclastic resorption

Bone resorption by osteoclasts is a multistep process, which is initiated by the proliferation of immature osteoclast precursors and finished by the degradation of the organic and inorganic phases of bone by the mature resorptive cells.

The initial event in bone degradation is the attachment of osteoclasts to the target matrix [65]. A number of cell surface glycoproteins have been identified as intercellular adhesion molecules. These have been classified into three major molecular families; the immunoglobulin (Ig) superfamily, the integrin superfamily and the cadherin family [66-70].

Once attached to the bone, the cell generates an isolated extracellular microenvironment between itself and the bone surface [71] in the sealing zone. This microenvironment, rich in filamentous actin (F-actin) and largely devoid of organelles, is organized as a ring surrounding the ruffled membrane [52]. The F-actin in the sealing zone localizes itself within the plasma membrane protrusions, known as podosomes [72].

Both the mineral and organic components of the bone matrix are degraded by osteoclasts within the resorption lacunae underneath ruffled border membranes [66]. The inorganic bone matrix is dissolved by the acidic environment, revealing the organic collagen network. Carbonic anhydrase II (CA-II) is a cytoplasmic enzyme hydrolyzing carbon dioxide into bicarbonate and protons [73-75]. CA-II is suggested to be the main source of protons for the acidification of the resorption lacuna [76-78]. A vacuolar-type proton pump, V-ATPase, is present in high amounts within the membranes of a population of intracellular vesicles. V-ATPase transports the protons generated by CA-II into these vesicles, which are then transported and fused to the ruffled border membrane [76-80] releasing their proton content to the lacuna. Acidification of the extracellular resorption lacuna is completed by passive, potential-driven chloride transport [81]. The result of these ion transporting events is a secretion of HCl into the resorptive microenvironment, prompting a pH of 4.5 [71]. This acidic milieu first mobilizes bone minerals; subsequently, the demineralized organic component of the bone is degraded by a lysosomal protease, Cathepsin K [82]. When the osteoclast stops resorption and moves away from the resorption lacuna, phagocytes clean up the remains and make room for osteoblasts to begin bone formation in the newly formed resorption cavity [83].

Only recently it has been shown that osteoclasts remove the degradation products of the bone matrix from the resorption lacuna by transcytosis [84,85]. The degradation products, both organic and inorganic, are endocytosed to the resorbing osteoclast, transported through the cell in large vesicles and finally released into the extracellular space. The bone-specific

enzyme TRACP is located in cytoplasmic vesicles, which fuse to the transcytotic vesicles and participate in destroying the endocytosed material in the transcytotic route [86].

Basic characteristic of RANKL, RANK and OPG and their roles in bone metabolism

The identification of the factors involved in osteoclast development began with OPG [59,87]. OPG (Figure 1.6C) is a member of the tumor necrosis factor receptor (TNFR) superfamily and is highly expressed in adult lung, heart, kidney, liver, spleen, thymus, prostate, ovary, small intestine, thyroid, lymph node, trachea, adrenal gland, testis and bone marrow [88]. Molecular binding experiments showed that OPG associates with RANKL and functions as a decoy receptor [62]. The expression of OPG is directly or indirectly modulated by a large number of factors; including TNF-α, interleukins, sex steroids, growth hormone, IGF-1, the macrophage colony stimulating factor, thyroid, parathyroid hormones, vitamin D and lipoproteins [89-92].

The receptor for RANKL is RANK (Figure 1.6A), a type I transmembrane protein originally cloned from dendritic cells [93]. Although OPG functions as a dimer, RANK, like other members of the tumor necrosis factor receptor (TNFR) superfamily, assembles into functional trimers [88]. RANK is ubiquitously expressed in skeletal muscle, thymus, liver, colon, small intestine, adrenal gland, osteoclast, mammary gland epithelial cells, prostate and pancreas [93-96].

Four groups independently cloned RANKL (Figure 1.6B) as an apoptosis regulatory gene, a ligand for RANK and a binding partner of OPG [61,62,93,95]. The RANKL gene gives rise to splice variants that encode two forms of type II transmembrane proteins and one form of a secreted protein [97]. Although a high RANKL expression can be found in lymph nodes, thymus and lung: only low levels of RANKL can be detected in spleen, bone marrow, peripheral blood, leukocytes, heart, placenta, skeletal muscle, stomach and the thyroid [61,62,93-95]. In addition, RANKL expression is induced in mammary gland epithelial cells in pregnancy [96], activated T-cells [93,98,99] and malignant tumor cells [100]. 1a,25$(OH)_2D_3$, parathyroid hormone, TNF-α, IL-1 and IL-11 promote RANKL synthesis [101]. Increased RANKL expression has been defined in a wide variety of conditions characterized by osteolysis: osteoporosis, malignant bone disease (especially myeloma), Paget's disease and rheumatoid arthritis [102].

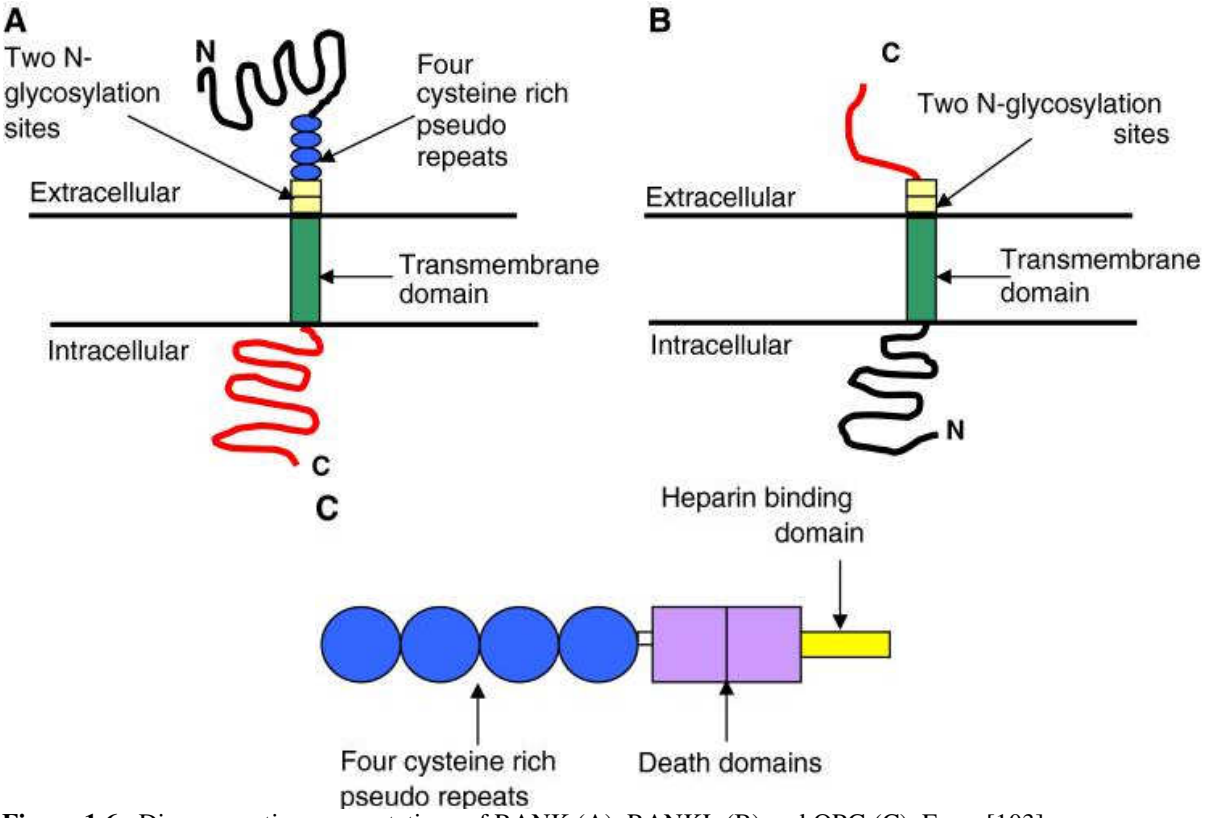

Figure 1.6.: Diagrammatic representations of RANK (A), RANKL (B) and OPG (C). From [103].

The biological roles of these factors in bone metabolism have been studied by the analysis of gene knock out and transgenic mice. OPG-deficient mice show severe osteoporosis accompanied by increased osteoclast differentiation and activation [104,105]. In contrast, transgenic mice over-expressing rat OPG show osteopetrosis associated with a decrease of osteoclasts [87]. RANKL- and RANK-deficient mice show the typical osteopetrosis, defective tooth eruption and lacking osteoclasts, but they retain the normal osteoclast progenitors [87,98,106,107]. Over-expression of RANKL in transgenic mice induces severe osteoporosis [104]. In line with mouse models, mutations in RANK and OPG have been identified in patients with bone disorders [108].

1.3 GENE TRANSFER TECHNOLOGIES

Gene transfer is the process of introducing foreign DNA into host cells aiming to affect the expression of the protein of interest. The gene transfer can occur *in vitro* in the cell culture or *in vivo* in living organisms.

An ideal gene delivery method needs to meet 3 major criteria: it should protect the transgene against degradation by nucleases in intercellular matrices, it should bring the transgene across the plasma membrane and into the nucleus of target cells and it should have no detrimental effects on the transgene expression [109].

Many different methods of gene delivery have been developed for various types of cells and tissues, from bacterial to mammalian. Generally, the methods can be divided into two categories, viral and non-viral [110]. Virus mediated gene delivery utilizes the ability of a virus to inject its DNA or RNA inside a host cell. A gene that is intended for delivery is packaged into a viral particle [111]. Non-viral methods include physical and chemical methods. All of these methods have advantages and disadvantages depending on the purpose of the study. Selection of an appropriate gene transfer vector requires careful consideration of a number of factors, including: duration of protein expression required for efficacy (transient versus long term), target cells (ease of transduction, receptor expression, dividing versus non-dividing), route of gene delivery (*ex vivo* versus *in vivo*), maximum threshold of vector-induced immune response acceptable for the host and desired temporal regulation of transgene expression (inducible versus constitutive) [112]. A wide variety of viral and non-viral systems have been investigated for their utility as gene delivery vehicles. It is unlikely that one vector technology will be ubiquitously appropriate for all scenarios, so vehicles should be optimized for the specific indications [113].

1.3.1. Viral methods of gene transfer

Viruses are parasitic particles, which are capable of efficiently infecting host cells with genetic sequences. This property has been exploited to develop recombinant viral vectors as delivery vehicles, capable of prolonged transgene expression and markedly higher transduction efficiency. In general, the viral genome is based on DNA or RNA, encased in a protein capsid and in some cases surrounded by a phospholipid bilayer [112]. Viruses are typically rendered replication-incompetent by deletion of a significant portion of the genetic sequence essential for viral propagation and replacement of this sequence with a transgene expression cassette [112]. All viruses bind to their hosts and introduce their genetic material into the host cell as part of their replication cycle. Viral vectors are able to mediate gene transfer with high efficiency and the possibility of long-term gene expression. The acute immune response, immunogenicity and insertion mutagenesis have raised serious safety concerns about some commonly used viral vectors [114]. The limitation in the size of the transgene that recombinant viruses can carry and issues related to the production of viral vectors present additional practical challenges [114].

Numerous viral vectors with divergent properties have been studied for gene transfer, the most common include; retrovirus, adenovirus, adeno-associated virus and lentivirus [113].

Lentiviral vectors

Lentiviruses, such as human immunodeficiency virus type 1 (HIV-1), are a specialized class of retrovirus, which are capable of infecting non-dividing cells. The ability of these vectors to transduce macrophages, hematopoietic stem cells, neurons and hepatocytes with high efficiency has prompted an increased interest in the use of this technology for gene transfer [115-117]. Although the integration sites of lentiviral vector are significantly more limited than other retroviral family classes [118], this feature markedly decreases the risk of oncogene activation. Additionally, these vectors carry safety risks associated with their pathogenic HIV origin and the possibility that recombination events could lead to replication-competent viruses [119].

1.3.2. Nonviral methods of gene transfer

Nonviral vectors typically consist of plasmid DNA alone or in combination with a carrier (e.g. liposomes, cationic polymers and porous scaffolds) that is non-specifically taken up by cells through endogenous endocytic mechanisms [120].

Methods of nonviral gene delivery have also been explored using physical (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery). Physical approaches, including needle injection [121], electroporation [122], gene gun [123,124], ultrasound [125] and hydrodynamic delivery [126,127], employ a physical force that permeates the cell membrane and facilitates intracellular gene transfer. The chemical approaches [128,129] use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells.

Non-viral methods present certain advantages over viral methods, with simple large-scale production and low host immunogenicity. Conventional systems are limited by low transfection efficiency, transient expression and non-selective cell targeting. Despite these limitations, nonviral vectors are easy to manufacture, able to incorporate large sequences of DNA and are cost-effective [130].

Although significant progress has been made in the basic science and applications of various nonviral gene delivery systems, the majority of nonviral approaches are still much less efficient than viral vectors, especially for *in vivo* gene delivery.

1.4 TRANSGENESIS OF LARGE ANIMALS

Transgenesis is the process of introducing an exogenous gene (transgene) into a living organism. Finally, that organism will exhibit a new property and transmit that property to its offspring. Transgenesis can be facilitated by gene transfer methods mentioned above.

The production of transgenic animals is a powerful tool used successfully in many animal systems to study early embryogenesis, organogenesis and adult tissues [131]. It is a particularly important technique for studying gene function and disease models like osteoporosis.

1.4.1 Nuclear transfer

Nuclear transfer (NT) is a delicate process which represents a major hurdle in the development of cloning technology [132].

In NT the nucleus, which contains the organism's DNA of a somatic cell is removed and the rest of the cell discarded. At the same time, the nucleus of an egg cell is removed. The nucleus of the somatic cell is then inserted into the enucleated egg cell. After being inserted into the egg, the somatic cell nucleus is reprogrammed by the host cell. The egg, now containing the nucleus of a somatic cell, is stimulated with a shock and will begin to divide (Figure 1.7) [133].

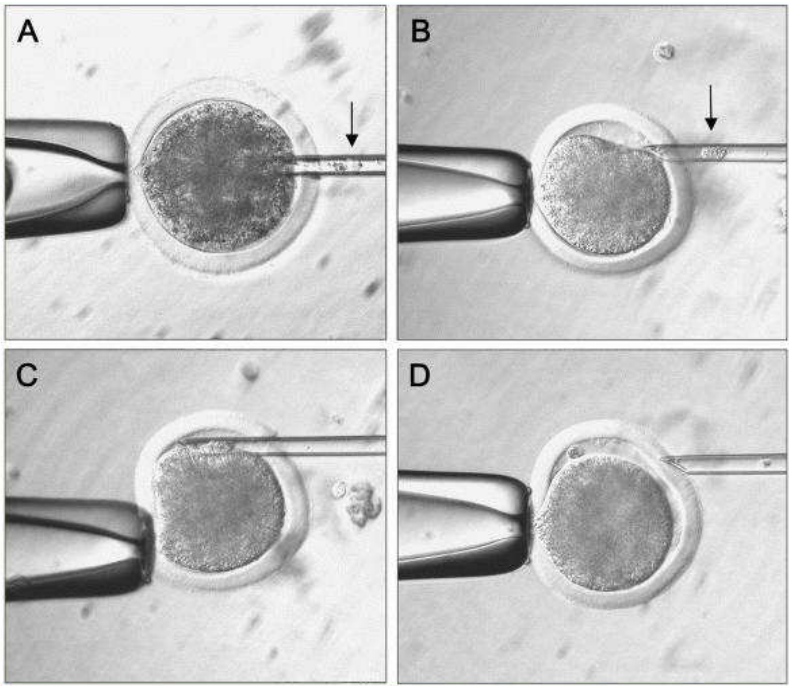

Figure 1.7.: (A) Enucleation of the oocyte at metaphase II; (B) Selection of donor cell; (C) Injection of donor cell into the perivitelline space. (D) Donor cell attached to the enucleated oocyte. From [133].

In order to begin the development process, the membranes separating the ooplast and the donor nucleus must be fused. This can be accomplished in two ways: (1) by the

administration of a brief electrical pulse, or (2) by chemical fusion. The fusion process also stimulates the embryonic development, which, if successful, results in the development of blastocysts that are transplanted into surrogate mothers [134]. After NT of a fully differentiated donor cell into a cytoplast, the resulting reconstruct can develop into an embryo and even a viable animal.

Offsprings have been obtained from an ever-growing list of species [135]. Nuclear transfer in mammals was not achieved until over three decades after the initial reports of Briggs and King on the production of adult frog clones using embryonic nuclei [132]. During the late 1980s and early 1990s, success in mammalian NT was limited mainly to domestic livestock (sheep, cattle). During this time period embryonic blastomeres, primarily from morula-staged embryos, were used as nuclear donors. At that time, embryonic blastomere NT did not work with mice [135].

1.4.2. Embryo transfer

Embryo transfer (ET) refers to a step in the process of *in vitro* fertilization whereby one or several embryos are placed into the uterus of the surrogate mother with the intent to establish a pregnancy.

After a NT, the developing clone is transferred at the blastocyst stage into a surrogate mother in whom the estrous cycle has been synchronized. After transfer of the embryo clone(s), the pregnancy is allowed to proceed normally; no additional hormones or special treatments are required to establish or maintain pregnancy. The surrogate mother is often chosen to be distinctively different from the donor animal with respect to some clearly visible trait [136].

1.4.3 Transgenic pigs

Since the birth of the first cloned sheep, more than 10 animal species have been cloned by somatic cell nuclear transfer [137]. The need for application of new embryo technologies in pig breeding is moderate when compared with other domestic species. Pigs have short reproductive cycles and large litters, so traditional breeding combined with artificial insemination offers the possibility for rapid genetic advancement [138]. Additionally, in contrast with traditional laboratory animals, pigs have a relatively long lifespan and their organ size, anatomy and physiology are similar to humans. Therefore, pigs can be studied and treated with methods and instruments that are used in human medicine. Pig organs may also be considered for xenotransplantation to replace diseased human organs temporarily or even permanently [139,140].

To produce transgenic pigs, the most promising past and present approaches, i.e. intracytoplasmic sperm injection (ICSI), pronuclear microinjection, somatic cell nuclear transfer (SCNT) and *in vitro* maturated oocytes as recipients are based on a reliable and efficient embryology background (Figure 1.8) [141].

The first piglet born after nuclear transfer was created using blastomeres of 4-cell stage embryos as donors [142].

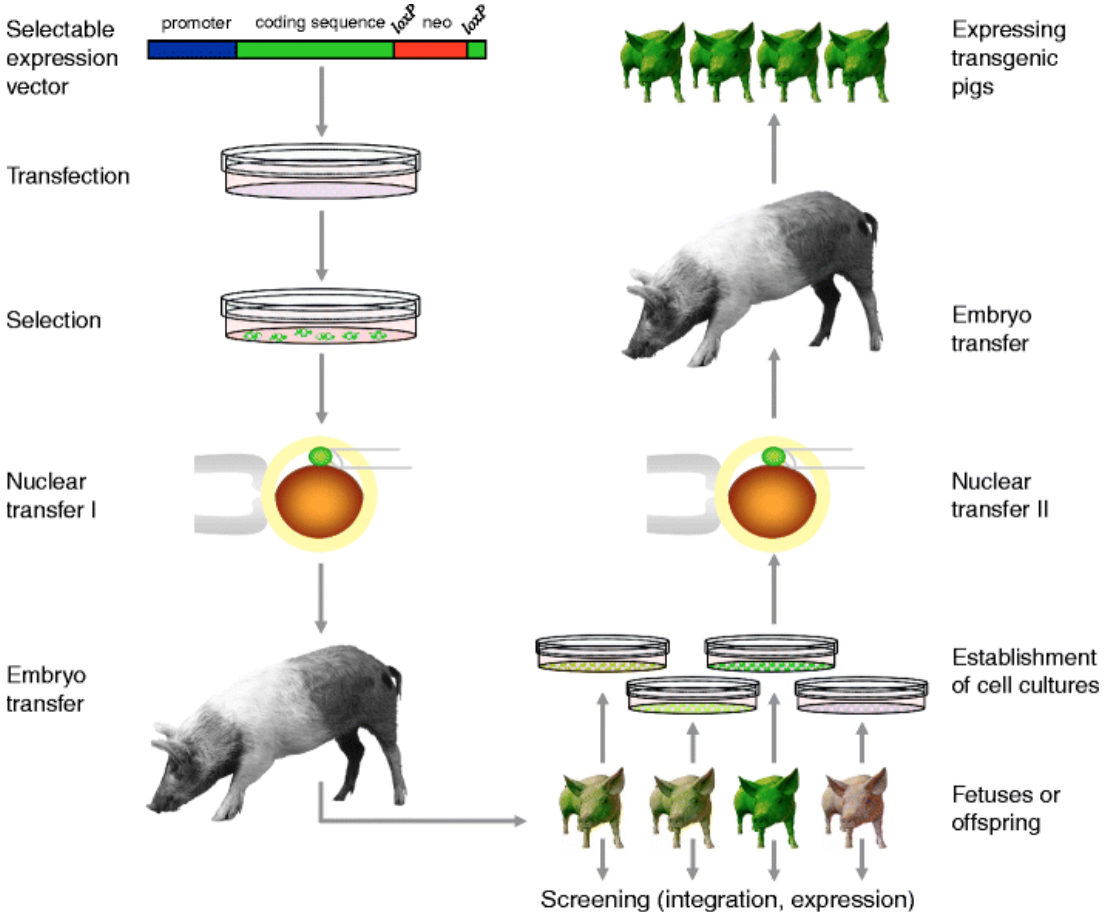

Figure 1.8.: Efficient production of transgenic pigs by using somatic cell nuclear transfer. An expression vector carrying a removable selection cassette is transfected into nuclear donor cells. After selection, the resulting transgenic cells are pooled and used for nuclear transfer. Pooling of cell colonies reduces the time in culture and allows the generation of independent founder fetuses/offspring in one litter. Cloned embryos are transferred to synchronized recipients. Depending on the expected onset and tissue specificity of transgene expression, pregnancies may be terminated to recover fetuses, or birth and early development of offspring is awaited. Fetuses or tissues from born offspring are processed for transgene integration and expression studies, while individual cell cultures are established for re-cloning of the fetuses/offspring with the most suitable integration/expression pattern. From [143].

1.5 INDUCIBLE TRANSGENE Tet SYSTEM

The unregulated over-expression of some proteins risks aberrant effects of uncontrolled cell signalling, including tumorigenesis [144,145] and protein over-production [146,147]. Inducible expression systems have been developed to address these concerns and to control the activity of genetically engineered cells [148]. These systems typically drive the transgene expression from inducible promoters, which are regulated by engineered and/or non-

mammalian transcription factors. The activity of these transcription factors is controlled by exogenous chemical agents, which permit ("ON" state) or repress ("OFF" state) the transgene expression.

The most widely characterized of these systems is the tetracycline inducible expression system, first described by Gossen and Bujard [149]. The original system utilizes the tetracycline-controlled transactivator (tTA), which is a fusion of the Tet repressor (TetR, found in *Escherichia coli*) and a VP16 activation domain (produced by the Herpes Simplex Virus). Gene expression is activated as a result of the binding of tTA to tetracycline response elements (TREs) located within an inducible promoter. In the absence of tetracycline (Tc), tTA binds the tet operon and activates the transcription of the downstream gene of interest. However, in the presence of tetracycline, the antibiotic binds to tTA, blocks the binding to the tet operon and subsequently transactivates it in a dose-dependent manner (Figure 1.9). The

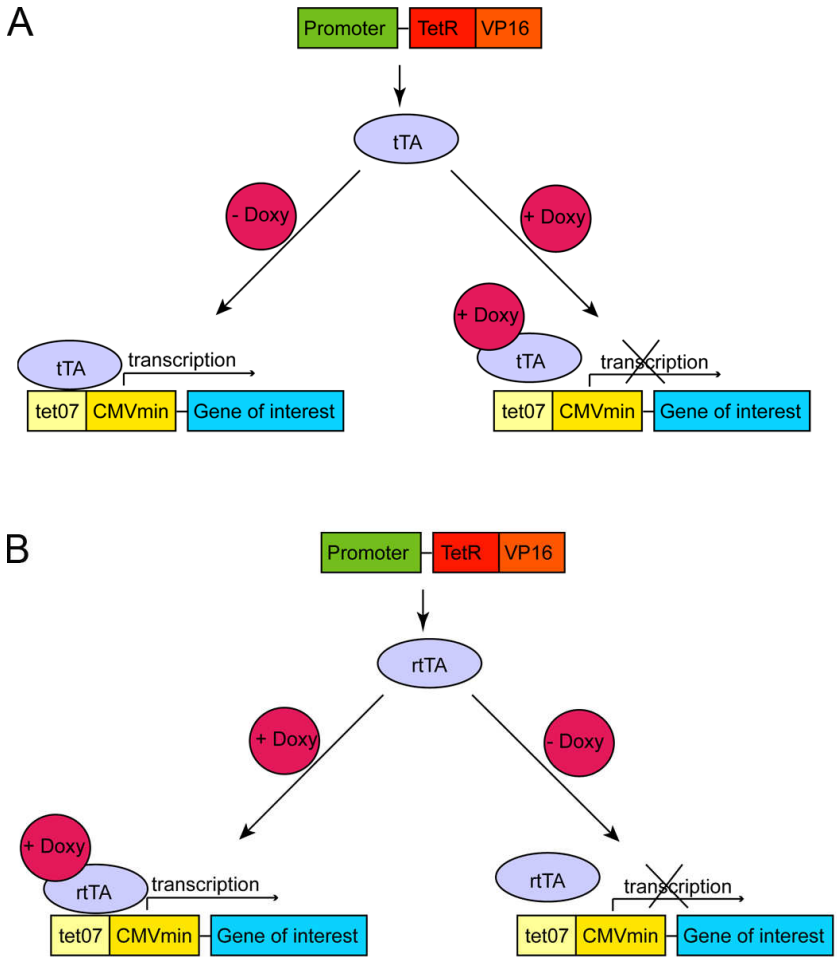

Figure 1.9.: The tetracycline system for mammalian transgenesis. Two expression cassettes need to be delivered to the target cell. One expresses the tTA/rtTA protein, which either binds to Tc analogue Doxycycline (Doxy) or the *tet* operator (*tetO7*) and the other expresses the therapeutic gene under the control of *tetO7*. Gene expression is conditional on the binding of tTA/rtTA to *tetO7* and hence on the absence of tetracycline. (A) Fusion of TetR to Herpes simplex VP16 yields a strong transcriptional activator tTA, whose activity can be switched off in the presence of the Doxycycline; (B) A mutant form of tTA, rtTA, however, which fails to act as a transactivator in the absence of Doxy, becomes active in the presence of Doxy and leads to transgene expression as a result of administration of Doxy.

tetracycline response element consists of 7 repeats of the 19 bp bacterial tet sequence separated by the spacer sequences. The TRE is usually placed upstream of a minimal promoter that has very low basal expression in the absence of bound Tet-Off (or Tet-On). This system is compatible *in vitro* by adding tetracycline to the cell culture media and *in vivo* by delivering a Tc via the drinking water. This Tet-Off version was later modified to a Tet-On system, in which transactivation increases with tetracycline concentration [150]. Despite the greater clinical relevance of the Tet-On derivative, the Tet-Off system is widely reported as having a greater degree of inducibility, including lower expression levels in the "OFF" state and higher expression levels in the "ON" state [151]. However, recent modifications of the Tet-On system have shown improved induction levels and lower basal activity in the "OFF" state [152,153].

The Tet system has advantages over other (like Cre, FRT and estrogen receptor) conditional gene expression systems. In the Tet system, the activation of the knock out of the gene is reversible once recombination is accomplished. The Tet system has very tight control on the expression. However, the Tet system, which depends on transcription and subsequent translation of a target gene, is not as fast-acting, which stabilizes the already-expressed target protein upon hormone administration [153].

1.6 PIGS AS MODEL ORGANISMS

It has been widely believed that pigs were first domesticated from the wild boars (*Sus scrofa*) approximately 9 000 years ago. Domestication, selective breeding and adaptation to diverse environments have led to the development of more than 250 breeds worldwide.

The pig is evolutionarily distinct from the primates and rodents [154]. An initial evolutionary analysis and the available human and mouse genome data revealed that for each of the types of orthologous sequences investigated, the pig is closer to humans than mice [140]. The genome of the pig comprises 18 autosomes, with X and Y sex chromosomes. The pig genome is of similar complexity, genetic information and size as the human genome and is estimated at 2.7 Gbp. In 1993, the public databases had only gathered approximately 600 pig sequences. In 2009 there were 1,850 working draft sequences from the pig genome that have been released to the public domain. In April 2011, the Swine Genome Sequencing Consortium released a mixed assembly of the whole porcine genome [155].

1.6.1 Pigs in human medical research

Livestock pig breeds and miniature pigs are relevant models in many fields of medical research [156]. The omnivores, human and pig, have a large number of similarities in anatomy, physiology, metabolism and pathology, e.g., they have a very similar gastrointestinal anatomy and function, the size of organs, pancreas morphology and metabolic regulation [143]. Pigs and other farm animals have been proposed as models to study human diseases, including non-insulin dependent diabetes, cancer and neurodegenerative disorders, because these require longer observation periods than those possible in mice [157]. The pig has also evolved as the major target species for producing xenografts to overcome the growing gap between demand and availability of appropriate human organs [157].

With regard to bone anatomy, morphology, healing and remodelling, the pig bone is considered to be closely representative of human one. While having a denser trabecular network, the pig has a human like lamellar bone structure. Furthermore, pigs have a similar rate of bone regeneration, cortical bone mineralization [158] and a close bone remodelling rates to humans [159]. Nevertheless, up to now no truly satisfying large animal model for osteoporosis exists [159].

2. MATERIALS AND METHODS

2.1 CELL BIOLOGY

2.1.1 Eukaryotic cells

The 293FT (HEK293FT) (Supplementary figure 1A) cell line was purchased from Invitrogen (Karlsruhe, Germany) and is a derive from primary embryonic human kidney 293F cell line transformed with human adenovirus type 5 DNA [160] which stably expresses the SV40 large T antigen. They have epithelial-like cell morphology and are growing in a monolayer. The 293FT cells are suitable host for generating lentiviral constructs [117] and are generally amenable to transfection using standard method. In addition, these cells have a Neomycin resistance and, therefore, should be maintained in medium containing Geneticin (Invitrogen).

Primary porcine fetal fibroblast (pFF) (Supplementary figure 1B), porcine ear fibroblast (pEF) (Supplementary figure 1C) and porcine kidney cell (pKC) (Supplementary figure 1D) were isolated using collagenase treatment by Annegret Wünsch at the Institute of Molecular Animal Breeding and Biotechnology (LMU, Prof. Eckhard Wolf). The pFFs and pEFs exhibit typical fibroblast-like morphology, i.e. spindle shaped (bipolar) or stellate (multipolar), while the pKCs contain a mixed population of cells with fibroblast-like and epithelial-like morphology. All cell types grow as adherent monolayer and were isolated from Swabian-Hall breed swine. Precisely, pFF-psRANKL and pFF-rtTA-psRANKL were isolated from a 27 and 40 days old fetus, respectively, while pEF-TRE-psRANKL and pKC-rtTA were isolated from a 1-28 days old newborn piglet form ear or kidney, respectively.

Porcine osteoclast precursor cells (Supplementary figure 1E) were isolated from bone marrow of German Landrace swine, which was withdrawn from the posterior iliac crest of the pelvic bone and centrifuged by density gradient method (see 2.8.1).

All cells are assayed and tested negative for HIV-1, hepatitis-B & C (human cells), mycoplasma, bacteria, yeast and fungi (human and porcine cells).

2.1.2 Media

Media for 293FT cells

293FT cells were maintained in complete growth media composed of Dulbecco's Modified Eagle Medium (D-MEM (high glucose, +Glutamine, +Pyruvate), PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Munich, Germany), 0.1 mM MEM Non-Essential Amino Acids (NEAA, Invitrogen), 1% Penicillin-Streptomycin solution (Pen-Strep, 100×, PAA), 2 mM L-glutamine (PAA) and 500 µg/ml Geneticin

(Invitrogen). Cells were preserved in freezing media composed of 90% complete growth media + 10% Dimethylsulfoxid (DMSO, Merck, Darmstadt, Germany).

Media for primary porcine cells

Complete growth media for porcine cells was composed of D-MEM (high glucose, +Glutamine, +Pyruvate, PAA) supplemented with 10% FBS (Sigma-Aldrich), 0.1 mM NEAA (Invitrogen), 1% Pen-Strep (PAA), 1mM Sodium pyruvate (Sigma-Aldrich), 1 mM L-Glutamine (PAA) and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich). For the rtTA or/and TRE-psRANKL-expressing cells, a 2% Tet System Approved Fetal Bovine Serum (Clontech, Saint-Germain-en-Laye, France) was used as supplement in culture media. For genetically modified cells during selection process, media used was composed of complete growth media supplemented with 10 µg/ml Blasticidin (PAA) for psRANKL-transgenic or 400 µg/ml Geneticin (Invitrogen) for rtTA-transgenic cells, respectively. Cells were preserved in freezing media composed of 90% FBS + 10% DMSO.

Media for porcine osteoclast precursor cells

Complete growth media for porcine osteoclast was composed of α-MEM-Glutamax (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich), 1% Pen-Strep (PAA), 20 ng/ml human soluble RANK-ligand (hsRANKL, PeproTech, Hamburg, Germany) and 5 ng/ml human macrophage-colony stimulating factor (M-CSF, Sigma-Aldrich).

2.1.3 Eukaryotic cell culture

Standard sterile cell culture plastic ware was purchased from Sarstedt (Nümbrecht, Germany). Cell culture vessels (Nunc, Langenselbold, Germany) used in this study were T-25, T-75 and T-225 cell culture flasks and 6-well culture dish. Sterile condition was maintained all along and cells were incubated at 37°C in a humidified 5% CO_2 incubator (Jouan, Thermo Scientific, Bremen, Germany).

Passaging

Upon reaching confluency (see *Cell culture conditions*) all medium was removed from the flask and the cells were washed once with phosphate buffered saline (PBS, PAA) to remove excess medium and serum. 1× Trypsin/EDTA solution (PAA) was added for maximum 5 minutes or until all cells are detached. The detached cells were washed off and resuspend in media.

A small portion of the cell suspension was taken to calculate cell density. In an eppendorf tube, cell suspension and trypan blue stain solution (Invitrogen) were mixed in 1:2 ratio. The trypan blue staining was used to determinate cell viability. 10 µl of this suspension was pipetted to the Neubauer cell counting chamber (Brand, Germany), the chamber was placed under the phase contrast microscope and the counting was performed. Cells viability should be at least 95% for healthy log-phase cultures. After the counting, a defined number of cells were seeded (for T-25 flask 125 000, for T-75 flask 375 000, for T-225 flask 1 125 000 and for 6-well dish 48 000 cells) or cryopreserved.

Freezing

For cryopreservation, a specific freezing media was prepared for each cell type (see 2.1.2). After cell trypsinization and counting (see *Passaging*), the cells were pelleted by centrifugation for 5 min at 500 g at room temperature. The supernatant was completely aspirated and the cell pellet was resuspend in pre-cooled to 4°C freezing media. Next, the cell suspensions were aliquoted in pre-labelled cryovials, which were then placed on dry ice and finally stored in liquid nitrogen tank.

Thawing

Cells were stored in liquid nitrogen and were thawed quickly in a 37°C water bath. Subsequently, the cells were transferred to a flask containing prewarmed, complete growth medium. The cells were incubated overnight at 37°C for allowing the cells to attach to the bottom of the flask. The next day, the medium was aspirated and replaced with fresh, complete medium.

Cell culture conditions

The human cell culture was performed according to the manufacturer's recommendations. 293FT cells were used for virus production and transfection and were cultured to maximum 20^{th} passage. The confluence of the monolayer before passaging was approx 90-95%. The media was exchange every third day and the cells were splitted in 1:8 ratio.

The primary porcine cells were used for over-expression experiments and generation of transgenic piglets and were cultured to maximum 10^{th} passage in a 0,02% collagen (SERVA Electrophoresis GmbH, Heidelberg, Germany) coated vessels. The confluence of the monolayer was 95-100% and media was change every second day. The splitting ratio was 1:3.

The osteoclast precursor cells were used for generation of the osteoclasts. The cells were not passaged and media was change every 4 days.
Wild type and genetically modified porcine cells were continuously passaged, observed and photographed under phase contrast microscope (Axiovert S100, Carl Zeiss, Jena, Germany) to check and document the morphology of cells.

Stimulation with Doxycycline
To induce psRANKL under the control of TRE promoter, a 1 µg/ml Doxycycline (Clontech) was added into cell culture. Cells were harvested after 3 days of Doxycycline stimulation.

2.1.4 Bacterial strains

For plasmid cloning and propagation, 2 type of *E. coli* competent cell were used:

- One Shot TOP10 Competent Cells (Invitrogen), which are derivates from DH10B *E. coli* strain. They provide high cloning efficiency and plasmid propagation and are ideal for high-copy number plasmids amplification.
- One Shot Stbl3 Chemically Competent Cells (Invitrogen) which are HB101 *E. coli* strain derivate, recommended for use with unstable inserts such as lentiviral DNA.

2.1.5 Bacterial culture media

The LB (Luria-Bertani) media (10% Bacto Tryptone, 10% NaCl and 5% Bacto Yeast extract) was adapted from [161]. All components of the media were mixed, autoclaved and immediately used or preserved at 4°C.
S.O.C. (Super Optimal broth with Catabolite repression) media (2% Bacto Tryptone, 0,5% Bacto Yeast extract, 0,2% 5M NaCl, 0,25% 1M KCl, 1% 1M $MgCl_2$, 1% 1M $MgSO_4$ and 2% 1M glucose) was purchased from Invitrogen and stored at 4°C.
For preparation of hard selective media, 15% agar was added to the LB media. After sterilization, the media was cooled to RT and depending on the plasmid resistance antibiotics, 100 µg/ml of Ampicillin (ROTH, Karlsruhe, Germany) or 50 µg/ml of Kanamycin (ROTH), were added. Media was then mixed by gentle shaking and poured into 10 cm bacterial Petri dishes (Greiner BioOne, Frickenhausen, Germany). The obtained LB-agar plates were stored at 4°C.

2.2 CLONING OF EXTRACELLULAR DOMAIN OF PORCINE RANKL cDNA

To isolate the extracellular domain of porcine RANKL cDNA, the oligonucleotide primers were designed based on the published human cDNA sequence (Supplementary table 1). Total RNA was isolated from pig osteoblast as a source for RNA and the reverse-transcription polymerase chain reaction (RT-PCR) products at the predicted size of about 700 base pairs (bp) were gel-purified (see 2.3.3) and cloned into the pCR2.1-TOPO cloning vector (Invitrogen). Next, to obtain secreted form of the pRANKL, the psRANKL cDNA from pCR2.1-TOPO plasmid was subcloned into the pSecTag ABC plasmid (Invitrogen) by sticky end BamHI/NotI ligation and sequenced (Sequiserve, Vaterstetten, Germany).

2.3 CLONING OF PLASMIDS

2.3.1 Plasmids and their cloning

pCR2.1-TOPO vector (Invitrogen) (Supplementary figure 2A) is designed for the cloning of PCR products directly from a PCR reaction. The vector include 3´-T overhangs for direct ligation of non-proofreading Taq-amplified PCR products, T7 promoter for *in vitro* RNA transcription and sequencing, M13 forward and reverse primer sites for sequencing, EcoRI sites flanking the PCR product insertion site for easy excision of inserts, Kanamycin and Ampicilin resistance genes for selection in *E. coli*, easy blue/white colony screening for selection of recombinants.

pSecTag2ABC (Invitrogen) (Supplementary figure 2B) is a mammalian expression vector designed for the secretion, purification and detection of fusion proteins. The vector has a large multiple cloning sites in three reading frames to simplify cloning in frame with the N-terminal secretion signal. The vector offer the following features: secretion signal from the V-J2-C region of the mouse Ig kappa-chain for efficient secretion of recombinant proteins, cytomegalovirus (CMV) promoter for high-level constitutive expression, C-terminal polyhistidine (6xHis) tag for rapid purification with nickel-chelating resin and detection with an anti-His (C-term) antibody, C-terminal c-myc epitope for detection with an anti-myc antibody, bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability, SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigen, Zeocin resistance gene for cost-effective selection in mammalian cells.

pENTR11 (Invitrogen) (Supplementary figure 2C) is a pUC-based plasmid allowing cloning DNA sequences using restriction endonucleases and ligase to create a Gateway entry clone. The resulting entry clone is ready for recombination with a destination vector to create an expression clone. It contains attL1 and attL2 sites for site-specific recombination of the entry clone with a Gateway destination vector to ensure cloning of the gene of interest in the correct orientation for expression, Kozak consensus sequence for efficient translation initiation in eukaryotic systems, ribosome binding site for efficient translation initiation in prokaryotic systems, rrnB transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*, pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*, Kanamycin resistance gene for selection in *E. coli*, the ccdB/Chloramphenicol fusion gene located between the two attL sites for counterselection.

pLenti6/V5-DEST (Invitrogen) (Supplementary figure 2D) is a pUC-based plasmid used for production of pseudotype lentivirus for effective transduction of dividing and non-dividing mammalian cells. The vector provides a stable and long-term expression of DNA sequence into the host cells. It include a multiple biosafety features such as psi packaging signal for viral packaging [162], HIV Rev response element for Rev-dependent nuclear export of unspliced viral mRNA [163] and ΔU3 elements. The vector contains Rous Sarcoma Virus enhancer/promoter for Tat-independent production of viral mRNA in the producer cell line [163], modified HIV-1 5′ and 3′ Long Terminal Repeats for viral packaging and reverse transcription of the viral mRNA [164], CMV promoter for constitutive expression of the gene of interest from a viral promoter, two recombination sites, *att*R1 and *att*R2, Chloramphenicol resistance gene for counterscreening, the *ccd*B gene for negative selection, C-terminal V5 epitope for detection of the recombinant protein of interest [165], SV40 promoter driving expression of Blasticidin, Blasticidin [166,167] resistance gene for selection in *E. coli* and mammalian cells, Ampicillin resistance gene for selection in *E. coli* and pUC origin for high-copy replication of the plasmid in *E. coli*

pTet-On (Clontech) (Supplementary figure 2E) express the reverse tet-responsive transcriptional activator (rtTA) from the strong immediate early promoter of cytomegalovirus. rtTA is a fusion of amino acids 1-207 of the tet repressor (TetR) and the negatively charged C-terminal activation domain (130 amino acids) of the VP16 protein of the herpes simplex virus. The plasmid pTet-On was originally described as pUHD17-1neo by [150] and is used to develop stable Tet-On cell line.

After a vector that contains a gene under the control of a tet-responsive element is transfected into a Tet-On cell line, the rtTA protein is only capable of binding the to TRE when bound by Doxycycline. Thus the introduction of Doxycycline to the system initiates the transcription of the genetic product.

pTRE-Tight (Clontech) (Supplementary figure 2F) contains TRE-based promoter P_{tight}, when used in Tet-On system, the controlled expression of gene of interest is responsive to the rtTA regulatory protein. P_{tight} contains a modified tet-responsive element (TRE), which consists of 7 direct repeats of a 36-bp sequence that contains the 19-bp tet operator sequence (tetO). The TRE_{mod} is just upstream of the minimal CMV promoter, which lacks the enhancer that is part of the complete CMV promoter. Consequently, P_{tight} is silent in the absence of binding of rtTA to the tetO sequences. The characteristics for functional optimization are the 7 tetO sequences that are closer together and no potential binding sites of endogenous transcription factors. As a result, P_{tight} demonstrates greater inducibility coupled with extremely low basal activity and supports the very highest levels of Tet transactivation. $pTRE_{tight}$ was derivate from pTRE, originally described as pUHD10-3.

pENTR11-psRANKL (Supplementary figure 3) is a pENTR11 vector containing cDNA sequence of psRANKL obtained by subcloning the extracellular domain of pRANKL cDNA and Igκ-leader sequence from pSecTagABC-psRANKL into pENTR11 (Invitrogen) using XhoI/SalI restriction sites.

pLenti6/V5-psRANKL (Supplementary figure 4) is a pLenti6/V5-DEST vector into which extracellular domain of pRANKL cDNA and Igκ-leader sequence from pENTR11-psRANKL were transferred by using Gateway LR Clonase (Invitrogen). The correct sequence of the resulting pLenti6-psRANKL was confirmed by sequencing (Sequiserve) and used for lentiviral production.

pLenti6/V5-rtTA-psRANKL (Neo) (Figure 3.9A) is a lentiviral vector obtained by subcloning the floxed Neomycin resistance gene from lox2Neo plasmid into pLenti6/V5-rtTA-psRANKL (Bla) (cloned for other purposes). The correct sequence of the resulting pLenti6/V5-rtTA-psRANKL (Neo) was confirmed by sequencing (Sequiserve) and used for electroporation.

pCAG-rtTA (Supplementary figure 5) is a mammalian expression vector having a chicken β-actin promoter for high-copy constitute expression in mammalian cell lines, reverse tet-responsive transcriptional activator (rtTA) and Neomycin resistance gene floxed with two cassettes for selection in mammalian cells. rtTA cDNA sequence is fused to bovine growth hormone poly A for high mRNA stability. This vector was designed by Nikolai Klymiuk (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf).

pTRE-psRANKL (Supplementary figure 6) is a mammalian expression vector having pRANKL cDNA sequence upon regulation of TRE-based promoter P_{tight} and floxed Blasticidin resistance gene. psRANKL cDNA sequence is fused to bovine growth hormone poly A for high mRNA stability. In combination with pCAG-rtTA vector and Doxycycline, the expression of psRANKL from pTRE-psRANKL vector is achieved. This vector was designed by Nikolai Klymiuk (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf).

2.3.2 Restriction, ligation and recombination reactions

Restriction, ligation and recombination reactions were performed according to the manufacturers' descriptions.

Briefly, for the restriction reaction 10 µg of the plasmid were digested in the presence of 1× buffer (NEB, Schwalbach, Germany) and 2-3 U of restriction enzymes (NEB) at 37°C over night. The reaction was stopped by heat inactivation for 20 minutes at 60°C or by gel purification (see 2.3.3).

In a 20 µl of the ligation reaction 50 ng of linearized destination vector was combined with a 3-fold molar excess of linearized insert, mixed with 2× Quick Ligation buffer (NEB) and 1 µl of Quick T4 DNA Ligase (NEB) and incubated for 5 minutes at 25°C, followed by cooling of the reaction mixture on ice. A 10 nM of ligated plasmid was used for transformation into TOP10 competent bacterial cells (see 2.4.1).

For the recombination reaction, a mixture of 300 ng of pENTR11 plasmid, 300 ng pLenti6/V5-DEST and 5× LR Clonase reaction buffer with 4 µl from LR Clonase (Invitrogen) were incubated for 4 hours at room temperature. The reaction was stopped by addition of 2 µl Proteinase K and incubation for 10 min at 37°C.

2.3.3 Gel purification

The DNA extraction from agarose gel was performed according to the manufacturer's description using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Briefly, the desired DNA fragment was excised from agarose gel, weighted, mixed with 2 times volume of NT buffer and dissolved at 50°C for 10 minutes. Next, the DNA was bind to the column, washed and finally eluted in 30 µl of prewarmed NE buffer. The purified DNA fragment was used for further cloning steps.

2.3.4 Analyzing of clones

6 or more bacterial colonies were picked and cultured over night at 37°C with 225 rpm constant shaking in 5 ml LB medium containing antibiotics.

Plasmid DNA (pDNA) was isolated using GenElute Plasmid Miniprep Kit (Sigma-Aldrich) and following the manufacturer's instructions. Briefly, the cells were pelleted, resuspended and lysed for 3 min at RT. The lysis was stopped with the neutralization solution and cell debris was pelleted by centrifugation. The supernatant was loaded on previously equilibrated column and washed twice with washing buffer. The pDNA was eluted in elution buffer (5mM Tris-HCl buffer pH8.0). The pDNA was digested with restriction enzymes to confirm the presence and orientation of the insert as well as the integrity of the vector (see 2.3.2).

2.3.5 Maxi-prep of the plasmids

From the correct clones, a bigger amount of pDNA was isolated by HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany) for the pENTR11 clone or EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) for pLenti6/V5 clones. The kits were used according to the manufacturer's instructions. Briefly, the bacteria were pelleted and lysed and the cell debris was precipitate and filtrated. After the filtration the bacterial lysate was loaded on a column, washed, eluted and the pDNA was precipitated with isopropanol and pelleted by centrifugation. The pDNA pellet was washed with 70% ethanol, air-dried and resuspended in elution buffer (5mM Tris-HCl buffer pH8.0). The pDNA concentration was photometrically (Biophotometer, Eppendorf, Hamburg, Germany) measured and the integrity of vector was analyzed by digestion. Finally, the vector was sequenced to confirm that the gene of interest is in frame with the C-terminal tag using the CMV forward primer 5′-CGCAAATGGGCGGTAGGCGTG-3′ and V5 epitope reverse primer 5′-ACCGAGGAGAGGGTTAGGGAT-3′ by Sequiserve (Vaterstetten, Germany).

2.4 GENE TRANSFER

2.4.1 Transformation of bacteria

The final step of cloning procedure finished with bacterial transformation of desired plasmids. For the transformation were used two types of *E. coli* strains and protocol according to the manufacturer.

For the transformation of lentiviral vectors into the Stbl3 competent cells, 5 µl of the recombination/ligation reaction was added to the cells and incubated for 30 minutes on ice. Next, the cells were heat-shocked for 45 seconds at 42°C and incubated at 37°C with 225 rpm constant shaking for 1 hour in 250 µl S.O.C. media (Invitrogen). Finally, the cells were plated on Ampicillin LB agar plates and left to grow for 12 hours at 37°C to obtain bacterial clones.

For the transformation of TOP10 competent cells, the same protocol was used, except that the cells were heat-shocked for 30 seconds at 42°C.

2.4.2 Lentivirus production

For lentivirus production the ViraPower™ Lentiviral Expression System from Invitrogen was used. This system allows creation of a replication-incompetent HIV-1-based lentivirus that is used to deliver and express gene of interest in either dividing or non-dividing mammalian cells. The major components of the system include: an expression plasmid containing the gene of interest under the control of a constitutive promoters and elements that allow packaging of the construct into virions (*e.g.,* 5′ and 3′ LTRs, Ψ packaging signal); an optimized mix of the three packaging plasmids (pLP1, pLP2 and pLP/VSVG) that supply the structural and replication proteins *in trans* that are required to produce the lentivirus; the 293FT cell line, which allows production of lentivirus following cotransfection of the expression plasmid and the plasmids in the packaging mix.

Briefly, cotransfection of the ViraPower™ Packaging Mix and the pLenti vector-containing gene of interest into 293FT cells produce a replication-incompetent lentivirus, which will be used to transduce a mammalian cell line. Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus [168,168] and stably integrated into the host genome [162,170]. After the lentiviral construct has integrated into the genome, the use of antibiotic selection generates a stable cell line for long-term expression studies.

For production of the viruses an established protocol based on the manufacturer's recommendations was used. Briefly, 24 x10^6 of 293FT cells were resuspended in Opti–MEM media (Invitrogen). Three plasmids pLP1, pLP2 and pLP/VSVG from ViraPower lentiviral packaging mix (Invitrogen) responsible for amplification and packaging of the viral particles

were mixed in ratio 3:1 with pLenti plasmids in Opti-MEM media and Lipofectamine 2000 (Invitrogen). The cells were resuspended in the media containing plasmids–Lipofectamine 2000 complex and incubated in cell culture incubator at 37°C/5%CO_2 for 6 hours. After incubation time, the media was replaced with complete culture media and the cells were further cultured for 48 hours. Virus-containing media were harvested, filtered though 0.22 µm filter and stored in aliquots at -80°C. All viral material was produced in S2-laboratory and stored at Max-von-Pettenkofer-Institute, Virology Section, University of Munich leaded by Prof. Dr. med. Ulrich Koszinowski.

2.4.3 Transduction of target cells

For transduction, the cells were incubated for minimum of 30 min with 16 µg/ml Polybrene (Sigma-Aldrich) in a growth media. Next, viral supernatant was added in a 1:2 dilution in normal growth media. One day after transduction, the medium was replaced by fresh, nonviral medium and successfully transduced cells were selected with 10 µg/ml Blasticidin (Invitrogen) for 10 days. Finally, the cells were tested for a presence of viral particles by using HIV-1 p24 ELISA kit (PerkinElmer, Wiesenbaden, Germany). Only virus-free cells were transfer to S1-laboratory and used in the following experiments.

2.4.4 Transfection

For transfection of the cell an established protocol based on Lipofectamine 2000 Reagent (Invitrogen) was used. Briefly, 90-95% confluent cell layer and 1 µg of the plasmid were used for 6-well dish. Next, the DNA plasmid and Lipofectamine were diluted in Opti-MEM media (Invitrogen) without serum in separated tubes and incubated for 5 minutes at room temperature. After incubation, DNA and Lipofectamine were mixed in ratio 3:1 and incubated for further 20 minutes at room temperature. Finally, the complexes were added to the cells and incubated in cell culture incubator at 37°C/5%CO_2 for 6 hours. After incubation time, the media was replaced with complete culture media and the cells were further cultured for further experiment.

2.4.5 Electroporation

For introduction of desired genes into the primary porcine cells, the Amaxa Nucleofector technology (Lonza, Cologne, Germany) was used. This procedure was preformed by Annegret Wünsch at the Institute of Molecular Animal Breeding and Biotechnology (LMU, Prof. Eckhard Wolf).

2.5 GENOMIC ANALYSIS

Genomic analysis, of isolation, Southern blot and genomic PCR, were performed by Nikolai Klymiuk at the Institute of molecular animal breeding and biotechnology (LMU, Prof. Eckhard Wolf).

2.6 TRANSCRIPTOMIC ANALYSIS

2.6.1 Isolation of total RNA

From the cells

Isolation of total RNA from cells was performed with RNeasy Mini kit (Qiagen). Briefly, the cells were washed with PBS, trpysinized and lyses with RLT buffer and 1% ß-mercaptoethanol. The cell lysates were then filtrated through QIAshredder spin columns, mixed with isovolume of 70% ethanol and loaded on RNeasy spin columns. Contaminants from genomic DNA were digested with 10U of DNAse (RNAse free DNAse Set, Qiagen) for 15 min at RT. Finally, columns were washed twice with washing solution and dried by centrifugation at maximum speed. High-quality total RNA was eluted in RNase-free water and measured spectrophotometrically (Biophotometer, Eppendorf) for evaluation of the RNA concentration and purity. Furthermore, the quality of the RNA was checked by gel electrophoresis. Briefly, 500 ng of the RNA was mixed with loading dye, denaturated for 10 min at 70°C, placed on ice for 3 min and loaded on the 1% agarose gel. The good RNA quality was evaluated if showed 2 bands on the gel (18S and 28S).

From the tissues

Isolation of total RNA from tissues was performed with RNeasy Mini kit (Qiagen). Briefly, to isolate the RNA, 30 mg of the frozen tissue was weighted, disrupted using a mortar and pestle in liquid nitrogen and homogenized by RLT buffer and 1% ß-marcaptoethanol using a QIAshredder spin column following the procedure described above.

2.6.2 cDNA synthesis

The cDNA synthesis was performed with Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocol. Briefly, 1 µg of total RNA, random primers and 10 mM dNTPs were heated for 15 min at 65°C. The denatured RNA was added to a mixture of PCR buffer, 15 U of reverse transcriptase, 40 U of RNAse inhibitor and 0.1 M DTT and the mixture was incubated for 1 hour at 50°C.

2.6.3 RT-PCR

A specific amount of cDNA (determined by the expression of GAPDH) was added to a master mix containing 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primers (Supplementary table 1) and 1 U Taq DNA polymerase (Invitrogen). The PCR reactions were run on MG Research PCR machine (BioRad, Munich, Germany). Normally, the PCR programs had 3 min 95°C, 30 cycles of amplification as each cycle consisted of denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and elongation (72°C for 60 sec) steps, 10 min 72°C, 4°C forever. The amplified products were analyzed on 2% agarose gels and visualized by ethidium bromide. As a reference for the correct size of the amplified product a 100 bp molecular weight standard (Invitrogen) was used. Pictures with different exposure times were taken by using a gel imaging system (Vilber Lourmat Deutschland GmbH, Hochdorf Riß, Germany).

To be sure that RNA is pure from DNA contaminations; the PCR was also preformed on RNA samples.

2.6.4 Gel electrophoresis

To prepare the gel for the electrophoresis, the agarose powder was weighted to obtain a desired percentage and completely dissolved in 1×TAE buffer by heating. Next, the gel solution was left to cool down, 0,003% of ethidium bromide was added and gel was poured into the gel tray with well comb fixed properly. After the gel has been cooled completely and solidified, the comb was removed and the tray was inserted properly into the gel chamber. The 1×TAE buffer was poured into the chamber to cover the gel and fill the wells. Finally, 10 µl (for RNA and DNA) or 20 µl (for cDNA) of samples/loading dye solution were pipetted into the appropriate wells of the gel and the gel was run at 80-120 V (according to the size of the gel) for 45 minutes.

2.7 PROTEOMIC ANALYSIS

2.7.1 Histology

Cell plating and fixation

$1x10^5$ of the each cell type were plated on glass slides and were cultured in complete media for maximum of 3 days. After this the media was removed, cells were rinsed with PBS (w Ca/Mg, PAA) and fixed with 4% PFA (Merck) for 10 min at RT. After the fixation the slides were washed with PBS, air-dried and immediately used or stored at -20°C for up to 2 weeks.

Immunofluorescence staining

The fixed cells were rehydrated in PBS (3x5 min at RT, PAA) and permeabilized with 0.2% Triton X-100/PBS (Sigma) for 15 min. Image enhancer solution (Invitrogen), which reduce the unspecific binding of secondary antibodies, was applied for 45 min. Blocking was performed with 2% BSA/PBS + 1.5% goat serum (Dianova, Hamburg, Germany) for 2 hours and it followed by addition of primary antibodies (Supplementary table 2) for 1 hours at 37°C. After PBS washing (3x5 min at RT), secondary antibodies were added for 30 min at room temperature. Finally, the slides were washed with PBS (3x5 min) and nuclear contrastaining was performed with a 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) in dilution of 1:10 000 in blocking solution. Following a PBS washing (3x5 min) the slides were mounted with Mowiol anti-fading media (6 g glycerol, 2.4 g Mowiol, 12 ml 0,2 M Tris-HCl pH 8.5, 0.024 g DABCO and 6 ml H_2O). Negative controls for antibody were carried out on the same slide by omitting the primary antibody. The stained slides were stored over night at 4°C. Pictures with different magnification were taken on Axioskope2 microscope (Carl Zeiss MicroImaging GmbH).

DAB staining

For DAB staining, the slides were washed with phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100/PBS for 15 min and endogenous peroxidase activity was quenched by application of 3% hydrogen peroxide for 30 minutes at room temperature. Blocking solution consisting of 2% BSA/PBS + 1.5% goat serum + 1.5% horse serum (Dianova) was applied to the slides for 2 hours at RT and it followed by incubation of primary antibodies (Supplementary table 2) overnight at 4°C. Incubation with secondary biotinylated goat-anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was performed for 1 h at 37°C followed by application of avidin-biotin complex for half hour at room temperature. Diaminiobenzidene (DAB, Vector Laboratories) was used as the colorizing agent. Following PBS washing (3x5 min) the slides were mounted with DPX anti-fading media (Sigma-Aldrich). Negative controls for antibody were carried out on the same slide by omitting the primary antibody. The stained slides were stored over night at room temperature. Pictures with different magnification were taken on Axioskope2 microscope (Carl Zeiss MicroImaging GmbH).

2.7.2 Western blotting

Protein extraction

For protein extraction from the cells, RIPA extraction buffer composed of 0.1% (*w*/*v*) sodium dodecyl sulfate (SDS), 1% (*w*/*v*) Na-DOC, 1% (*v*/*v*) Triton X-100, 50 mM Tris–HCl (pH 8,2), 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA; pH 8.0) and 20 mM NaF containing a mixture of protease inhibitors (Complete Mini, EDTA-free, Roche, Penzberg, Germany) was used. After typsinization and washing, the cells were lysed in the above buffer by incubation on ice for 30 min. After incubation cells were disrupted by sonication and incubated on ice for additional 30 min. The insoluble cell debris was removed by centrifugation at 10 000 g for 10 min at 4°C. The supernatant containing protein was aliquoted and stored at -80°C.

For protein analysis from supernatant, the media was not change for at least 2 days. Next, the supernatant was collected and filtered through 0,22 µm pore size filter (Milipore, Schwalbach, Germany) to remove cell debris. Finally the supernatant was aliquoted and stored at -20°C.

For the protein extraction from the tissues, the same protocol as the one used for the cells was used, except that 0,6 g of the tissue was disrupted using mortar and pestle in liquid nitrogen and lysed in RIPA buffer.

BCA assay

The total protein amount from cells, supernatants and tissues was measured by using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) by ELISA. Briefly, the protein concentration was determined based on a chemical reaction where BCA interact with the cuprous cations that are reduced by the proteins in an alkaline media. As a result an intense purple-coloured reaction is observed and the intensity of the color correlates with the protein concentration. The protein amount was calculated using a standard curve that is built by serial BSA dilutions. The measurements are performed at 450 nm on microtitre-plate reader (Thermo Scientific).

SDS-PAGE

Protein extracts were mixed with 4×Laemmli buffer (200 mM Tris-HCl pH 6.8, 40% glycerol, 10% SDS, 30% 2-mercaptoethanol, 0.02% bromphenolblue and 0.2 M DTT) and were boiled for 5 min at 99°C. The protein mixtures were spined down and loaded on 12% acrylamide gels (BioRad). The electrophoresis was performed in 1×running buffer formulated from 0.25 M Tris-base pH 8.3, 1% SDS and 1.92 M glycine. For a molecular weight standard, Seeblue

plus 2 protein marker (Invitrogen) was used. The electrophoresis was run at 60 for 1 gel or 100 mA for 2 gels. The procedure continued protein transfer to PVDF membrane (Roche) (see *Protein transfer*).

Protein transfer

The protein transfer was performed by using the vertical "wet" transfer method. In brief, gel loaded with proteins was equilibrated for 15 to 20 min in 1×blotting solution containing 250 mM Tris-base and 1.92 mM glycine. During this equilibration time, the PVDF membrane was incubated in methanol, rinsed in water and soaked in 1×blotting buffer. The Western blot setup was then assembled. The protein transfer was performed over night with 30 V at 4°C. After the transfer, the gel was preceded with Coomassie staining and immunodetection.

Coomassie staining

The gel was first incubated in isopropanol fixation solution consisting of 10% acetic acid and 25% isopropanol (Merck) and then in Coomassie staining solution formulated from 7% acetic acid, 40% methanol (Merck) and 0.025% Coomassie brilliant blue (ROTH). In this solution the gel was boiled for 30 sec and gently shaken for 20 min at RT. Destaining solution consisting of 7% acetic acid and 40% methanol was applied to the gel for 12 hours with gentle shaking. This staining was used to analyze the quality loading and transfer of the protein extracts.

Protein immunodetection

The PVDF membrane was incubated with blocking solution consisting of 5% skim milk/TBS-T20 for 1,5 hour with gentle shaking. Then, primary antibody (Supplementary table 2), diluted in the blocking solution, was applied with vigorous shaking at 4°C over night. The membrane was washed 4x7 min with TBS-T20 washing solution consisting of 1 mM Tris-base pH 7.4, 150 mM NaCl and 0.05% Tween20 and secondary antibody, diluted in the blocking solution, was applied to the membrane for 1 hour with vigorous shaking. Finally, the membrane was again washed 4x7 min with TBS-T20 and proteins were visualized by using chemiluminescent ECLplus solution (GE Healthcare, Munich, Germany) and detection film (Lumi-film chemiluminescent, Roche) using detector and fixator solution (Merck).

2.7.3 ELISAs

sRANKL ELISA

To determine the amount of RANKL protein secreted in the medium, concentration of the protein in the cell culture supernatant was measured. A sRANKL enzyme-linked immunosorbent assay (ELISA) was performed using the Biomedica sRANKL ELISA kit (Vienna, Austria). A standard curve was generated using two-fold serial dilutions ranging from 200 to 25 pg/ml.

Cathepsin K ELISA

To determine the amount of Cathepsin K produced by the active osteoclast, we measured the concentration of the protein in the serum of wild type and transgenic pig. A Cathepsin K enzyme-linked immunosorbent assay (ELISA) was performed using the Biomedica Cathepsin K ELISA kit. A standard curve was generated using two-fold serial dilutions ranging from 300 to 11 pmol/l.

CrossLaps ELISA

For the quantification of degradation products of C-terminal telopeptides of Type-l collagen (CTX) during bone resorption, we measured the concentration of the CTX protein in the serum of wild type and transgenic pig. A CrossLaps enzyme-linked immunosorbent assay (ELISA) was performed using the Nordic Bioscience Serum CrossLaps ELISA kit (Herlev, Denmark). A standard curve was generated using two-fold serial dilutions ranging from 2 to 0,114 ng/ml.

2.8 OSTEOCLAST FORMATION AND ANALYSIS

2.8.1 Isolation of porcine osteoclast precursor cells by density gradient centrifugation

Bone marrow was withdrawn from the posterior iliac crest of the pelvic bone from wild type swine. To prevent clotting, bone marrow was treated with 1% sodium-heparin and stored on ice before processing. Bone marrow was subjected to isolation of mononuclear cells, which contain the osteoclastic precursor cells. Density gradient centrifugation was used to separate the mononuclear precursor cells from other elements in blood. Therefore, the bone marrow was diluted 1:4 with PBS and carefully layered on top of the Ficoll–Paque. The tubes were centrifuged with 350 g at 20°C for 30 min. The mononuclear hematopoietic precursors accumulating at the interface between PBS and Ficoll-Paque were transferred in 50 ml-tubes

resulting ~10 ml of mononuclear cells. These tubes were filled with PBS and centrifuged with 350 g at 20°C for 10 min. Pellets were washed with PBS and resuspended in growth medium. Cell solution of 10 µl were stained with 990 µl Trypan-blue to assess viability and counted in a Neubauer counting chamber. To dispose the culture of contaminating lymphocytes, the cells were purified for adherence by stimulation with 5 ng/ml M-CSF (human recombinant Macrophage-Colony Stimulating Factor, Sigma-Aldrich). Therefore, the non-adherent cells were washed off at day four of culture, so that only the adherent monocytes were used for further experiments.

2.8.2 Osteoclastic cell culture

Porcine osteoclast precursor cells isolated from bone marrow were cultured for initially 4 days in growth medium supplemented with 5 ng/ml M-CSF (Sigma-Aldrich). Next, the cells were stimulated for further 14 days with 20 ng/ml RANKL (recombinant human soluble RANK Ligand, PeproTech) to generate osteoclasts.

2.8.3 Osteoclasts visualization

TRACP assay

For TRACP assay experiment, the cells were cultured in 6-well dish and after 14 days of stimulation with 5 ng/ml M-CSF (Sigma-Aldrich) and 20 ng/ml RANKL (Peprotech), fixed with 10% glutaraldehyde solution (Sigma-Aldrich) for 15 minutes at 37° and washed 2 times with PBS. The fixed cells were stained in TRACP-staining solution for 30 min at 37°C. TRACP staining solution consisted of 5 mg Naphtol AS-MX Phosphate and 15 mg Fast Red Violett LB Salt in 50 ml TRACP-buffer (0,2 M sodium-acetate, 0,2 M acetic acid, 0,3 M sodium-tartrate). Mature osteoclasts were defined as highly TRACP-positive cells containing 3 or more nuclei. Photomicrographs were taken with axiocam magnetic resonance on an Axiovert-microscope (Carl Zeiss MicroImaging GmbH).

Phalloidin staining

For phalloidin staining osteoclastic precursor cells were cultured on sterile glass cover slides and stimulated to obtain osteoclast. The slides were fixed with 4% PFA at room temperature for 10 min, permeablizated with 0,1% Triton-X (Sigma-Aldrich) for 15 minutes at room temperature and stained with Alexa Fluor 488-phalloidin in 1:20 dilution for 1 hour at 37°C. Nuclei were stained with propidium iodide (Sigma-Aldrich). Analysis was done with a confocal LSM 510 (Carl Zeiss MicroImaging GmbH) equipped with Plan-Apochromat

63×/1.4 Oil DIC. Propidium iodide and Alexa Fluor 488-phalloidin (excitation 488 and 543) were detected in dual-channel mode using LP560nm and BP505-530nm emission filters.

Concanavalin A staining

For Concanavalin A staining osteoclastic precursor cells were cultured on sterile glass cover slides and stimulated to obtain osteoclast. The slides were fixed with 4% PFA at room temperature for 10 min and stained with Concanavalin A antibody (24 µg/ml, Invitrogen) for 45 minutes at room temperature in the dark. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in dilution of 1:10 000 in PBS solution and the analysis was done with a Axioskope2 microscope (Carl Zeiss MicroImaging GmbH).

Giemsa staining

For Giemsa staining, osteoclastic precursor cells were cultured in 6-well dishes and stimulated to obtain osteoclast. The cells were fixed with 4% PFA at room temperature for 10 min and stained with 5% Giemsa´s stain solution (Gibco, Karlsruhe, Germany) for 3 hours at 37°C. Following a H_2O washing (3x5 min) the dishes were differentiated with 0,5% aqueous acetic acid solution for 30 seconds and finally covered with water. Pictures with different magnification were taken on Axioskope2 microscope (Carl Zeiss MicroImaging GmbH).

2.8.4 *In vitro* osteoclastogenesis assays

To determinate whether the psRANKL is capable of inducing osteoclastogenesis, the osteoclastic precursor cells were seeded in 6-well dish and cultured in medium supplemented with 5 ng/ml M-CSF (Sigma-Aldrich) for 4 days. At day 5, osteoclastic precursor cells were stimulated with 20 ng/ml hsRANKL (hsRANKL + M-CSF) or transfected with 1 µg pLenti6-psRANKL construct using Lipofectamine Reagent 2000 (Invitrogen) (see 2.4.4). Two negative cell controls were carried on, one cell control was stimulated with 5 ng/ml M-CSF (Sigma-Aldrich) and other was cultured in normal growth media without stimulants. Cultures were maintained for additional 3 or 7 days and proceeded further to confirm the identity of the osteoclasts.

To be sure that transfected osteoclastic precursor cells are expressing psRANKL protein, three days after transfection the supernatant from cells cultured with M-CSF alone and transfected with psRANKL + M-CSF was collected and psRANKL protein was measured by sRANKL ELISA (Biomedica) as mentioned above. The TRACP cytochemistry was preformed on day 7 after transfection in 6-well dish as described above. Mature osteoclasts with highly TRACP-

positive cells containing 3 or more nuclei were counted in the whole 6-well dish and per each condition under the Axiovert-microscope (Carl Zeiss MicroImaging GmbH).

For the detection of CA-II, RNA was isolated as described in 2.6.1 and reverse transcription was done (see 2.6.2) from cells cultured in 6-well dish for 7 days in medium, with 20 ng/ml hsRANKL + 5 ng/ml M-CSF, with 5 ng/ml M-CSF and transfected with psRANKL, respectively. The primer set for CA-II (Supplementary table 1) was used and PCR amplification was carried out. The PCR results were visualized on 2% agarose gel (see 2.6.4).

Resorption assay

For quantitative measure of bone resorption, 10 000 osteoclastic precursor cells were plated per well on 96-OsteoAssay human bone plate (Lonza) and cultured in medium, with 20 ng/ml hsRANKL + 5 ng/ml M-CSF, with 5 ng/ml M-CSF and 5 ng/ml M-CSF + 0,2 µg transfected with psRANKL, respectively. Two wells were left without cell for the background values for data calculation. At day 0 and day 3 after transfection, the supernatant was collected and used in CalciFluor assay (Lonza) to measure calcium that is released as a result of osteoclast-mediated resorptive activity. Briefly, after collecting the supernatant the calcium detection reagent was dissolved and pipetted in 96-well black-welled assay plate. Next, the probes and standards were added and the fluorescence was determinated by fluorimeter (Safire II, TECAN, Crailsheim, Germany) by excitation of 555λ and emission of 577λ. Finally, the calcium concentration of the samples was read out from the standard curve.

2.9 GENERATION OF TRANSGENIC ANIMALS

All procedures of generation transgenic pigs from oocyte and donor cell preparation over somatic cell nuclear transfer to the embryo transfer were performed by Barbara Keßler, Mayuko Kurome and Eleonore Schilling at the Institute of Molecular animal breeding and biotechnology (Prof. Eckhard Wolf) according to German laws, regulations and institutional guidelines. All animals subjected to this study displayed a good health status.

Tissue preparation

After sacrificing the animals, different organs were taken, cut into small pieces and snap frozen in liquid nitrogen. The frozen tissues were preserved at -80°C before processing.

Application of Doxycycline

Doxycycline was fed in the form of tablets (Doxy 200®, CT Arzneimittel GmbH, Berlin, Germany; one tablet contains 200 mg Doxycycline). For the first *in vivo* stimulation experiment, a daily dose of 3.2 mg/kg body weight was applied for periods of 10 days, on day 11 the dose was doubled and applied for further 10 days. For second stimulation experiment, a daily dose of 6,3 mg/kg body weight was applied for 10 days, on day 11 the dose was doubled and applied for further 10 days, on day 21 the dose was again doubled and applied for 10 days and on day 31 the dose was again doubled and applied for 10 days. In figures 3.22 and 3.23, 0,25X is indicating a dose of 3,2 mg/kg body weight, 0,5X a dose of 6,3 mg/kg body weight, 1X a dose of 12,5 mg/kg body weight, 2X a dose of 25 mg/kg body weight and 4X a dose of 50 mg/kg body weight, respectively. Blood samples were taken from the *Vena jugularis* prior to Doxycycline treatment and at different time points thereafter (on day 3, 6, 10) and were analyzed by sRANKL, Cathepsin K (only for first stimulation) and CrossLaps ELISA (only for first stimulation) (2.7.3).

2.10 MICROSCOPY

The microscopes and cameras used in this thesis are purchased from Carl Zeiss MicroImaging GmbH. Axiovert100 microscope was used for taking immunofluorescent microphotographs. Pictures were taken with 10× and 40× objectives and using AxioCam MRm black-white camera. Axiovert 40CFL was used for imaging of DAB-stained cells, TRACP-stained and Giemsa-stained osteoclast. Objectives having 5×, 10×, 20× and 40× magnifications were used. For taking pictures was used AxioCam ICc3 colour camera. Laser scan microscope (LSM 510, Carl Zeiss MicroImaging GmbH) was used for imaging of phalloidin-stained cells.

2.11 COMPUTIONAL SOFTWARES

In this thesis a number of specialized programs were used for processing and analyzing the obtained data. The charts were created by using GraphPad Prism 5 (Systat Software). Photomicrographs were processed with using AxioVision LE software (Carl Zeiss MicroImaging GmbH) and Adobe Photoshop CS2 program (Adobe Systems Incorporated). For cloning experiments, Clone Manager Suite 7 was used.

The following links were used:

- www.ncbi.nlm.nih.gov/sites/entrez - major source of publications,

- blast.ncbi.nlm.nih.gov/Blast.cgi - NCBI-BLAST for regions of similarity between biological sequences,
- www.ebi.ac.uk/Tools/clustalw/index.html - ClustalW for multiple sequence alignment,
- www.ch.EMBnet.org – Boxshade for pretty printing and shading of multiple-alignment files,
- www.cbs.dtu.dk/services/NetNGlyc - NetNGlyc 1.0 Server for prediction N-glycosylation sites in proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons and
- smart.embl-heidelberg.de - SMART Program (Simple Modular Architecture Research Tool) for the identification and annotation of genetically mobile domains and the analysis of domain architectures.

2.12 STATISTICS

The statistical relevance between groups was determinated using one-way analysis of variance (ANOVA), or by two-sample T-test on GraphPad Prism 5 (Systat Software). Graphs and bar charts show mean values and SEM. The ELISA enzymatic and colorimetric experiments, such as osteoclast quantification consisted of minimum 3 independent repeats, each performed in duplicates. PCR, western blot and staining results have been reproduced at least twice.
The figure bar charts represent mean and standard deviation of minimum three independent experiments. A value of $p<0.05$ was considered significant.

3. RESULTS

3.1 CLONING AND FUNCIONAL CHARACTERIZATION OF THE PROCINE EXTRACELLULAR DOMAIN OF RANKL (psRANKL)

3.1.1 Cloning and sequence analysis of extracellular domain of porcine sRANKL

To isolate a porcine extracellular domain of RANKL, the soluble functional form was cloned and over-expressed. For this purpose, the primers were constructed based on published extracellular domain of human RANKL cDNA sequence (NG_008990.1) obtained from BLAST search. Since RANKL is expressed by osteoblast, total RNA was extracted from this cell type and used as a template for RT-PCR for cloning porcine sRANKL (psRANKL). Using human RANKL-specific primers (Supplementary table 1), several potential psRANKL cDNAs were amplified by RT-PCR. Five clones were sequenced and exhibited the identical sequences. Finally, a cDNA of 731 bps and a protein of 243 amino acid residues with 28,2 kDa molecular weight was generated. Furthermore, the recently published gene sequence (GenBank Accession No. CU024906) and our cloned cDNA has 100% identity to the genome sequence. This fragment coding for the extracellular portion was amplified by PCR, gel-purified and subcloned into the expression vector having the Igk-leader signal sequence to obtain secreted form of porcine sRANKL. Sequencing confirmed that the insert formed correct open reading frame with the leader signal sequence of the parent plasmid. Finally, the extracellular region having Igk-leader signal and porcine sRANKL was subcloned into lentiviral pLenti6/V5-DEST vector generating a cDNA template of 842 bps and a protein of 280 amino acids with molecular weight of 31,7 kDa with predicted pI of 6,90. Amplicon was sequenced, published under GeneBank accession number DQ523616 and aligned to published human, rat and murine cDNA sequences. Nucleotide homology to the human sequence at the corresponding region is 87%. Homology between the respective region from *Mus muscullus* and *Rat norvegicus* sequences are 79%. The protein sequences of extracellular domain of porcine, human (GeneBank Acc. No.: AAB86811.1), rat (Q9ESE2) and murine (AAB86812.1) sRANKLs are compared in Figure 3.1A, which shows that porcine and human share 90% homology, porcine and rat 81% and porcine and mouse 80% homology, respectively. The homology between the species is strong along the entire sRANKL molecule and the strongest sequence similarity appears to be at their carboxyl regions. Furthermore, the glycosylation sites are conserved among all four sRANKL proteins. A highly identity

between porcine and human sequence suggest that the function of this gene could be highly conserved. In addition, phylogenic analysis showed that psRANKL is more related to human than to rat and murine (Figure 3.1B). To predict structural domains in psRANKL, the putative amino acid sequence was analyzed using SMART program. As shown in Figure 3.1C, a TNF domain is located at C-terminus of newly cloned psRANKL. Similar domain can be predicted in sRANKL from human, rat and mouse, indicating that the TNF domain is a common characteristic of RANKL from different species.

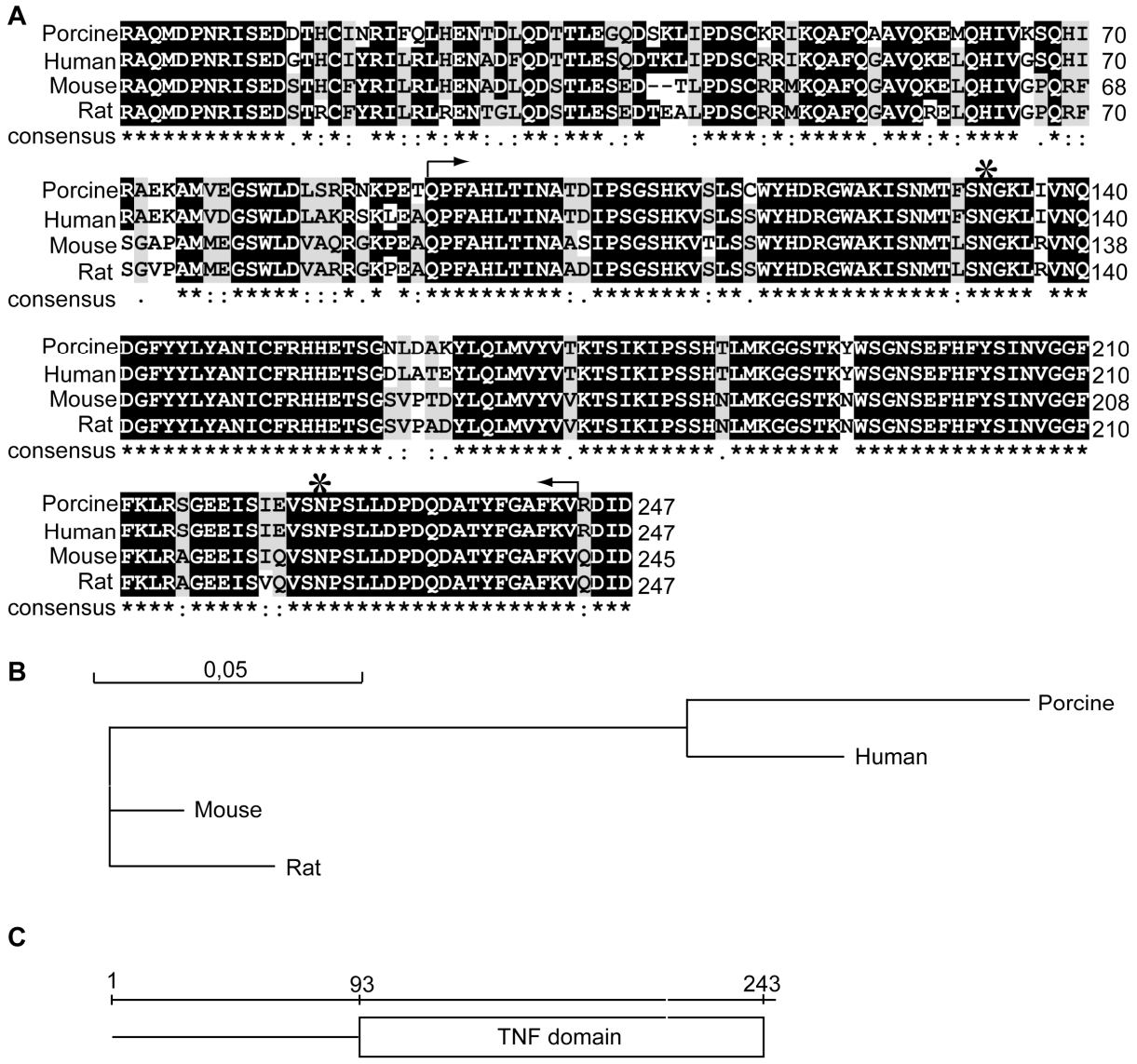

Figure 3.1.: Multiple alignment, phylogenetic tree and architecture of deduced amino acid sequences for sRANKLs. (A) Alignment of the predicted amino acid sequences of porcine (ABF81680), human (AAB86811), rat (Q9ESE2) and murine (AAB86812) sRANKL proteins. The number indicates the amino acid position. Identical amino acid residues are marked with black block and asteroids, while similar amino acid residues are marked with gray color and dots on consensus line. Conserved predicted N-glycosylation sites are indicated by asterisks and were determinated using the NetNGlyc 1.0 Server. Right/left arrow marks the beginning/end of the TNF-like core domain within extracellular region. (B) A phylogenetic tree of the amino acid sequences of porcine, human, murine and rat sRANKLs. The uprooted tree was built using the neighbour-joining method with program ClustalW based on the alignment of sRANKL protein sequences. The scale bar is 0,05 representing genetic distance. (C) Architecture of psRANKL predicted by SMART. The TNF domain is showed boxed. The number indicates the amino acid position.

3.1.2 Over-expression of porcine sRANKL in porcine fetal fibroblast

To generate stable over-expressing porcine fetal fibroblast (pFF), expression construct pLenti6/V5-psRANKL (Supplementary figure 4) was used to generate lentivirus, which was used to transduce the porcine cells. As a control for over-expression, the wild type cells were carried out thought the whole experiment. Next, to examinate over-expression of psRANKL, RT-PCR, Western blot, ELISA and immunocytochemistry were performed.

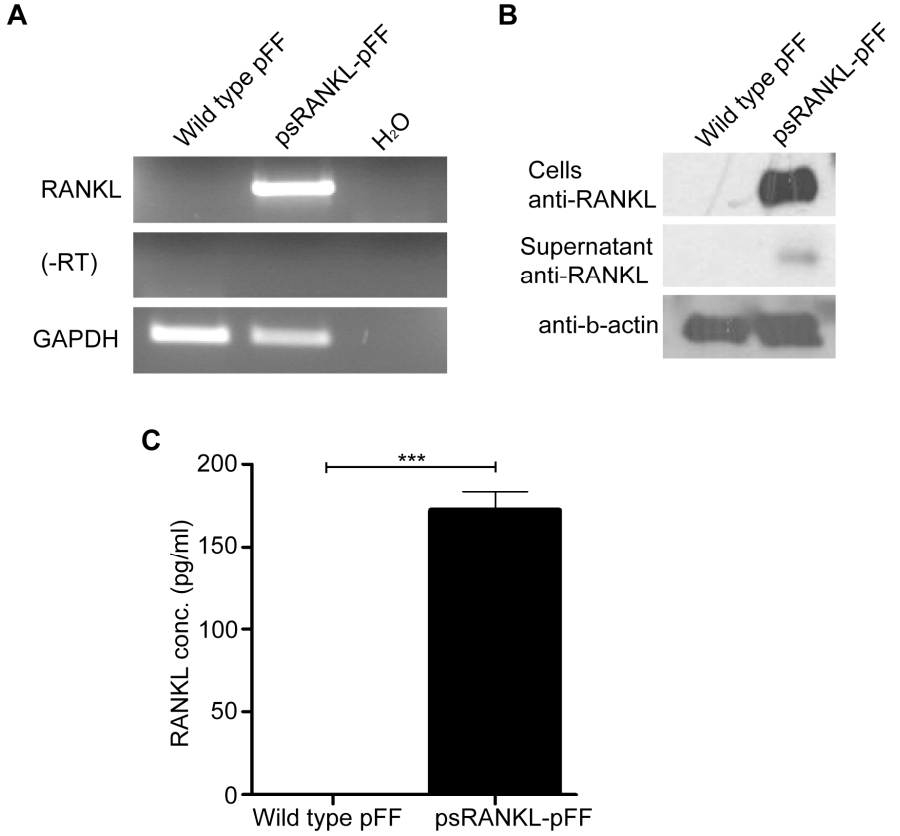

Figure 3.2.: Over-expression analysis of porcine sRANKL in wild type and porcine sRANKL-transduced pFFs on mRNA and protein level. (A) Total RNA from wild type and psRANKL-transduced pFFs was used for RT-PCR of psRANKL mRNA. Expression of psRANKL mRNA was detected in psRANKL-transduced pFF, while no expression was detected in untransduced cells. No contamination of genomic DNA was found in both samples performing PCR without RT. The expression of psRANKL was normalized to the expression of GAPDH gene. (B) Total protein and supernatant from wild type and psRANKL-transduced pFFs was used for Western blot analysis. A strong expression can be found on protein level in transduced cells, while there was no visible expression of psRANKL in untransduced cells. The blots were stripped and reprobed with β-actin to normalize for the amount of loaded protein. (C) sRANKL ELISA of the supernatant collected from wild type and psRANKL-transduced pFFs. A strong secretion can be found in transduced cells, while there was no secretion of psRANKL from wild type cells.

RANKL over-expression on mRNA level has been exanimate in the genetically modified porcine fetal fibroblast (psRANKL-pFF) and compared to the wild type cells. To be sure that only the transgene is amplified, the primers were designed in a way to bind on 3´ end of Igk-leader signal sequence and 5´ end of psRANKL sequence generating a single transcript of a 411 bps. RNA-control PCR was also performed, by omitting reverse transcriptase step, to exclude cDNA contamination. A strong expressional level of psRANKL mRNA was detected

in psRANKL-transduced cells, whereas no visible expression signal could be detected in untransduced wild type cells (Figure 3.2A). On a protein level, an over-expression of a psRANKL in transduced cells could be also seen comparing both cell types, using Western blot analysis. Western blot analysis of harvested media revealed a secreted psRANKL protein of about 30 kDa in psRANKL-pFF (Figure 3.2B). No band was found in untransduced cells. To quantify the amount of secreted psRANKL in the supernatant, a sRANKL-specific ELISA system of the supernatant from both cell types was performed and found a significant secretion of a psRANKL in transduced cells (Figure 3.2C). Therefore, the fusion protein psRANKL was expressed as expected in the soluble form. To further confirm these results and to show localization of psRANKL protein, two types of immunocytochemistry, DAB staining and immunofluorescence using an anti-RANKL-antibody as primary antibody, were preformed on psRANKL-pFF and wild type cells. In both immunocytochemistral analyses, psRANKL protein was primarily located around the nucleus but also in cytoplasm of the psRANKL-pFF, while no or very low background psRANKL expression was detected in untransduced pFFs (Figure 3.3A and B).

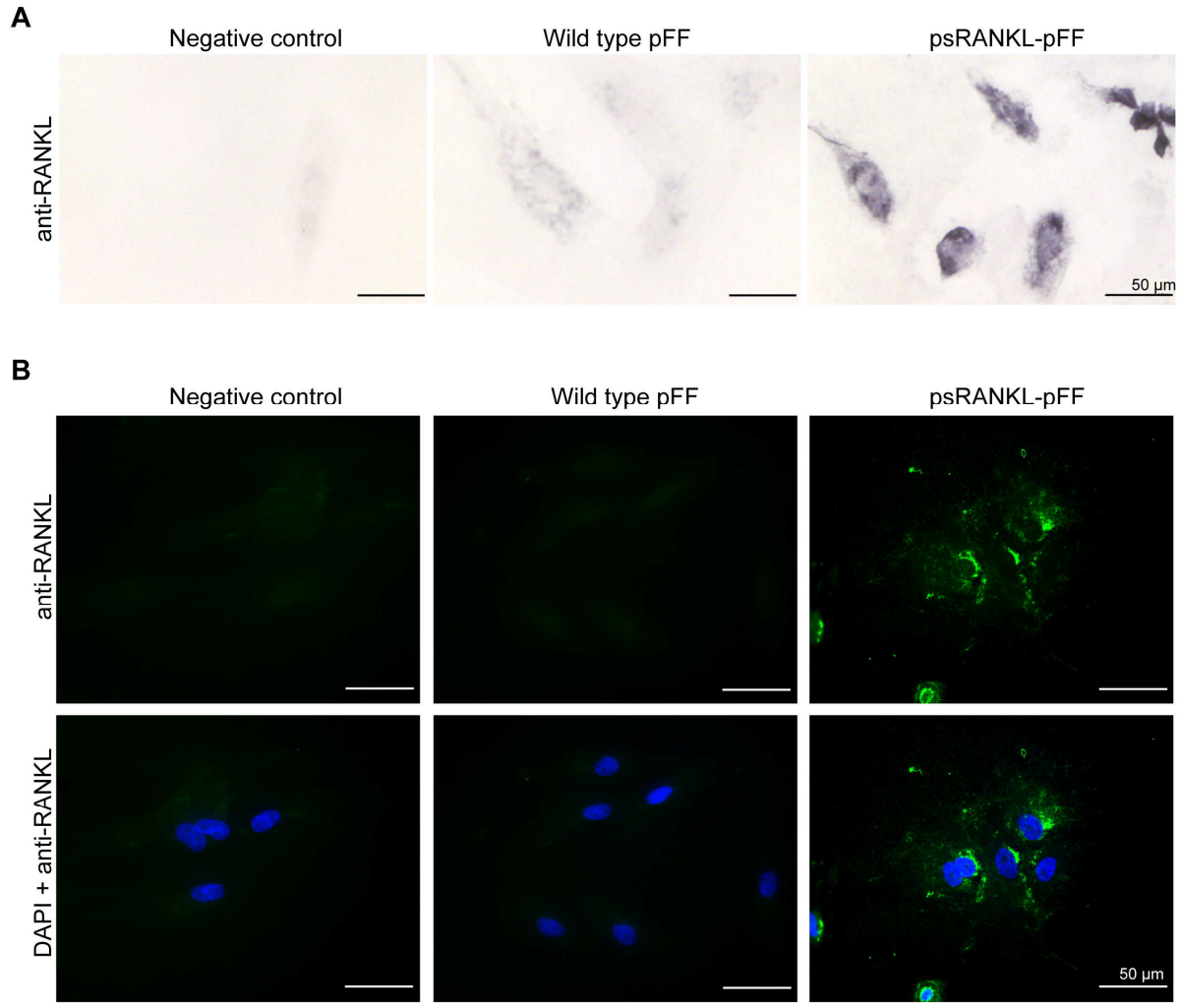

Figure 3.3.: Immunocytochemistral analysis of over-expression of psRANKL protein in wild type and psRANKL-transduced pFFs. (A) DAB-immunostaining of the cells. A strong positive signal indicated by the brown color reaction by the DAB chromagen was detected in transduced cells, while there was very low background signal in wild type cells. (B) Immunofluorescence staining of the cells. The negative control without primary anti-RANKL-antibody and the wild type pFF show hardly fluorescence signal. A strong Alexa Fluor 488 signal (green) in transduced pFF indicates a high expression of psRANKL. Green: RANKL staining; blue: DAPI nuclear counterstaining.

3.1.3 Characterization of porcine osteoclasts

Induction of multinucleated giant cells by treatment of monocytes with hsRANKL and M-CSF

To obtain osteoclast cultures and to characterize them, porcine osteoclastic precursor cells were treated with 20 ng/ml hsRANKL and 5 ng/ml M-CSF for 14 days. The cultured osteoclastic precursor cells from bone marrow were of typical spindle-shaped morphology (Supplementary figure 1E). First osteoclast appeared in culture on day 3-6 after stimulation. The osteoclasts´ culture had a morphology varied from small multinucleated cells localized within a dense fibroblast/stromal cell layer to giant cell that had lost contact to the surrounding cell layer (Figure 3.4A). To ensure, that the generated cells were indeed osteoclasts, they were tested for important specific characteristics. First the morphology of the resulting cells (multinuclearity, formation of actin rings with podosomes) was analyzed. A

confluent layer of multinucleated cells was observed by light microscope (Figure 3.4B). The increase in osteoclast numbers was accompanied by the appearance of multinucleated cells with typical osteoclast morphology: large size, flat morphology, multinucleation and intense staining for TRACP, an osteoclast marker (Figure 3.4C and D). The identity of the osteoclast

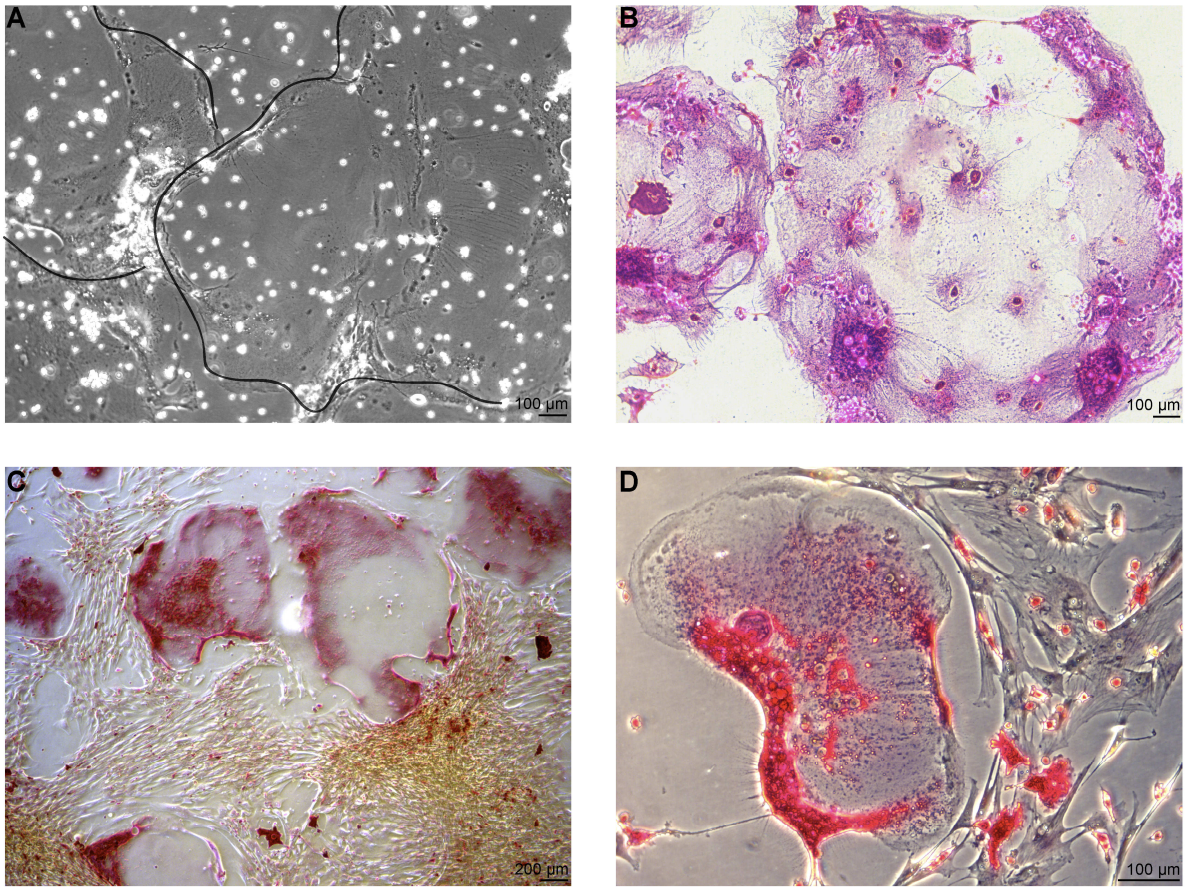

Figure 3.4.: Osteoclast differentiation in cultures stimulated with hsRANKL and M-CSF for 14 days, their morphology, Giemsa and TRACP staining. (A) Phase-contrast figure showing typical osteoclast morphology: large size, flat appearance, and multinucleation. Marked black lines are indicating the border of the osteoclasts. (B) Giemsa staining of porcine osteoclast under light microscopy. (C & D) TRACP staining of porcine osteoclast under light microscopy. The osteoclasts are showing strong staining for TRACP**.**

was further confirmed by staining of the actin by confocal microscopy. Osteoclastic precursor cells were cultured on glass slides and induced with hsRANKL and M-CSF to differentiate for 14 days. Next, they were stained for phalloidin actin ring (Figure 3.5A). The treatment with hsRANKL and M-CSF led to large multinucleated cells with a smooth periphery lined by a band of tightly packed podosomes forming a belt. These podosomes, small cylindrical actin structures, showed an increasingly dense phalloidin staining. Furthermore, the podosomes were also visualized by Concanavalin A staining showing that the podosomes had bright, fluorescent patches that exactly corresponded to the areas bordered by bright lines seen under bright-field microscopy (Figure 3.5B).

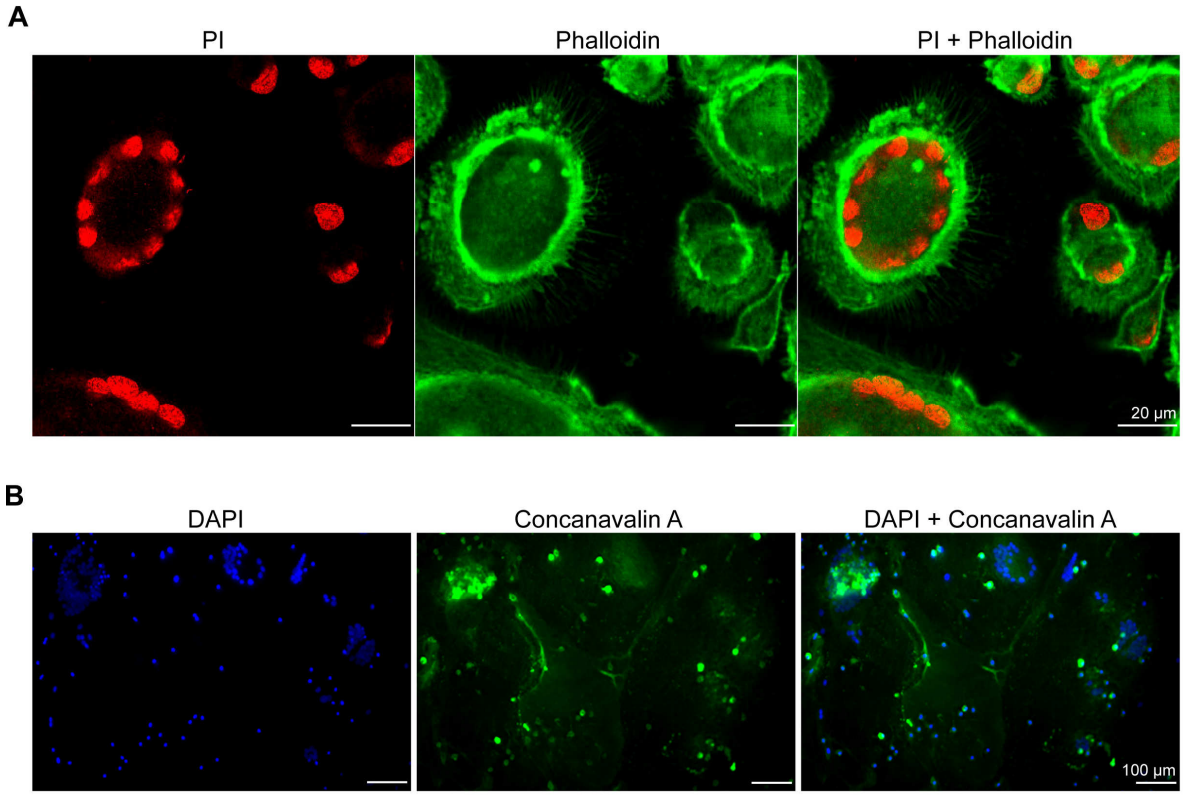

Figure 3.5.: Osteoclast differentiation in cultures stimulated with hsRANKL and M-CSF for 14 days, Phalloidin and Concanavalin A staining. (A) Phalloidin staining of porcine osteoclast showing F-actin rings at the periphery of the cells and podosomes. Green: phalloidin; red: propidium iodide (PI) nuclear counterstaining. (B) Concanavalin A staining of porcine osteoclast showing glycoprotein containing mannose chains on the membrane. Blue: DAPI; green: Concanavalin A.

3.1.4 Morphological characterization of porcine osteoclast generated by psRANKL

To be sure that newly cloned porcine sRANKL is able to induce osteoclasts, the psRANKL cDNA was introduced using a transfection method into porcine osteoclastic precursor cells. Next, the morphology of the osteoclast formed by psRANKL and M-CSF was compared with the morphology of the osteoclast induced by hsRANKL and M-CSF.

As shown in Figure 3.6A, the osteoclasts´ culture generated by hsRANKL + M-CSF had larger osteoclasts, while this generated by psRANKL + M-CSF had small multinucleated cells localized within a dense fibroblast/stromal cell layer. Next, both cell cultures were visualized by Giemsa and TRACP staining (Figure 3.6B and C), showing bigger osteoclast with highly positive TRACP staining for hsRANKL + M-CSF culture, while smaller osteoclasts with low TRACP expression for psRANKL + M-CSF culture. Both cultured cell showed multinuclearity.

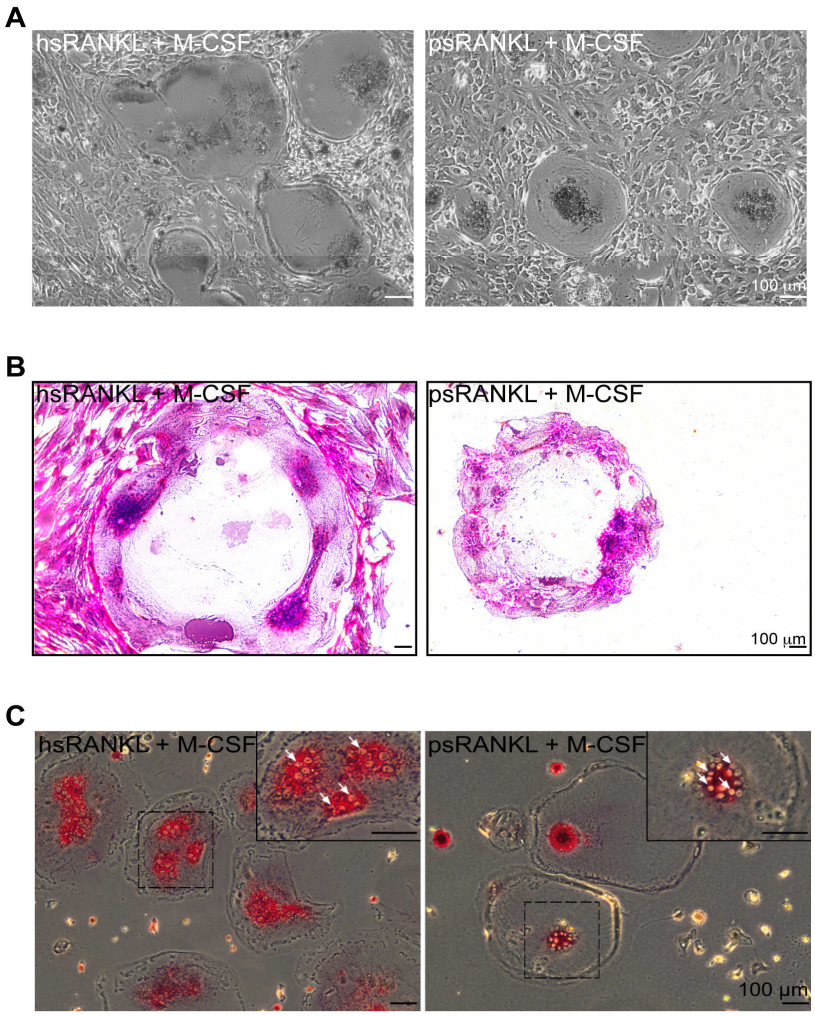

Figure 3.6.: Osteoclast differentiation in cultures transfected with psRANKL and stimulated with hsRANKL and M-CSF for 7 days, their morphology, Giemsa and TRACP staining. (A) Phase-contrast figure showing typical osteoclast morphology. (B) Giemsa staining of porcine osteoclast under light microscopy. (C) TRACP staining of porcine osteoclast under light microscopy. The insets show $TRACP^{+}$ cells in higher magnification. Note the multinuclearity of the cells (white arrows).

To visualized podosomes, both cell cultures were stained for Phalloidin and Concanavalin A (Figure 3.7A and B). The treatment with hsRANKL + M-CSF led to large multinucleated cells with a smooth periphery lined by a band of tightly packed podosomes forming a belt. While treatment with psRANKL + M-CSF led to smaller multinucleated cells showing a periphery line of podosomes.

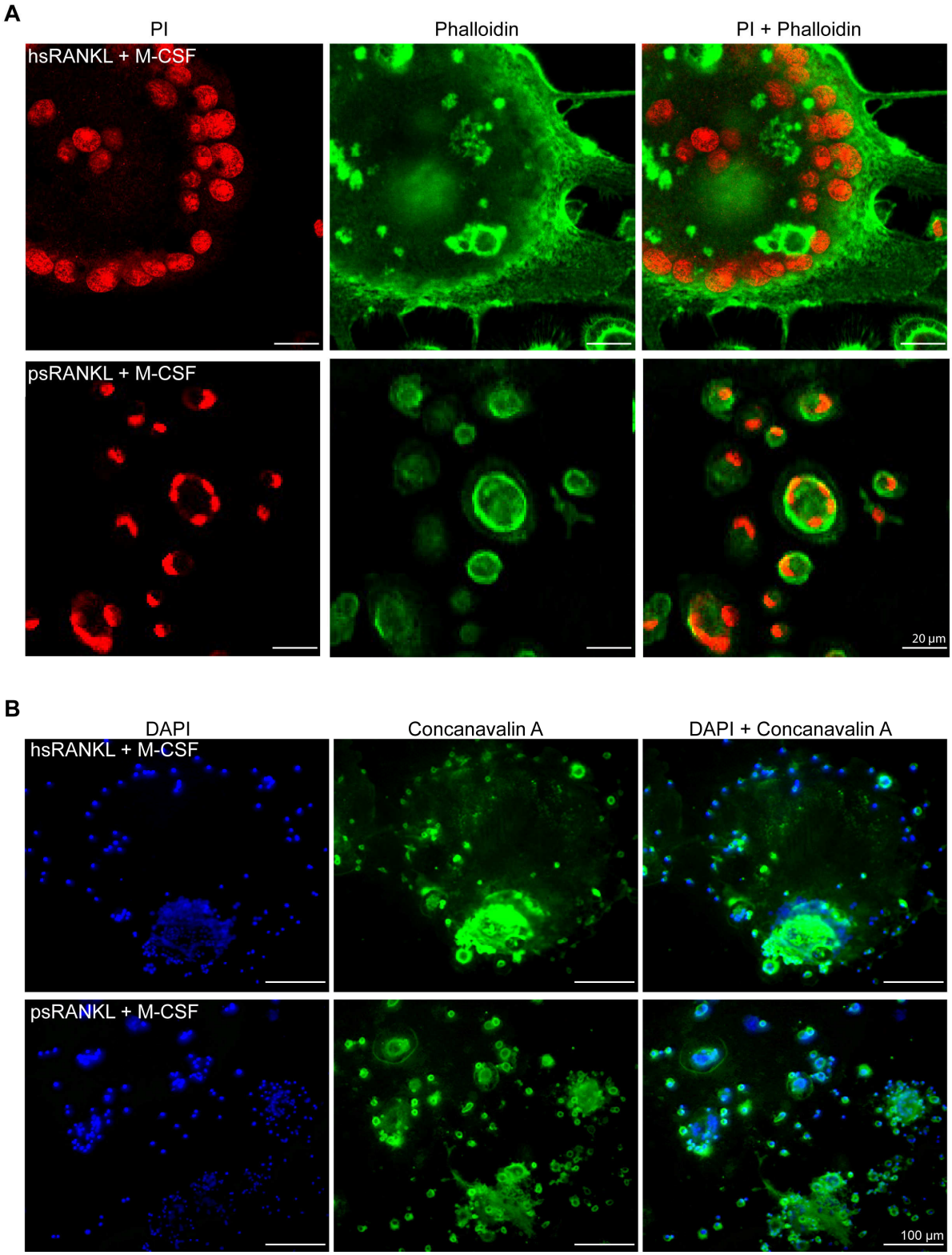

Figure 3.7.: Phalloidin and Concanavalin A staining of the osteoclasts stimulated by transfected psRANKL and by addition of hsRANKL and M-CSF. (A) Phalloidin staining of porcine osteoclast showing F-actin rings at the periphery of the cells and podosomes. Green: phalloidin; red: PI. (B) Concanavalin A staining of porcine osteoclast showing glycoprotein containing mannose chains on the membrane. Blue: DAPI; green: Concanavalin A.

3.1.5 *In vitro* osteoclastogenesis with psRANKL by transfection and bone resorption of psRANKL induced osteoclasts

To continue functional characterization of newly cloned porcine sRANKL, psRANKL was expressed in osteoclastic precursor cells by transfection and tested for its ability to induce osteoclast formation *in vitro*. Given that M-CSF is also implicated in osteoclast formation, cells cultured only in presence of M-CSF (as a negative control) were used to eliminate any possible contamination from M-CSF or endogenous pathway. The cells were also treated with hsRANKL + M-CSF (as a positive control) and cultured in medium without any stimulants (as a null control).

To test if the osteoclastic precursor cells are expressing recombinant psRANKL, on day 3 after transfection, sRANKL ELISA was preformed. The ELISA showed a significant over-expression of psRANKL of about 92 pg/ml in transfected cells compared to the cells cultured in presence with M-CSF which showed very low detectable level of expression (Figure 3.8A). After 7 days of incubation, the osteoclastic precursor cells were stained for TRACP. As shown in Figure 3.8B, the cytochemistral analysis revealed that transfection of osteoclastic precursor cells with psRANKL had the ability to induce the formation of $TRACP^+$ multinuclear cells. M-CSF alone had lesser degree of osteoclast inductivity, whereas hsRANKL + M-CSF had the strong effect on osteoclast inductivity. No $TRACP^+$ cells were observed in cultures when porcine osteoclastic precursor cells were cultured in medium. These results indicate that the recombinant psRANKL is a functional protein. The numbers of $TRACP^+$ multinuclear cells were scored using light microscopy. As shown in Figure 3.8C, introduction of psRANKL induced osteoclasts. The average number of cells per 6-well in the presence of hsRANKL + M-CSF was 25,50, transfected with psRANKL + M-CSF 22,25, in the presence of M-CSF 8 and in medium 1,25, respectively. These results confirm that recombinant psRANKL is able to induce osteoclast in greater number than M-CSF alone.

Next, these cells were tested for expression of the carbonic anhydrase type II (CA-II), another osteoclastic marker that is related to the bone resorbing ability of osteoclasts. Cultures treated with hsRANKL + M-CSF or transfected with psRANKL showed a strong expression of CA-II in RT-PCR. In contrast, M-CSF alone exhibited significaly lower expression levels of CA-II. No basal expression of CA-II was found in cultures incubated with medium alone (Figure 3.8D).

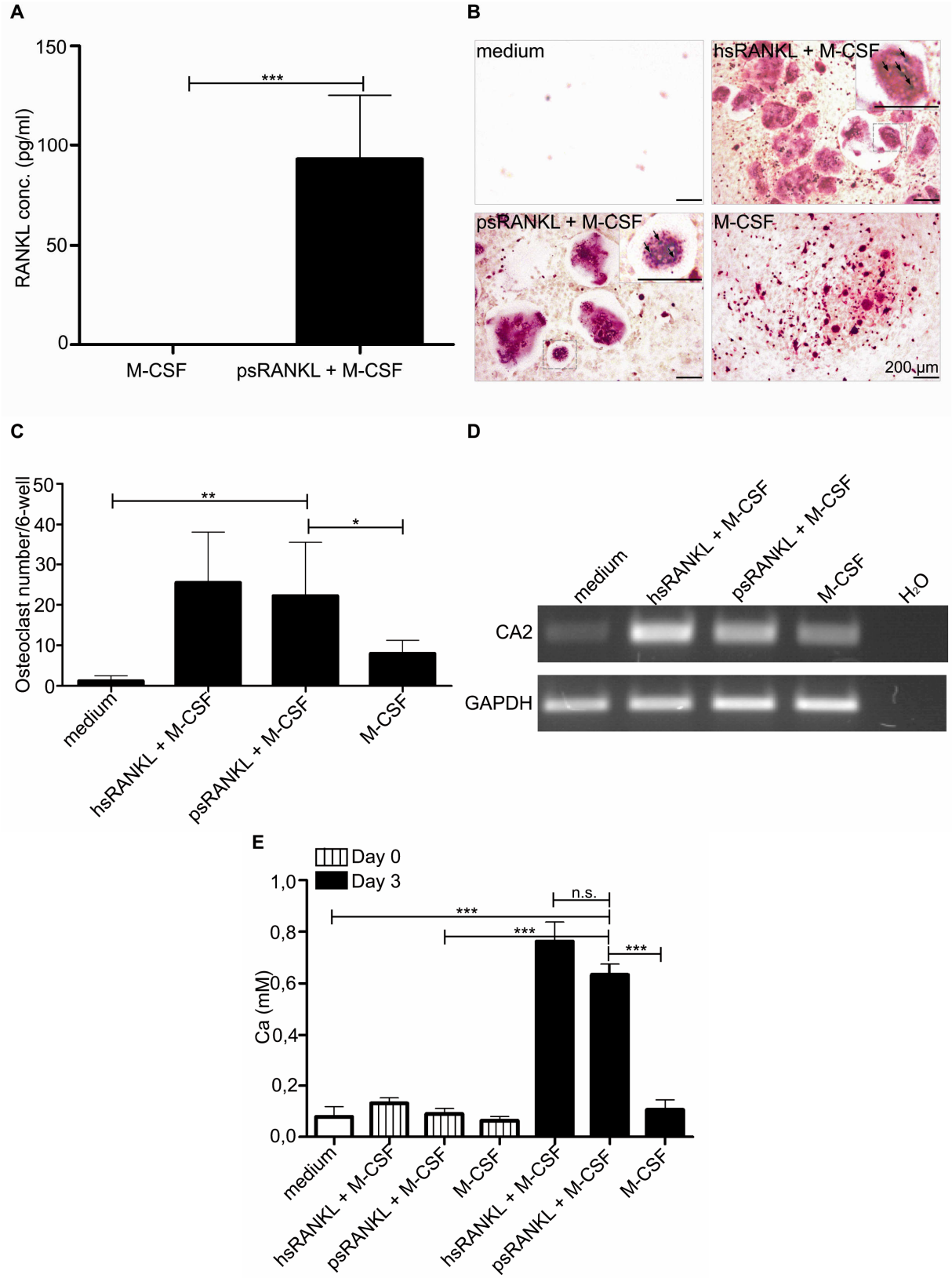

Figure 3.8.: Effects of extracellular domain of psRANKL protein on osteoclastogenesis. Osteoclastic precursor cells were transfected with psRANKL expression plasmid. The untransfected or transfected cells were untreated or treated with hsRANKL + M-CSF and M-CSF alone, respectively and cultured for 3 or 7 days. (A) sRANKL ELISA on day 3 after transfection showing over-expression of psRANKL from transfected cells compared to the untransfected cells cultured in presence with M-CSF alone. (B) Osteoclast began to form at day 3 and the cultures were stained for TRACP activity at day 7. A strong TRACP signal was observed in osteoclast cultured in presence of hsRANKL + M-CSF and transfected with psRANKL, compared to lesser or no $TRACP^+$ cell cultures with M-CSF or media, respectively. A close-up view of $TRACP^+$ cells with several nuclei (black

arrows) is shown. (C) Total number of $TRACP^+$ multinucleated cells was scored after 7 days of incubation. In the cultures treated with hsRANKL + M-CSF and transfected with psRANKL, the higher number of osteoclast was found whereas the less or very low number was found in cultured treated with M-CSF or medium alone, respectively. (D) RT-PCR analysis of CA-II in osteoclasts after 7 days of incubation. Total cellular RNA was extracted from cells cultured in media alone or in media supplemented with M-CSF, hsRANKL + M-CSF and psRANKL + M-CSF and RT-PCR was performed using CA-II-specific primers. The signal could be detected in cultures treated with hsRANKL + M-CSF, psRANKL + M-CSF, and M-CSF alone, while no signal could be found in cultures cultured in medium. (E) Bone resorption assay. Untransfected and transfected precursor cells were plated on bone slices and cultures were untreated or treated with hsRANKL + M-CSF and M-CSF alone for 3 days. After osteoclast formed, the level of calcium released was measured showing that osteoclast cultured with hsRANKL + M-CSF and psRANKL + M-CSF were able to resorb bone, while that cultured in medium or with M-CSF alone were not able.

To determinate the biological effects of psRANKL, osteoclastic precursor cells were plated on bone plate and transfected with recombinant psRANKL. After 3 days, the supernatant was taken for the measurement of calcium levels by CalciFluor assay as a measurement of osteoclast activity (Figure 3.8E). psRANKL increased calcium level (from 0.1 mM to 0.7 mM) compared to cells cultured in presence with M-CSF alone. These results indicated that the cloned region is functionally active and generates bone-resorbing osteoclasts.

3.2 GENERATION OF DOUBLE-TRANSGENIC rtTA-psRANKL PIGS

3.2.1 Establishment of an inducible Tet-On system

A tandem construct containing rtTA under the control of CMV promoter and psRANKL under the tetracycline regulated promoter was cloned. To obtain such construct, first, the target-specific cDNAs were cloned in pLenti6/V5-rtTA-psRANKL (Bla) plasmid by ligation reaction. Next, the Blasticidin selection marker from pLenti6/V5-rtTA-psRANKL (Bla) plasmid was exchanged with floxed Neomycin resistance gene from lox2Neo plasmid (Figure 3.9A). To verify the cloning strategy, a digestion analysis of pLenti6/V5-rtTA-psRANKL (Neo) was performed by cutting out the insert from the backbone (Figure 3.9B). The correct clone was also sequenced to verify the quality of the rtTA, TRE and psRANKL sequences. Finally, the plasmid pLenti6/V5-rtTA-psRANKL (Neo) was linearized by PvuI digestion. This linearized fragment, having the proper digestion pattern and correct sequence was used for the next step – electroporation into porcine fetal fibroblast.

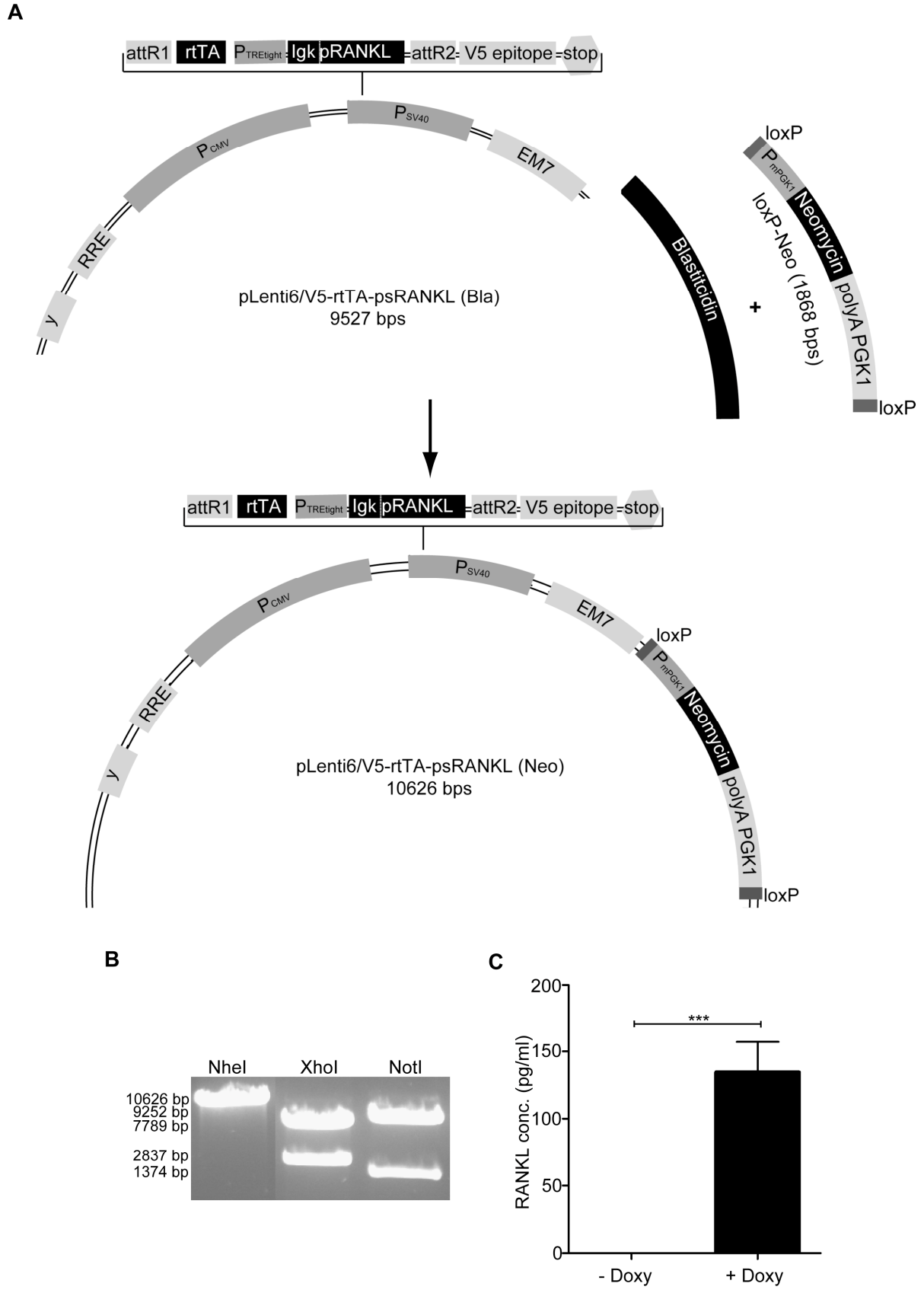

Figure 3.9.: Establishment of inducible system, its cloning and functional analysis. (A) A schematic representation of the cloning steps of pLenti6/V5-rtTA-psRANKL (Neo) plasmid. (B) Digestion results of pLenti6/V5-rtTA-psRANKL (Neo) plasmid with different restriction enzymes showing correct digestion pattern. (C) sRANKL ELISA. 293FT cells were transfected with linearized pLenti6/V5-rtTA-psRANKL (Neo) plasmid and stimulated or unstimulated with Doxycycline. After 3 days of stimulation, supernatants from stimulated and

unstimuled cells were collected and used to assay psRANKL induction. The induction of psRANKL was found in transfected and stimulated cells compared to unstimuled ones.

To verify if the pLenti6/V5-rtTA-psRANKL (Neo) plasmid is inducible, the 293FT cells were transfected and stimulated with Doxycycline for 3 days. A sRANKL ELISA of supernatant from transfected cell stimulated and unstimulated, respectively, was performed. The result showed high inducibility of the psRANKL upon stimulation with Doxycycline and no background signal without stimulant (Figure 3.9C).

3.2.2 Molecular characterization of double-transgenic porcine fetuses

With the goal to generate transgenic pigs, the pFFs were electroporated with pLenti6/V5-rtTA-psRANKL (Neo) plasmid, which were finally used for nuclear transfer. After embryo transfer and 40 days of gestation, 3 porcine fetuses were obtained (Figure 3.10A). All fetuses had good organ developed according to the period of gestation.

When DNA was isolated from ear fibroblast from all three fetuses and hybridized with radiolabeled rtTA and psRANKL DNA, hybridizing bands were seen, demonstrating the presence of genes in two transgenic animals, fetus 2 and 3. Fetus 1 didn't show the integration of the rtTA or/and psRANKL into its genome (Figure 3.10B). In this respect, the fetus 1 is used as isogenetic control for further experiments. To compare which one of the two transgenic fetuses are expressing rtTA and psRANKL at higher level, the ear fibroblasts and tissues like heart, liver, brain and bone, were used for further characterization on mRNA and protein level (only cells).

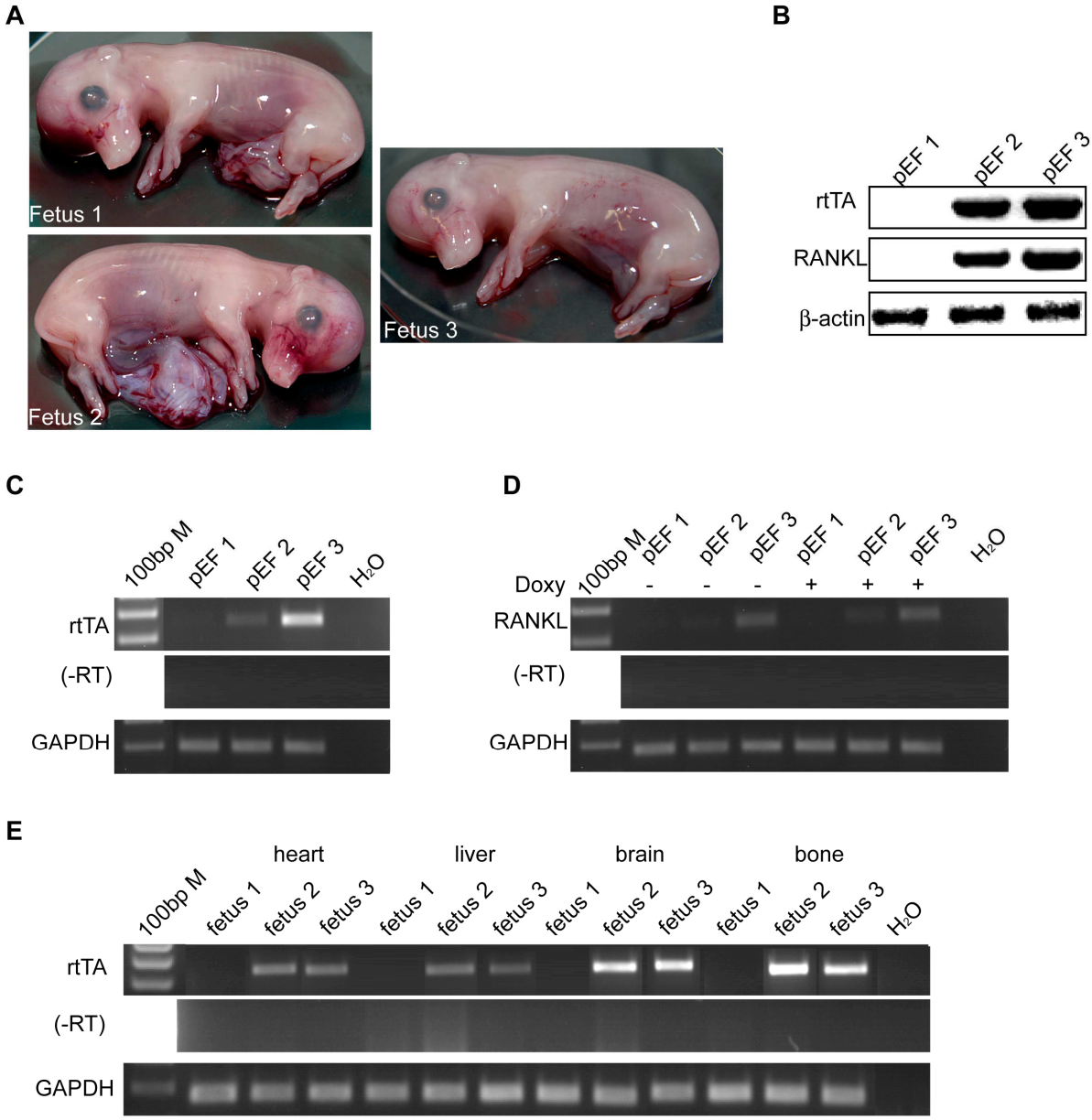

Figure 3.10.: Molecular characterization of double transgenic rtTA-psRANKL porcine fetuses 1, 2 and 3 on genomic and mRNA level. (A) Figure showing three obtained fetuses at 40 days of gestation. Figures were kindly provided by Eleonore Schilling (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). (B) Genomic analysis of the ear fibroblast isolated from three fetuses. Fetus 2 and 3 are transgenic for both genes while fetus 1 in not transgenic. Figure is kindly provided by Nikolai Klymiuk (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). (C) RT-PCR analysis of rtTA mRNA from ear fibroblast from all three fetuses. PCR results showed an over-expression of rtTA mRNA in fetus 2 and 3 but not in fetus 1. (D) RT-PCR analysis of psRANKL mRNA from ear fibroblast from all three fetuses after Doxycycline stimulation. No psRANKL expression was found in cell from fetus 1 and 2, while fetus 3 showed induction psRANKL mRNA. (E) RT-PCR analysis of rtTA expression in heart, liver, brain and bone isolated from three fetuses. No rtTA expression was found in any organs from fetus 1, while fetus 2 and 3 showed lower expression of rtTA in heart and liver and higher expression in brain and bone. In (C), (D) and (E) –RT is indicating a PCR performed on RNA probes to exclude the potential contamination from cloned cDNA sequence.

Using mRNA isolated from ear fibroblast, the rtTA expression was verified. As expected fetus 1 didn't show any expression of rtTA mRNA, the fetus 2 showed weak rtTA expression, while fetus 3 showed stronger expression of rtTA (Figure 3.10C). No contamination of cDNA was evident in all probes. To test the inducibility of the psRANKL mRNA, the ear fibroblasts

were stimulated with Doxycycline for 3 days. This experiment showed that only the fetus 3 has an expression of psRANKL mRNA in uninduced and induced state (Figure 3.10D). In both transgenic fetuses the expression of rtTA on mRNA in different tissues was detectable while as expected fetus 1 didn't show any signal of expression (Figure 3.10E). The expression was stronger in brain and bone and weaker in heart and liver from both transgenic fetuses. These results are summarized in table 3.1.

		rtTA	psRANKL
FETUS 1	pFF	-	-
	pFF + Doxy	n.d.	-
	Heart	-	n.d.
	Liver	-	n.d.
	Brain	-	n.d.
	Bone	-	n.d.
FETUS 2	pFF	+	-
	pFF + Doxy	n.d.	-
	Heart	+	n.d.
	Liver	+	n.d.
	Brain	++	n.d.
	Bone	++	n.d.
FETUS 3	pFF	++	++
	pFF + Doxy	n.d.	++
	Heart	+	n.d.
	Liver	+	n.d.
	Brain	++	n.d.
	Bone	++	n.d.

Table 3.1.: Summary of expressional results of double transgenic rtTA-psRANKL porcine fetuses 1, 2 and 3 on mRNA level. -: no expression; +: good expression; ++: strong expression; n.d.: not determinated.

The expression of rtTA in the cells was also analyzed on protein level by two immunostaining procedures. Immunofluorescence staining as well DAB-staining showed low rtTA expression in ear fibroblast of fetus 2 and high expression in fetus 3, while no expression in fetus 1 (Figure 3.11 A and B).

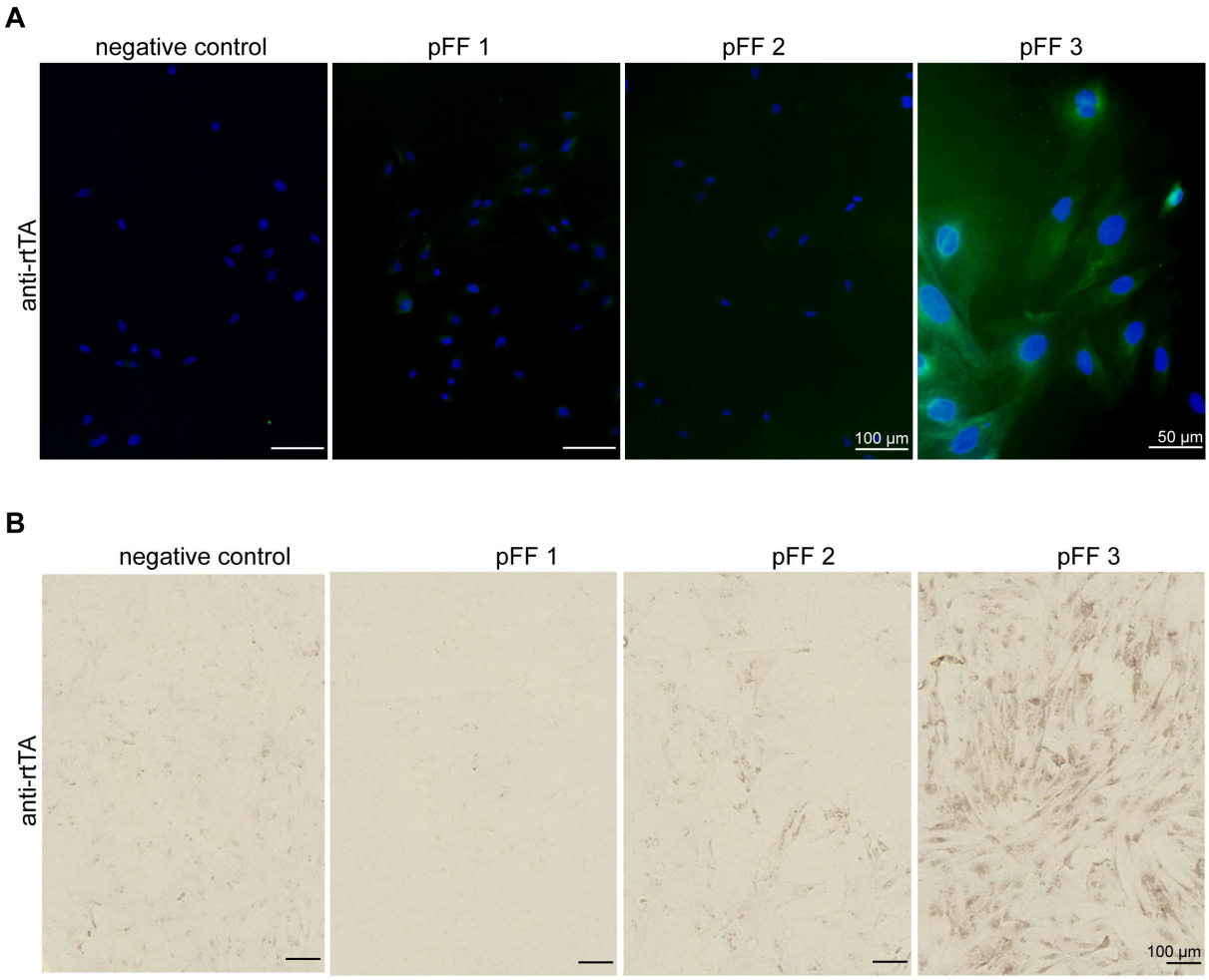

Figure 3.11.: Immunocytochemistral analysis of over-expression of rtTA protein in the fibroblasts of three fetuses. (A) Immunofluorescence staining of the cells. The negative controls without primary anti-rtTA-antibody and the pFF 1 show hardly fluorescence signal. A strong Alexa Fluor 488 signal (green) in two transgenic pFFs indicates an expression of rtTA. Green: rtTA staining; blue: DAPI. (B) DAB-immunostaining of the cells. A strong positive signal indicated by the brown color was detected in pFF 3 cells, low signal in pFF 2, while there was very low background signal in pFF 1 cells.

3.2.3 Generation of double transgenic piglets from the cells isolated from fetus 3

The ear fibroblasts from the fetus 3 were further used for a re-cloning of the animals, i.e. in a new nuclear and embryo transfer, wherefrom, 2 piglets (piglets 9878 and 9879) were originated. Unfortunately, both piglets were born dead (Figure 3.12A). From the piglet 9878, ear fibroblast and bone marrow cells (pBM) could be isolated, while from the piglet 9879 only bone marrow cells (Figure 3.12B). These cells and tissues (heart, lung, spleen, liver, kidney, muscle, tail and skin) were used for further expressional analysis.

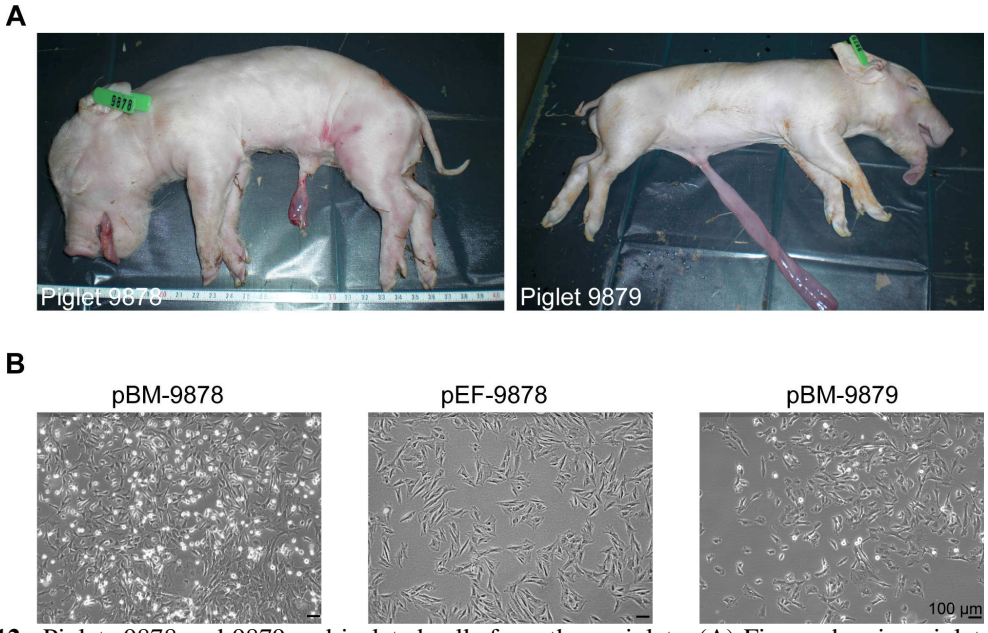

Figure 3.12.: Piglets 9878 and 9879 and isolated cells from these piglets. (A) Figure showing piglets 9878 and 9879 after birth. Both piglets were born. Figures are kindly provided by Eleonore Schilling (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). (B) Isolated cells from both piglets. From piglet 9878 bone marrow and ear fibroblast could be isolated, while from piglet 9879 only bone marrow cells.

3.2.4 Molecular characterization of double transgenic piglets 9878 and 9879 originated from fetus 3

Expression analysis started with RNA isolation from different tissues from both piglets. As shown in Figure 3.13A, the RNA quality was good for lung and spleen of both fetuses showing the 2 bands of 18 and 26S. While the RNA isolated from heart, liver, kidney, muscle, tail and skin from both piglets was degraded.

Nevertheless, rtTA RT-PCR and RNA-control PCR on tissues from both piglets were performed (Figure 3.13B). Even though, the rtTA over-expression could be proved in all tissues from both fetuses compared to the wild type animal, the rtTA could be also seen in a RNA control PCR, demonstrating cDNA contamination in RNA probes.

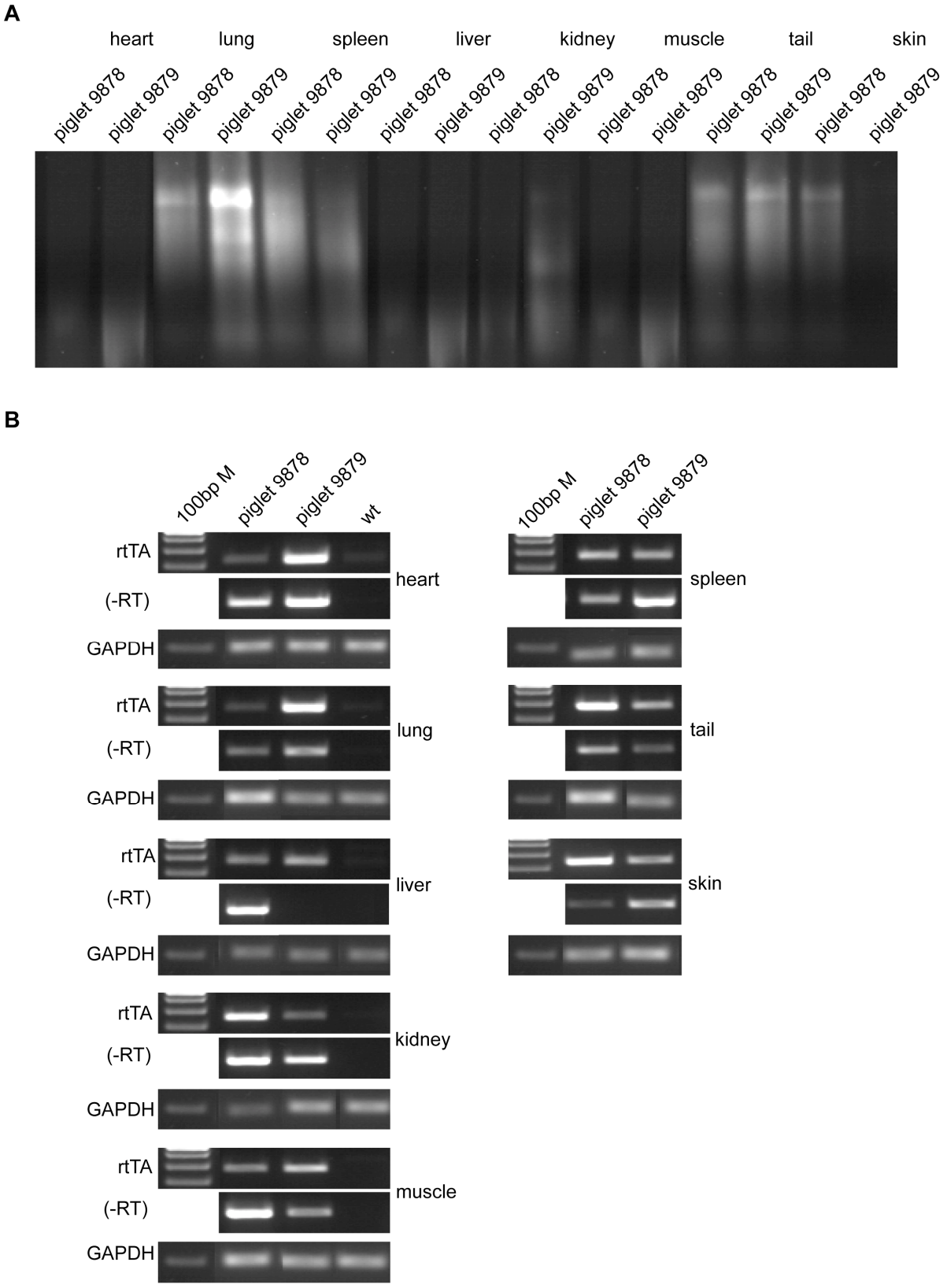

Figure 3.13.: Molecular characterization of double transgenic rtTA-psRANKL porcine fetuses 9878 and 9879 ´s tissues on mRNA level. (A) RNA quality control gel showing good RNA quality from lung and spleen from both piglets and degraded RNA from heart, liver, kidney, muscle, tail and skin of both piglets. (B) RT-PCR and RNA-control-PCR analysis of rtTA expression in heart, lung, spleen, liver, kidney, muscle, tail and skin isolated from two piglets. Since there was also a band in the without reverse transcription PCR, genomic DNA contamination could not be excluded.

To avoid the problem of the contaminated RNA, the RNA was isolated from the cell culture. The isolation of RNA from ear fibroblast and bone marrow cell showed good RNA quality. Next, the cells from both piglets were stimulated with Doxycycline for 3 days and tested for rtTA and psRANKL expression. rtTA RT-PCR showed a clearly over-expression of the transactivator in each cell type, showing stronger expression in pBM-9879 and weaker in pBM-9878 and pEF-9878. No cDNA contamination was obvious. The induction of the psRANKL mRNA can be shown only in the pEF-9878, while pEF-9879 and pBM-9878 didn't show any psRANKL mRNA induction, rather basal psRANKL expression. In this case also the cDNA contamination could not be demonstrated (Figure 3.14).

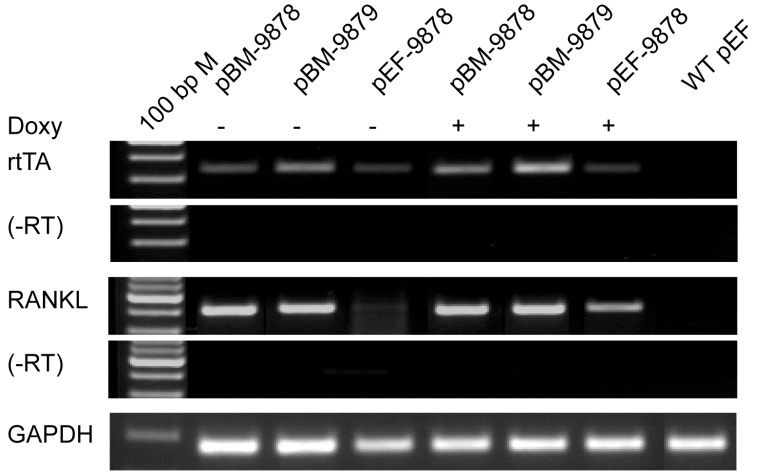

Figure 3.14.: Molecular characterization of double transgenic rtTA-psRANKL porcine fetuses 9878 and 9879´s cells on mRNA level. RT-PCR and RNA-control-PCR (-RT) analysis of rtTA and psRANKL expression in isolated cells from two piglets. Both piglets showed over-expression of rtTA in every cell analyzed. The rtTA band could not be seen in RNA-control-PCR. After 3 days of Doxycycline stimulation, the induction of the psRANKL mRNA could be showed only in the pEF-9878, while pEF-9879 and pBM-9878 didn't show any psRANKL mRNA induction. No cDNA contamination could be demonstrated.

3.3 GENERATION OF rtTA TRANSGENIC PIGS AND KIDNEY CELL LINE FROM rtTA-PIGLET 9894

Initially, the expression vector CAG-rtTA (Supplementary figure 6) was cloned containing rtTA cDNA under the control of constitutive chicken β-actin promoter. Another characteristic of this vector is the floxed and removable Neomycin resistance cassette, meaning that after the electroporation and selection over 2 passages, a stable transfected cell clone could be obtained. Cloning of this plasmid was performed by Nikolai Klymiuk (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf).

After electroporation of the porcine fetal fibroblast with the CAG-rtTA vector, the cells were selected using Neomycin and used for nuclear and embryo transfer. After the pregnancy of 117 days, 5 piglets (piglet 9890, 9891, 9892, 9894 and 9895) were born (Figure 3.15A). The piglet 9890 died soon after the birth. Piglet 9894 was euthanized within the second day due to

poor vitality, but a full set of tissue samples was preserved. The remaining three piglets (piglet 9891, 9892 and 9895, Figure 3.15B) were clinically healthy and developed normally but were euthanized at the age of 4 weeks to preserve a comprehensive set of tissues (heart, lung, spleen, live, kidney, muscle and skin) for genotyping as well as for transgene expression studies on the RNA and protein level. In addition primary cell cultures were established from the kidneys to serve as nuclear donor cells for second round SCNT experiments.

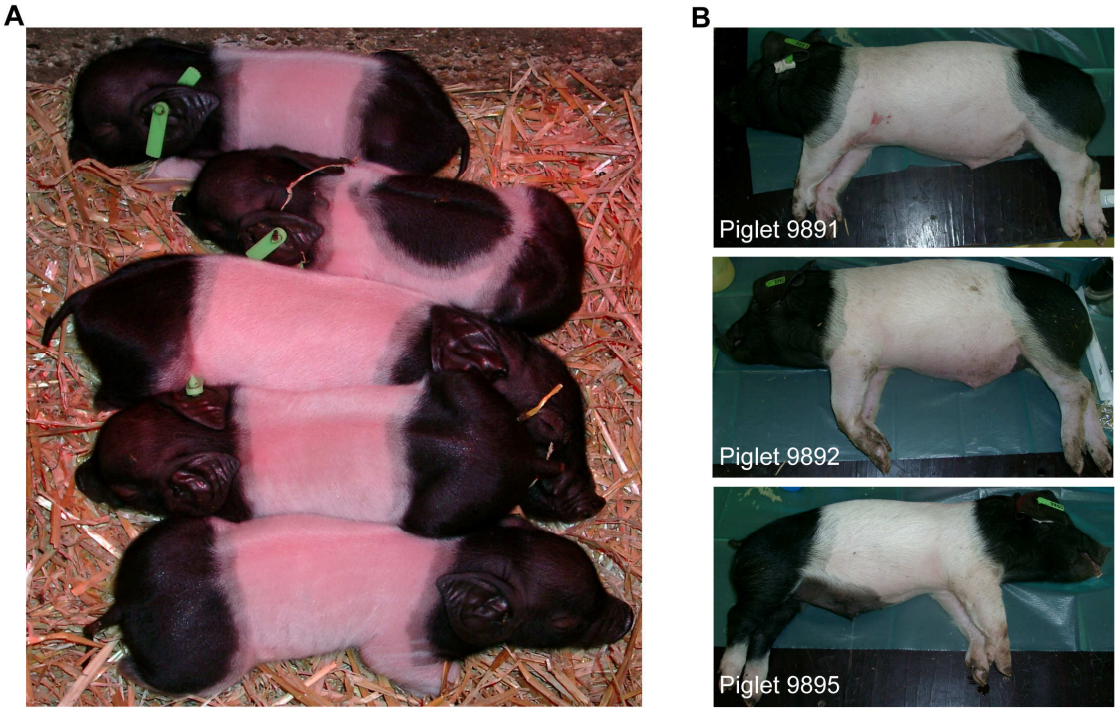

Figure 3.15.: rtTA-transgenic piglets. (A) Figure showing 5 living piglets 9890, 9891, 9892, 9894 and 9895 after birth. (B) Piglets 9891, 9892 and 9895 were sacrificed 28 days after the birth. Both figures are kindly provided by Eleonore Schilling (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf).

The expression analysis of the rtTA began with RNA isolation from different tissues of all piglets. The quality of RNA was good from all samples (Figure 3.16A). Next, the over-expression of rtTA on mRNA level was tested in those tissues. RT-PCR analysis revealed ubiquitous rtTA-specific RNA expression in all five founder animals. Precisely, in heart, liver, kidney, spleen and skin strong differences could have not be seen in expressional pattern between the animals, while the piglet 9895 showed lower expression in lung and muscle and piglet 9892 in muscle. Appropriate negative controls (wild type animal) gave the predicted results, as well as the RNA-control PCR (Figure 3.16B).

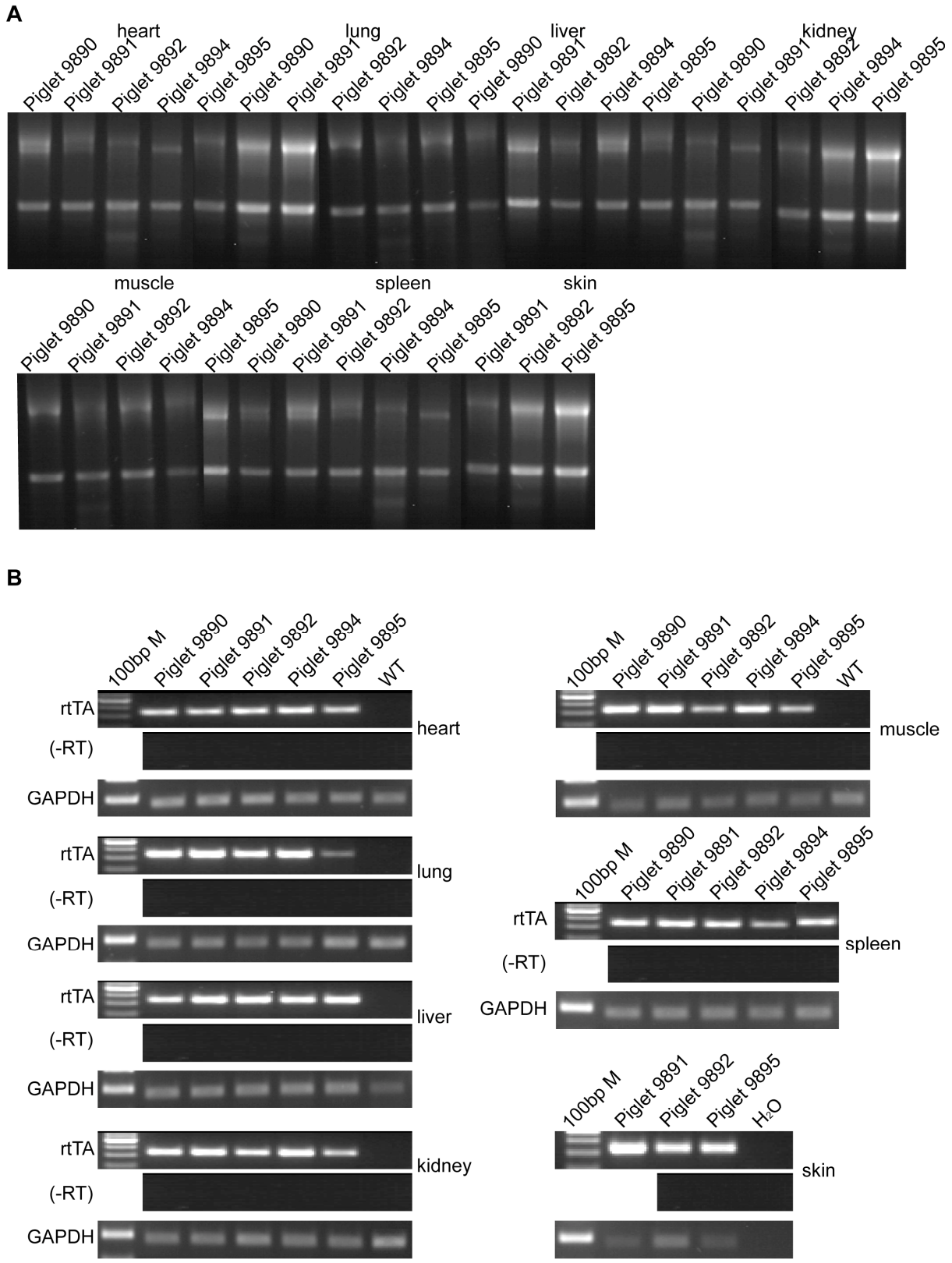

Figure 3.16.: Molecular characterization of rtTA-transgenic piglets 9890, 9891, 9892, 9894 and 9895´s tissues on mRNA level. (A) RNA quality control gel from the tissues showing good RNA quality from all piglets. (B) RT-PCR and RNA-control-PCR (-RT) analysis of rtTA expression of obtained organs from five piglets. All animals showed over-expression of rtTA in every tissue analyzed. The rtTA band could not be seen in RNA-control-PCR.

To confirm over-expression on a protein level, a Western blot analysis on muscle, liver and lung was performed. As evidenced by SDS-PAGE, piglet 9890 and 9894 showed a band of approximately 27 kDa in muscle (Figure 3.17A), which meets the size of the rtTA protein. All animals showed a protein band in liver and only piglet 9891 and 9894 showed it in lung (Figure 3.17B and C). The corresponding negative (wild type animal) and positive (pFF-rtTA) controls gave predicted results.

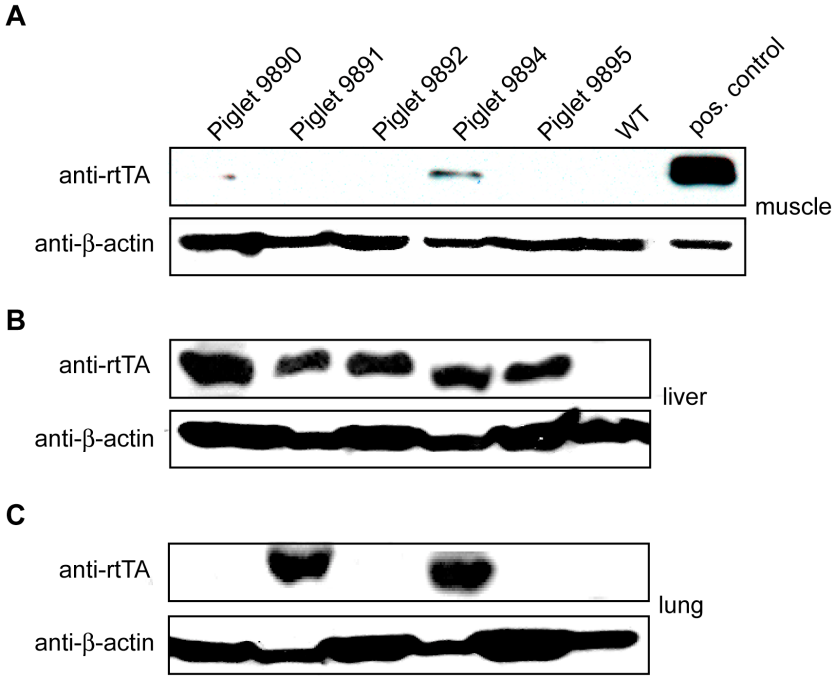

Figure 3.17.: Molecular characterization of rtTA-transgenic piglets 9890, 9891, 9892, 9894 and 9895´s tissues on protein level. Total protein from each of the transgenic and wild type piglets and rtTA-pFF was used for Western blot analysis. A strong expression can be found on protein level in positive control (rtTA-pFF), in muscle (A) of piglet 9890 and 9894, in liver (B) of all transgenic animals and in lung (C) of 9891 and 9894 piglets, while there was no visible expression of rtTA in wild type animal. The blots were stripped and reprobed with β-actin to normalize for the amount of loaded protein.

Additionally, the kidney cells from all transgenic piglets were isolated and analysed for rtTA over-expression. The RT-PCR analysis showed clearly over-expression of rtTA mRNA in all cell lines. No cDNA contamination was present (Figure 3.18A). On protein level, a corresponding band of 27 kDa, can be showed in each kidney cells from all piglets, while wild type didn't show any band (Figure 3.18B).

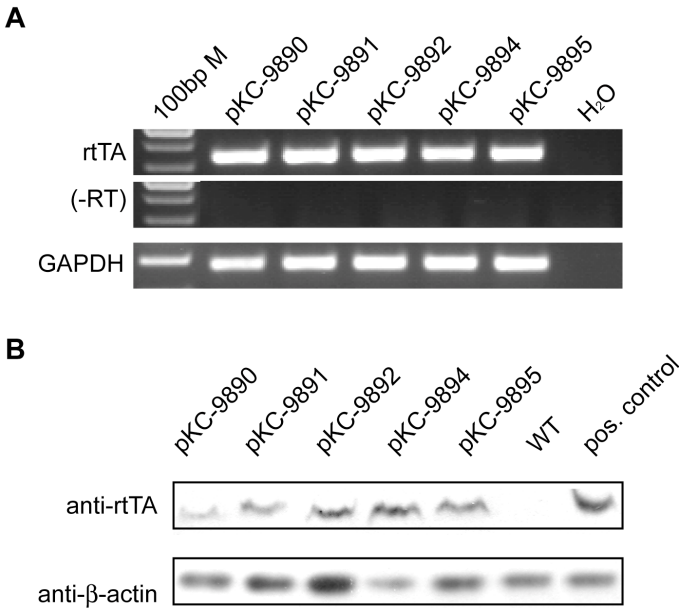

Figure 3.18.: Molecular characterization of rtTA-transgenic piglets 9890, 9891, 9892, 9894 and 9895´s kidney cells on mRNA and protein level. (A) RT-PCR and RNA-control-PCR (-RT) analysis of rtTA expression of isolated kidney cells from five piglets. All animals showed strong over-expression of rtTA in analyzed cells. The rtTA band could not be seen in RNA-control-PCR. (B) Total protein from each of the transgenic kidney cells and wild type pKC was used for Western blot analysis. A strong expression can be found on protein level in kidney cells of all piglets, while there was no visible expression of rtTA in wild type cells. The blots were stripped and reprobed with β-actin to normalize for the amount of loaded protein.

3.3.1 Electroporation of kidney cells from rtTA-piglets with TRE-psRANKL vector and its characterization

To confirm that rtTA expressed in transgenic pigs is functionally active, primary kidney cells from all founders were nucleofected with a TRE-controlled expression vector for porcine sRANKL (Supplementary figure 7). The cloning of this vector was performed by Nikolai Klymiuk (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). After generation of stable transfected cell clones, cells were exposed to Doxycycline (1 µg/ml) for 72 h.

First, the rtTA over-expression was analyzed in kidney cells. The kidney cells from all animals showed an over-expression of rtTA on mRNA level. Next, the induction of psRANKL expression was analyzed and the RT-PCR showed an induction of psRANKL in kidney cells from piglet 9890 and 9894, even though the kidney cells from piglet 9894 showed stronger induction then the cells from 9890 piglet. Both porcine cell lines didn't show any basal expression. The cells from piglet 9891, 9892 and 9895 didn't show an induction or basal expression of psRANKL mRNA. No cDNA contamination could be demonstrated in each kidney cells (Figure 3.19A). To confirm these results, the Western blot analysis was performed using kidney cells and supernatant. As shown in Figure 3.19B, an induction of psRANKL can be seen in cells from piglet 9890, 9891 and 9894, while only in the

supernatant from piglet 9894 the rtTA protein band can be seen. The cells from piglet 9892 and 9895 didn't show an inducibility of psRANKL protein. Finally, culture supernatants were harvested before and after Doxycycline stimulation and analyzed by specific ELISA for the presence of sRANKL. Non-stimulated TRE-psRANKL nucleofected cells from rtTA transgenic founders did not secrete detectable amounts of sRANKL into the culture medium, however, a marked increase in sRANKL secretion was observed after Doxycycline stimulation of cells from founders 9890, 9891 and 9894 (Figure 3.19C).

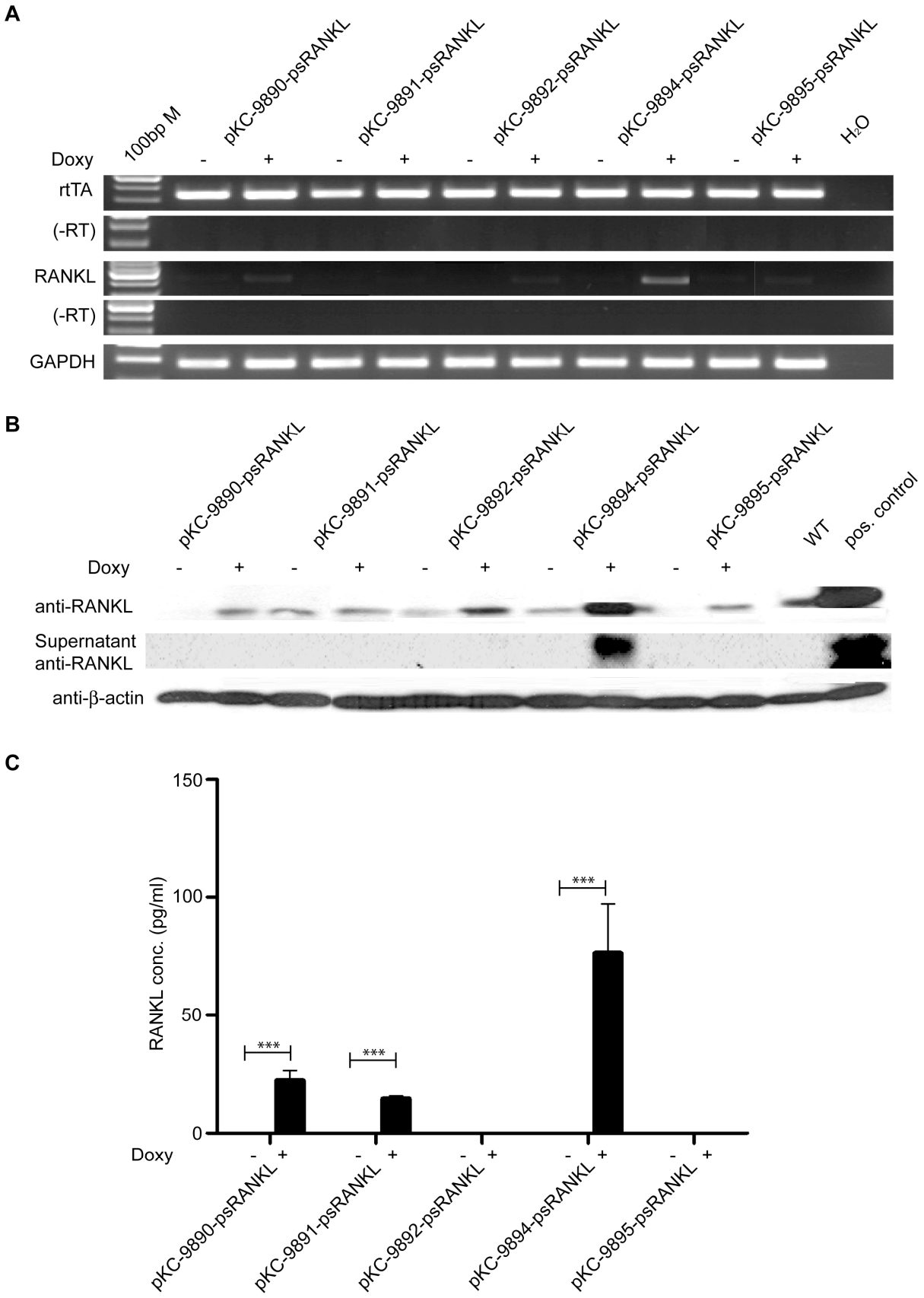

Figure 3.19.: Molecular characterization of rtTA-transgenic piglets 9890, 9891, 9892, 9894 and 9895´s kidney cells electroporated with TRE-psRANKL construct on mRNA and protein level. (A) RT-PCR and RNA-control-PCR (-RT) analysis of rtTA and psRANKL expression of isolated kidney cells from five piglets and subsequently electroporated with TRE-psRANKL vector. All cell lines showed strong over-expression of rtTA. The psRANKL RT-PCR showed an induction of psRANKL upon Doxycycline stimulation in kidney cells from piglet 9890 and 9894. Both porcine cell lines didn't show any basal expression. The cells from piglet 9891, 9892

and 9895 didn't show an induction or basal expression of psRANKL mRNA. The rtTA and psRANKL band can not be seen in RNA-control-PCR. (B) Total protein and supernatant from isolated kidney cells from five piglets and subsequently electroporated with TRE-psRANKL vector, wild type and psRANKL-pFF was used for Western blot analysis upon Doxycycline stimulation. An induction of psRANKL can be seen in cells from piglet 9890, 9891 and 9894, while only in the supernatant from piglet 9894 the rtTA protein band can be seen. The cells from piglet 9892 and 9895 didn't show an inducibility of psRANKL protein. The blots were stripped and reprobed with β-actin to normalize for the amount of loaded protein. (C) sRANKL ELISA of the supernatant collected from isolated kidney cells from five piglets and subsequently electroporated with TRE-psRANKL vector upon Doxycycline stimulation for 3 days. A strong secretion can be found in kidney cells of piglet 9894, 9891 and 9892 while in all other cells was no secretion of psRANKL.

3.4 GENERATION OF rtTA-psRANKL TRANSGENIC PIGS

Based on the results obtained above, the kidney cells from rtTA-TRE-psRANKL-piglet 9894 were then used for SCNT. After the pregnancy, 4 piglets were born, piglets 9958, 9959, 9961 and 9962. The piglets 9958 was born dead, piglet 9962 died 2 days after the birth (Figure 3.20A), while piglets 9959 and 9961 are still alive (Figure 3.20B). From all piglets the ear fibroblasts were isolated (Figure 3.20C) and used for expression analysis.

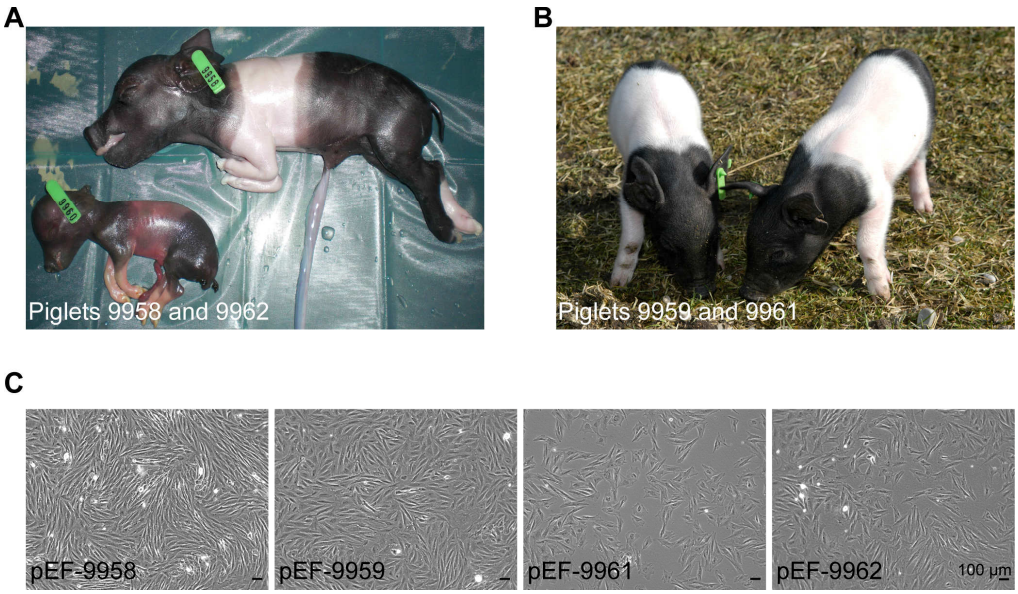

Figure 3.20.: rtTA-psRANKL transgenic piglets after birth and their cells. (A) In this figure, 2 dead piglets 9958 and 9962 are shown after birth. Figure is kindly provided by Eleonore Schilling (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). (B) Figure showing 2 live piglets 9959 and 9961. Figure is kindly provided by Eleonore Schilling (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). (C) Isolated ear fibroblasts from four piglets.

The ear fibroblasts isolated from four obtained piglets were used for expressional analysis. First, to test if the TRE-psRANKL construct was integrated into the genome, the ear fibroblasts were used in genomic PCR analysis which showed that piglets 9958, 9961 and 9962 were transgenic for psRANKL, while piglet 9959 not (Figure 3.21A). Next, the cells were used in a rtTA RT-PCR that showed that cells from all piglets are over-expressing transactivator (Figure 3.21B). No cDNA contamination can be demonstrated. Furthermore, the cells were stimulated with Doxycycline for 3 days to test the induction of psRANKL and

analyzed with PCR and Western blot. Unfortunately, no psRANKL induction could be seen on mRNA level as well as on protein level (Figure 3.21B and C).

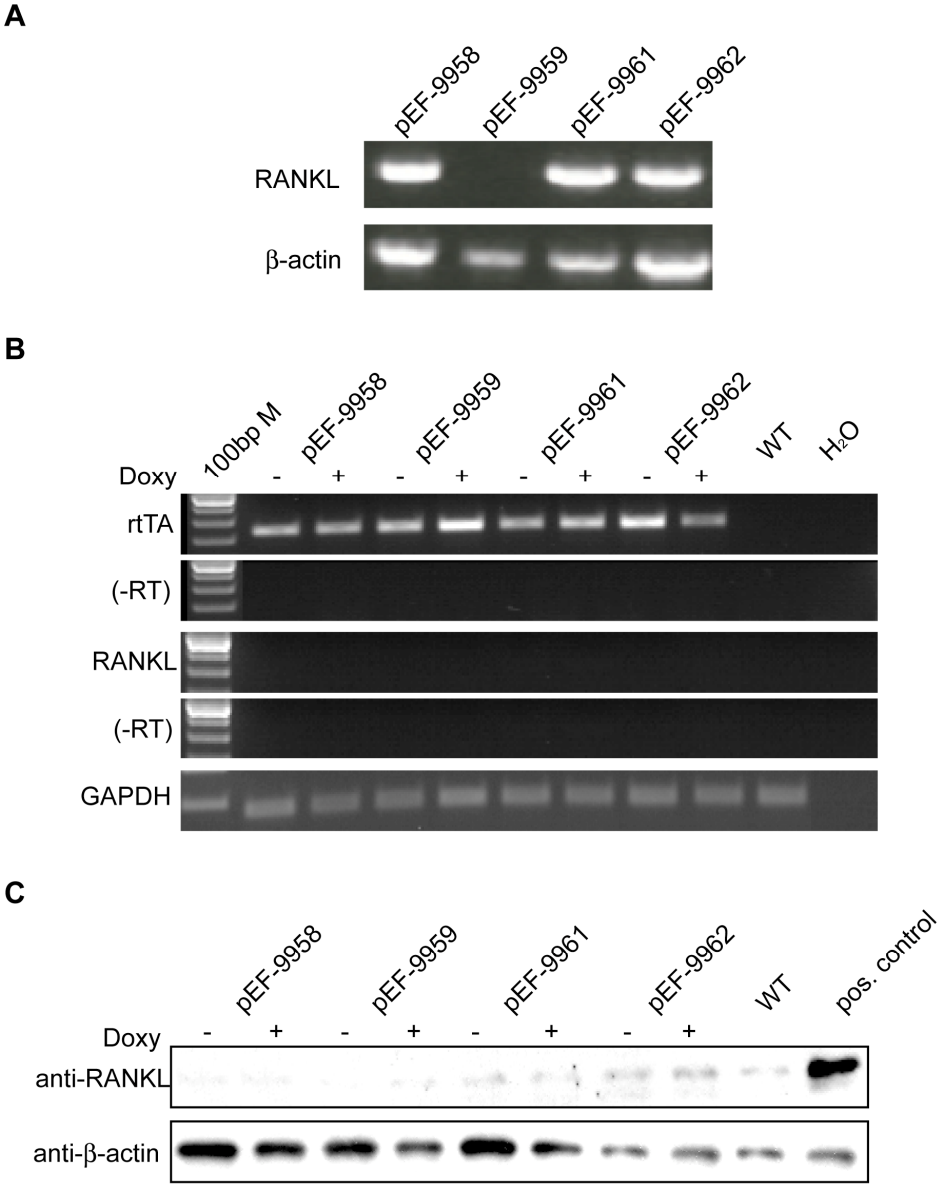

Figure 3.21.: Molecular characterization of rtTA-psRANKL-transgenic piglets 9958, 9959, 9961 and 9962´s ear fibroblasts on genomic, mRNA and protein level. (A) Genomic PCR of the ear fibroblast isolated from four piglets. Piglets 9958, 9961 and 9962 are transgenic for psRANKL gene, while piglet 9959 is not transgenic. The figure is kindly provided by Nikolai Klymiuk (Institute of molecular animal breeding and biotechnology, Prof. Eckhard Wolf). (B) RT-PCR and RNA-control-PCR analysis of rtTA and psRANKL expression of isolated ear fibroblasts from four piglets. All animals showed strong over-expression of rtTA in analyzed cells. The psRANKL RT-PCR didn't show an induction of psRANKL upon Doxycycline stimulation in the cells from all piglets. No psRANKL basal expression can be shown in all piglets. The rtTA and psRANKL band can not be seen in RNA-control-PCR. (C) Total protein from isolated cells from four piglets, wild type and psRANKL-pFF was used for Western blot analysis upon Doxycycline stimulation. An induction of psRANKL can not be seen in any cells. The cells from piglets 9961 and 9962 are showing basal psRANKL protein expression as seen in a wild type cells. The blots were stripped and reprobed with β-actin to normalize for the amount of loaded protein.

3.4.1 *In vivo* Doxycycline stimulation of piglets 9959 and 9961

The surviving founder animals 9959 (control, only rtTA transgene) and 9961 (rtTA and psRANKL transgenes) were examined for induction of the transgene *in vivo*. A first Doxycyline stimulation experiment was performed at age of 10 weeks with a dose of 3.2 and 6.3 mg/kg body weight for 10 days in transgenic boar 9961 and a wild-type control 9959. The induction of RANKL was investigated in animals at day 0, 3, 6 and 10, using ELISA for sRANKL, while bone resorption was studied by ELISA for Cathepsin K and Crosslaps.

As showed in Figure 3.22A, systematic application of Doxycycline was able to increase significantly psRANKL plasma levels in double transgenic piglet 9961, while control piglet 9959 didn't show an induction of psRANKL during stimulation. This increase of circulating psRANKL was already seen 3 days after onset of stimulation. When the Doxycycline concentration was doubled, the concentration of psRANKL was also doubled. Interestingly, increased plasma levels of psRANKL were followed by an increase in serum Cathepsin K concentrations, a marker of stimulated osteoclast activity (Figure 3.22B). This level of Cathepsin K in the transgenic piglet 9961 is decreasing with increasing Doxycycline concentration, while the level in the control animal 9959 remained at the same level during the whole experimental period. The expression of CTX did not show differences in two animals (Figure 3.22C).

For determination of dose-dependent psRANKL induction, we started a second stimulation experiment in 5-month-old animals, using four different doses of Doxycyline (6.3, 12.5, 25 and 50 mg/kg body weight per day) for 10 days each in 9961 piglet and a wild-type control. Blood samples were taken from the jugular vein at day 3, 6 and 10 for each dose of Doxycycline and RANKL concentrations in plasma were quantified by specific ELISA. A Doxy dose-dependent increase in RANKL concentration in plasma up to >150 pg/ml was revealed for the transgenic pig, whereas plasma RANKL concentrations in the equally treated control pig ranged around the detection limit of the ELISA (Figure 3.23A). As the ELISA was not able to discriminate between transgenic and endogenous RANKL, we tested the concentration of the protein in plasma from 10 wild-type pigs of similar age. In all samples RANKL concentrations were around the detection limit of the ELISA (Figure 3.23B).

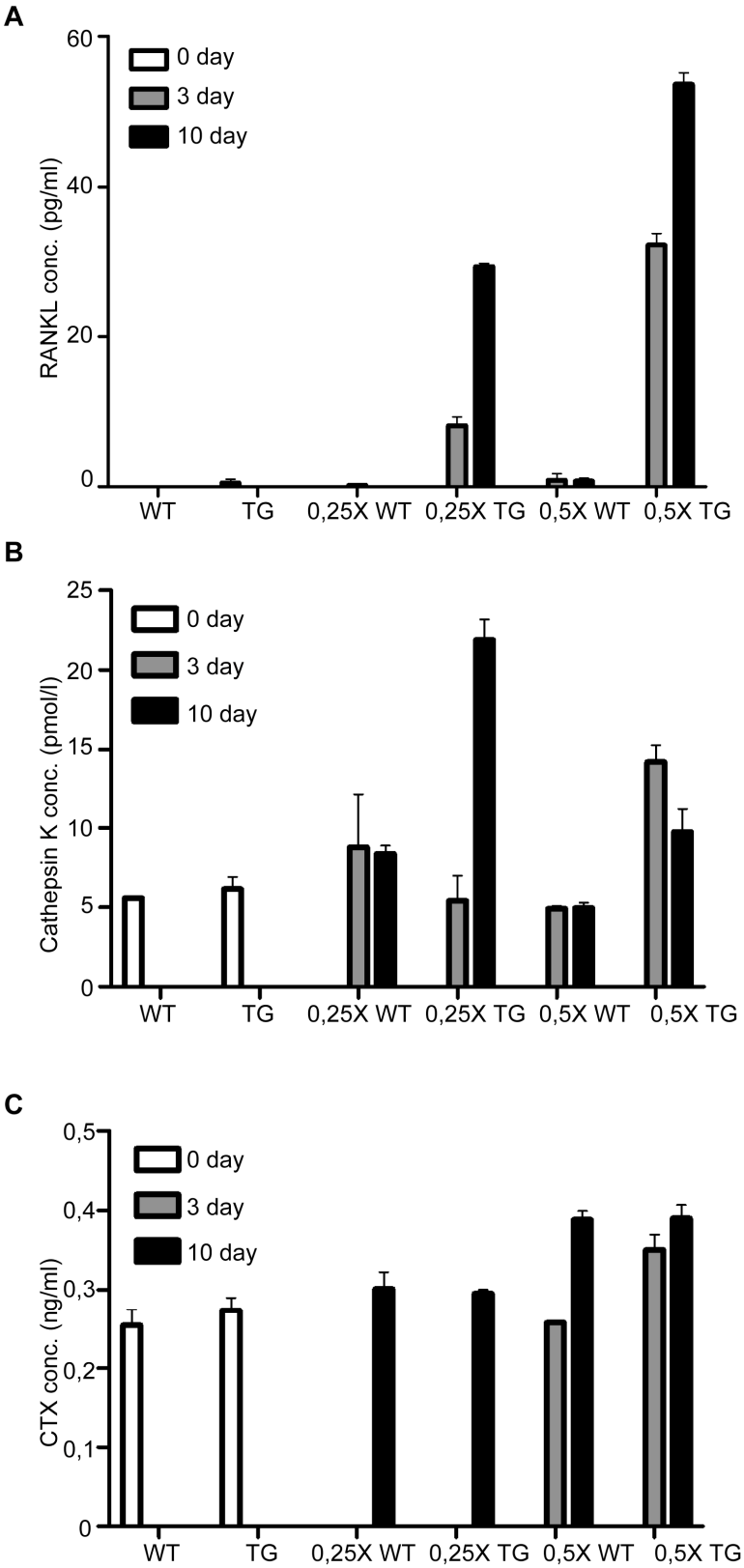

Figure 3.22.: Molecular characterization of resorption markers in two piglets 9959 and 9961 after Doxycycline stimulation for 0, 3, 6 and 10 days with 2 different Doxy concentrations, 0,25X and 0,5X (see Application of Doxycycline). (A) sRANKL ELISA of the plasma collected from transgenic 9961 and control 9959 piglets after Doxycycline stimulation for 0, 3, 6 and 10 days. A strong secretion can be found in piglet 9961 starting from day 3 and increasing, while in piglet 9959 no secretion of psRANKL was found. (B) Cathepsin K ELISA of the plasma collected from transgenic 9961 and control 9959 piglets after Doxycycline stimulation for 0, 3, 6 and 10 days. A significantly higher release of Cathepsin K can be found in piglet 9961 from day 6, while the level of Cathepsin K in the control animal 9959 remained at the same level during the experimental time. (C) CTX

ELISA of the serum collected from transgenic 9961 and control 9959 piglets after Doxycycline stimulation for 0, 3, 6 and 10 days. No obvious expressional pattern could be seen in both animals.

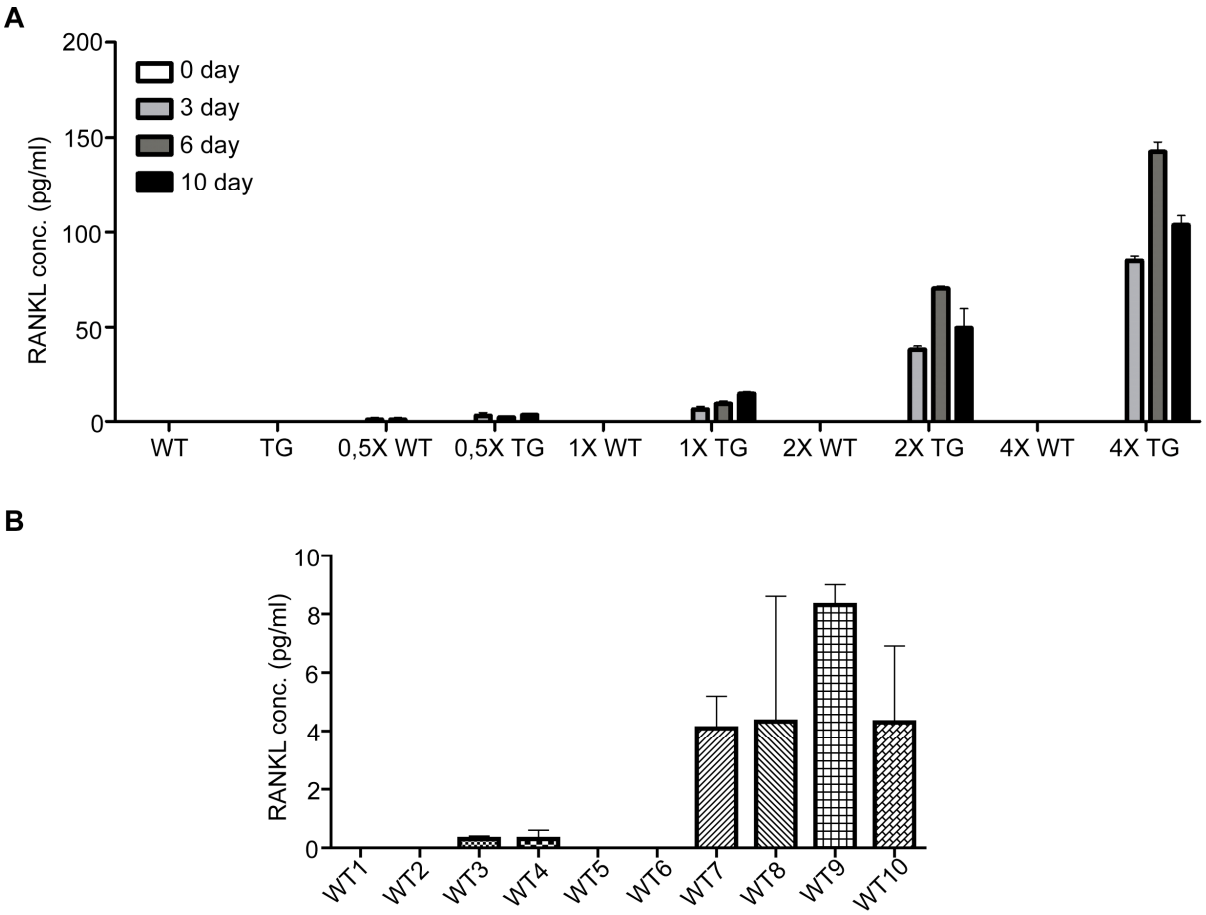

Figure 3.23.: Dose dependence characterization of psRANKL in two piglets 9959 and 9961 after Doxycycline stimulation for 0, 3, 6 and 10 days. 0,5X, 1X, 2X and 4X are indicating different concentration of Doxy (see Application of Doxycycline). (A) sRANKL ELISA of the plasma collected from transgenic 9961 and control 9959 piglets after Doxycycline stimulation for 0, 3, 6 and 10 days. A strong secretion can be found in piglet 9961 starting from day 3, increasing at day 6 and decreasing at day 10, while in piglet 9959 no secretion of psRANKL was found. The same pattern of secretion can be found in all dose of Doxy used. (B) sRANKL ELISA in plasma collected from 10 different wild type pigs. Low basal RANKL expression could be detected in 6 wild type pigs.

4. DISCUSSION

Understanding the bone biology is important for the development of a successful treatment of bone diseases, such as osteoporosis. Literature shows that cytokines and their signalling pathways, like RANK/RANKL/OPG, play an essential role in the regulation of normal bone remodelling [171]. Alteration in this system causes an imbalance between bone resorption and bone formation. RANKL is thought to play a crucial role in the development of post-menopausal osteoporosis [172]. In addition, the clinical relevance of RANKL is also supported by the finding that blocking RANKL by an inhibitory antibody (Denosumab) prevents osteoporosis and fractures in postmenopausal women [173]. Understanding the specific role of RANKL in bone biology is thus essential for understanding the disease mechanisms and important for developing new therapeutic strategies [174]. Therefore, in this thesis, the specific cytokine pathway of RANKL was studied. For practical reasons rodent models have been used most frequently in medical research. Nevertheless, the porcine bone anatomy and physiology is related much closer to humans. Therefore, porcine animal models would be more suitable for studying bone physiology and diseases *in vivo* [143,175]. For this purpose we used *Sus scrofa* (pig) as a large animal model, in which we expressed a soluble RANKL under the Tet-On regulation. Having this large animal model system will help us to better understand the development of the osteoporosis. In addition, this is the first genetic large animal model for osteoporosis with regulated gene expression.

4.1 OVER-EXPRESSION AND FUNCIONALTY OF THE EXTRACELLULAR DOMAIN OF PORCINE sRANKL

4.1.1 Sequence analysis suggests that sRANKLs are conserved

Initially, RANKL was considered to be a membrane-bound protein and required a cell to cell contact for fulfilling its biological roles [58,61,62,93,176]. A decade ago, a study by Lum *et al*. [177] has shown that mouse RANKL, like TNF-α, can be shed by the metalloprotease disintegrin TACE. As a result, the soluble RANKL form is released into the circulation with a potent effect on osteoclastogenesis. Our initial goal was to produce a functional porcine extracellular domain of RANKL (sRANKL) with high level of secretion. Therefore, taking into account the results of Lum *et al*. [177], we cloned the porcine sRANKL without the N-terminal transmembrane domain. Instead, we added an N-terminal Igκ-leader sequence to facilitate the secretion of the new fusion protein.

Next, by the use of human RANKL-specific primers, we were able to amplify a part of the porcine gene. The sequence analysis showed a considerable nucleotide homology to human,

murine and rat sequences, suggesting that this gene is highly conserved between species. Indeed, within the phylogenetic tree created, the porcine sRANKL is evolutionary closer to the human. Even though, pigs are evolutionary distinct from primates and rodents, they are closer related to humans than to mice and rats. Because of this relationship, pigs have already shown a very high anatomical, physiological, metabolic and pathological similarity to humans [178,179]. So, because of its proximity to the humans we found that using *Sus scrofa* as a large animal model to analyze the osteoporotic progression is more suitable approach.

Already cloned RANKL proteins are considered to be type II transmembrane glycoprotein belonging to the TNF superfamily. The ligands of the TNF superfamily are characterized by a conserved C-terminal domain called the TNF homology domain (THD) [101]. This domain is responsible for the formation of functional homotrimer and for the specific receptor binding. Despite their varied actions, members of the TNF ligand and receptor families have remarkably similar structures and their mode of interaction is conserved. Indeed, alignment of porcine with human, mouse and rat sRANKL revealed nearly identical amino acid sequence within the THD domain, suggesting that this domain is highly conserved [180]. The homology of the glycosylated site within THD domain was also conserved in all four aligned sequences. Therefore, the homology of the sequence suggests that newly cloned porcine sRANKL belongs to the TNF superfamily. Consistent with this observation, one could conclude that porcine sRANKL will have a similar role like other ligands of the TNF superfamily. In general, the ligands of the TNF superfamily have pivotal roles in the organization and function of the immune system [181,182], which was an evolutionary advancement in vertebrate. In addition, RANKL and its receptor RANK have not got a critical function in bone homeostasis, but they are also important in the terminal differentiation of the lactating mammary gland [183].

4.1.2 Lentiviral gene transfer successfully over-expresses porcine sRANKL at a high level

In order to achieve an over-expression in the cells, few techniques can be employed. Each of them has its advantages and drawbacks. For initial screening and analysis the cell transfection can be used, as it provides excellent modulation efficiency. The major disadvantage of this system is it very low integration chance. Next, stable transduction can be achieved by the use of different virus based technologies – retroviral, adeno-associated viruses (AAV) and lentiviral. Bosch *et al*. [184] showed that transduction with adeno-associated viruses had a higher transfection efficiency when compared to nonintegrating plasmids, in porcine

mesenchymal stem cells and skin fibroblasts. Nevertheless, retro- and AAV viruses have a potential mutagenesis risk and in many cases methylation-dependent gene silencing can occur [185,186]. Lentiviruses are considered as a promising alternative to the original oncogenic retrovirus, because they resist the methylation-dependent gene silencing, have a consistently higher transduction rates and stably integrate into the host genome [187]. In the study of Ezashi *et al*. it was shown that porcine fetal fibroblasts can be transduced by the use of a lentiviral delivering system [188]. They also showed that these fibroblasts were successfully transduced by lentiviruses containing human genes, like OCT4, SOX2, KLF4 and c-MYC, in order to generate pluripotent stem cells [188]. Our first observation of the lentiviral psRANKL-transduced pFFs showed no visible morphological or proliferation changes in comparison to the wild type control. Despite of the guarantee of stable long-term high gene expression detected *in vitro,* eventually *in vivo* limitations can occur [189].

Once we proved that the porcine cells produced increased levels of psRANKL mRNA and protein, we next, quantitatively analyzed the porcine sRANKL release in the cell culture supernatants, using an ELISA known to detect human sRANKL [190]. Elevated sRANKL-concentrations have been detected by ELISA in the supernatants of the pig bone marrow stromal cells stimulated with $1\alpha2,5(OH)_2D_3$ [190]. Indeed, the sRANKL level was significantly higher in cells transduced with porcine sRANKL lentiviruses. Furthermore, a light microscopy analysis clearly showed that psRANKL was presented in cell cytoplasm, most likely in the place of its biosynthesis. Additionally, with the cytoplasm staining, we also detected psRANKL along the plasma membrane of the porcine sRANKL-pFF.

Taken together, we can conclude that using a lentiviral delivery system leads to a significant increase in the expression and production of active psRANKL.

4.1.3 Porcine osteoclasts generated by human sRANKL show typical osteoclast morphology

Osteoclasts are one of the key cells responsible for the bone remodelling. Unfortunately, it is very difficult to access osteoclasts. This is mainly due to two properties of these cells. First, under physiologic conditions, the osteoclast is a very scarce cell type, covering only 1% of the bone surface [192]. Second, calcified tissue is hard to handle, which makes it nearly impossible to purify satisfying numbers of authentic cells for *in vit*ro studies. Thus, it is important to establish a system for generating of homogeneous osteoclast cultures [193-195]. However, human osteoclasts are currently generated by isolation of primary bone marrow cells that are adherent and lacking in nonadherent lymphoid cells. Stimulation of this mixed

population with M-CSF and sRANKL drives a proportion of these precursors to the monocyte-macrophage lineage and subsequently to mature osteoclasts [58,62,95,196].

Here, for the first time the ability of human soluble RANKL, together with human M-CSF, was demonstrated to induce osteoclastogenesis in porcine bone marrow-derived cells *in vitro*. The generated cells show the characteristics of osteoclasts: they were multinucleated and showed the typical cytoskeletal structures like the formation of actin rings and podosomes. Furthermore, they expressed molecular markers characteristic of osteoclast differentiation, like tartrate-resistant acid phosphatase (TRACP). Moreover, the morphology of the generated cells is consistent with human osteoclast morphology [197].

It is known that the cytoskeletal organization in porcine bone marrow-derived cells changes markedly during the differentiation into the osteoclast [198]. In this study, actin ring morphology was observed in primary porcine osteoclast cultures after treatment with a human sRANKL and M-CSF. Phalloidin and Concanavalin A staining showed that actin ring structures became dispersed already after treatment.

4.1.4 Porcine sRANKL generates osteoclasts with osteoclastic activity

After successful osteoclastogenesis of porcine bone marrow with human sRANKL and M-CSF, an *in vitro* differentiation study with porcine sRANKL was performed. We wanted to investigate the functional activity of the newly cloned protein. Therefore, we carried out the functional study of porcine sRANKL in a physiological cellular background by using porcine primary osteoclast precursor cells.

As with the human sRANKL, the truncated porcine sRANKL was sufficient to induce osteoclastogenesis. Cells transfected with porcine sRANKL generated nearly the same number of osteoclasts compared to cells cultivated in the presence of recombinant human sRANKL, although the concentration of human sRANKL in the supernatant was nearly 200-fold higher. When the osteoclastic precursor cells were incubated in a medium (containing FBS), only a small number of osteoclasts was counted. This may suggest that the serum supports the survival of mature osteoclasts to some degree [199].

The morphology of the generated osteoclasts was consistent with typical osteoclast morphology. The multinucleated cells display the typical cytoskeletal features like the formation of actin rings and the appearance of podosomal attachments. Furthermore, they express the specific osteoclastic markers like TRACP and CA-II.

We used CA-II as an osteoclastic marker. CA-II expression appears at an early stage of differentiation on osteoclasts [200]. mRNA for CA-II can be found prior to observing

multinucleated osteoclasts, suggesting that CA-II may be present on committed osteoclast progenitor [200]. Porcine and human sRANKL induced the expression of the CA-II, but there was also lower expression with M-CSF alone. Laitala *et al.* have shown that CA-II was expressed not only in osteoclasts but also in some mononuclear bone-derived cells [200]. A distinct group of mononuclear cells expressing CA-II might be the osteoclast precursors. In nonresorbing osteoclasts CA-II mRNA levels are low or even below the detection limit. When osteoclasts are activated for resorption, a rapid increase in the mRNA levels of CA-II occurs. The nonresorbing osteoclasts expressing CA-II mRNA could be starting resorption and similarly, osteoclasts associated with a lacuna but not expressing mRNA for CA-II, might just have finished bone resorption [200]. Furthermore, it is known that M-CSF is required for progenitor cells to differentiate into osteoclasts, but M-CSF on its own cannot complete osteoclastogenesis. Fuller and Chambers [201] reported that M-CSF prolonged the survival of mature osteoclasts, whereas in the absence of M-CSF, the number of osteoclasts were strikingly decreased. Therefore, the fact that we found an expression of the osteoclastic marker CA-II and TRACP-positive cells in the presence of only M-CSF, might be due to an endogenous RANKL production. Nevertheless, the osteoclasts formed in the presence of M-CSF alone were significantly less and smaller than with combined treatment with human or porcine sRANKL and M-CSF. On the other hand was M-CSF alone not sufficient to produce bone-resorbing osteoclasts. Only when porcine or human sRANKL was added, functional osteoclasts were generated.

These results not only reveal that the human sRANKL can differentiate porcine osteoclasts, but also indicate that the porcine sRANKL has similar function than the known human sRANKL.

4.2 rtTA-psRANKL TRANSGENIC FETUSES GENERATED BY ELECTROPORATION OF LENTIVIRAL CONSTRUCT

4.2.1 Porcine sRANKL is inducible in 293FT cells after Doxycycline stimulation in a Tet-On system

Traditional constitutive over-expression is often limited by its inability to precisely control the timing and level of gene expression, which are critical for the function of many genes. It is not uncommon for fate-determining genes to be toxic at inappropriate stages or even have different or complete opposite functions at different expression levels [202]. Thus, efficient conditional manipulation of transgene expression is necessary. The most common strategy to achieve conditional transgene expression is the use of the tetracycline-inducible gene

expression systems (Tet-Off or Tet-On), which has been extensively used in mammals [149,150]. This system utilizes transactivator (rtTA), transactivator responsible element (TRE) and a drug Doxycycline. Some major advantages of this system compared to the other gene regulation systems (ecdysone-regulated gene switch [203,204], the lac operator–repressor system [205] and the inducible GAL4/UAS system [206]) are a well-known safety profile, easy availability and good tissue penetration of the regulating drug. Moreover, bacterial origin of the Tet- elements assures minimal interference with endogenous physiological processes of the eukaryotic cells. For all these reasons we have chosen this system to obtain a regulated gene expression. Most importantly, the establishment of an inducible system was needed, because the constitutive sRANKL expression in a mouse model was embryonic lethal [105].

Gene transfer vectors derived from lentiviruses are widely used in molecular and cellular biology. Those vectors have the unique capacity to transmit one or few copies of a transgene into almost every cell type of vertebrates. The transferred genetic information is permanently integrated into the host cell chromosome [207]. At the same time, lentiviral vectors have emerged as the most efficient and versatile systems for somatic and germ-line gene transfer, because of their high transmissibility in quiescent cells [117,208]. In the present study, a Doxycycline-controlled system that allowed continuous expression of rtTA protein and regulated porcine sRANKL expression was generated. The use of such binary Tet-On system in animals usually requires the establishment of two transgenic lines [209,210]. Thus, to obtain a double-transgenic offspring the two transgenic lines must be crossed. The disadvantage of this approach is the lack of control of the appropriate copy number of either the transactivator or the responder line. This may result in an imbalanced expression of the transgene [211,212]. To avoid this problem we combined the two expression units of the Tet-On system on a single DNA fragment. Furthermore, in order to avoid multiple transfections of the target cells, which would increase the risk of insertional mutagenesis [2123,214], a single lentiviral vector, which delivered the expression cassettes for both, transactivator and transgene, was used. Such lentiviral vectors carrying the elements of the Tet system have been described but they make use of the Tet-Off system [2145,216]. Thus, we generated the first lenviral vector that make a use of the Tet-On system.

To characterize Doxycycline-regulated transgene expression of our vector, a single construct, carrying rtTA and porcine sRANKL as transgenes, was introduced into the cells and the expression of the transgene was studied. For this purpose, we used sRANKL ELISA and measured the expression of psRANKL upon Doxycycline stimulation. The ELISA showed no

signal in OFF state. Our results support the tight control of the gene expression, since, in the absence of Doxycycline, the psRANKL expression was at or below the detection level.

4.2.2 Ear fibroblasts of transgenic fetuses generated by electroporation of the lentiviral construct express rtTA and induce low porcine sRANKL expression upon the Doxycycline stimulation

In a first attempt to create a transgenic porcine model, a lentiviral gene transfer technology was used. This transfer technology had shown to be effective in bovine and porcine animal models [217]. In contrast to lentiviral transgenesis, the efficiency of DNA microinjection, the most widespread technology to generate transgenic animals [218], is low. In the mouse model, approximately 2.6% of the transgenic offspring per injected and transferred embryos, can be obtained. In the pig model the efficiency is even lower, ranging at 0.9% [219]. In contrast, the generation of transgenic pigs carrying the green fluorescent protein (GFP) gene by lentiviral gene transfer revealed 70% of the born animals being transgenic [217]. Therefore, lentiviral gene transfer was adapted to pigs and resulted in high proportions of transgenic offsprings [217,220].

When the expression of the rtTA-transgene was analyzed, the results showed that the heterologous CMV promoter is sufficient to direct the expression of rtTA mRNA, although the expression was variable within cell types and different tissues. This may be explained by an inappropriate copy number or restricted tissue specificity of the rtTA as shown in the study by Kues *et al.* [221]. The variable rtTA-expression levels observed within the cell lines and tissues may be perhaps attributed to the relative sensibility of the cytotoxic effects of rtTA [222].

Next, the porcine sRANKL mRNA expression upon Doxycycline stimulation was analyzed *in vitro*. A RT-PCR allowed the determination of the background expression of porcine sRANKL transgene in the absence of Doxycycline, showing a leakage of transcription from the minimal CMV promoter situated in the TRE-regulated promoter. This background expression led to a detectable porcine sRANKL activity in fibroblasts of fetus 3. Background activity in the OFF state has also been reported when the Doxycycline-regulated expression of luciferase was delivered by two lentivirus vectors [212]. This may suggest that the background activation of the minimal CMV promoter is due to transactivatory effects of the chromosomal environment into which the vector is integrated, or to transactivation by enhancer elements within the CMV promoter used to express the transactivator [223]. All these parameters could be under the influence of the chromatin status of cis-acting sequences

present in the vector (Tet-responsive elements and promoter driving the transcription of regulatory gene) and they are therefore dependent on the position of the integrated vector DNA in the transfected cell genome. Moreover, the design of such a vector has been difficult because of transcriptional interference occurring between the cis-acting sequences within the vector [31]. A study by Barde *et al.* [224] showed that relative orientations of the transcriptional cassettes, as well as their spacing within the vector, have an impact on both background activity levels and inducibility.

4.2.3 rtTA-psRANKL piglets originated from fetus 3 were born dead and their cells did not induce psRANKL

Since the cells from fetus 3 showed at least a psRANKL expression, they were used for a subsequently re-cloning of the animal.

Unexpectedly, the regulated phenotype resulted in a lethality of the newborns. This finding suggests that even a low over-expression of porcine sRANKL in the fetal stage causes the prenatal death confirming the results obtained by Mizuno *et al.* [105]. This is not surprising since RANKL is an essential regulator of osteoclastogenesis, lymphocyte development and mammary gland development [62,96,107]. Thus, the fetal death may be due to an abnormality in bone metabolism or in immune system.

From both dead animals, the heterogeneous cell population from bone marrow could be isolated, showing that cells in the bone are preserved from degradation and environmental changes, unlike ear fibroblast.

Since both animals were born dead, we were not able to establish the time point at which they died and the period that was needed for tissue-RNA degradation. For this reasons we performed a RNA control PCR that showed cDNA contamination in our RNA preparation, further confirming the statement that RNA was degraded already at the beginning of the preparation of the sample. Therefore, we can not prove whether the over-expression of rtTA is due to the transcriptional over-expression or the cDNA contamination. Thus, in order to avoid this problem in the further experiments, good quality RNA was isolated from cells and RT-PCR showed strong over-expression of rtTA. Furthermore, the psRANKL mRNA expression upon Doxycycline stimulation was analyzed. A RANKL-RT-PCR showed a high background expression of transgene in OFF state, indicating a leakage of transcription or interaction of the two expression cassettes as discussed above.

The lethal phenotype of this system prevented the study of the contribution of the psRANKL in living organism.

4.3 rtTA TRANSGENIC PIGS

Inducible transgene expression is an essential refinement of genetic engineering of large animals, particularly for the generation of biomedical models. Many expression regulation schemes have been proposed. Most extensively studied are vector systems containing promoters that respond to the binding of small molecules such as tetracycline [149] or rapamycin [225]. The transgene expression can be controlled through administration of the small molecular drugs [150,226]. Indeed, a tetracycline-regulation scheme, Tet-system, has been used successfully in numerous transgenic animal models, with a variety of transgenes targeted at various tissues and organs [210,227-232]. Nevertheless, the generation of these lines is traditionally a difficult task. Several factors are known to influence the efficiency of the Tet-system in transgenic animals, with the most important being the site integration of the transgene [233]. Moreover, the expressions of therapeutic genes including hGH and M-CSF were shown to be effectively controlled with such a Tet-system *in vitro* [234]. *In vivo*, Rizzuto *et al*. used a similar vector encoding the erythropoietin gene-transfected mouse muscle by electroporation. Erythropoietin expression was shown to be turned on and kept on with daily oral Doxycycline administration for 56 days [235].

4.3.1 rtTA-transgenic piglets express rtTA at high level in cells and organs

In contrast to a previous approach where the two components of a binary tetracycline-regulated expression system were combined on a single vector, we used a two-step approach and transferred the rtTA and TRE-psRANKL expression cassettes sequentially. This approach has a number of advantages: (1) transcriptional interference between adjacent expression cassettes [191] is avoided; (2) transgenic pigs with a suitable expression level and pattern of rtTA can be used as genetic basis for any TRE-controlled expression cassette; and (3) mendelian segregation of the rtTA and TRE-psRANKL expression cassettes generates all genetic constellations (non-transgenic, single-transgenic, double-transgenic) required for well-controlled experiments. Due to the large litter size in pigs all these genetic constellations are expected in a single litter. In detailed, this strategy included the cloning of two constructs which were introduced step by step to obtain double transgenic pigs. One construct expressed rtTA under a strong constitutive promoter and other expressed psRANKL under tetracycline-inducible promoter. The lentiviral construct was also aborted, since the study of Hofmann *et al.* [189] analyzed an epigenetic regulation of integrants in lentiviral transgenic animals *in vivo.* This study showed that one-third of the proviruses exhibited low or undetectable levels

of expression and a high degree of methylation of CpG dinucleotides, reminiscent of gene silencing. Furthermore, to generate high levels of rtTA expression, we decided to switch from the CMV promoter to the chicken β-actin (CAG) promoter. This idea is supported by previous studies in which it was revealed a mosaic expression of β-galactosidase within tissues was revealed when rtTA was under the control of a CMV promoter [209], but this problem has not occurred before when a strong tissue-specific promoter was used [210]. Moreover, this promoter has been used in previous experiments to express human CD59 and DAF in transgenic mice and pigs resulting in widespread tissue expressions [236,237].

In the first round of electroporation and nuclear transfer, rtTA-transgenic pig lines were produced. To select the most suitable rtTA transgenic founders, the animals were euthanized and systematically investigated for rtTA-specific RNA and protein expression in multiple tissues. These lines showed a strong and nearly ubiquitous expression of rtTA mRNA in the organs. However, the presence of rtTA transcripts does not necessarily mean that the rtTA transgene is expressed on the protein level. Unexpectedly, transgenic pigs carrying rtTA displayed confined expression in muscle and lung on protein level. These results are consistent with previous studies, which showed that transgene expression in transgenic animals may be different inbetween different tissues [238,239]. Mosaic expression patterns have also been described in Tet-transgenic mice and, in some cases, the highest expression levels were found in skeletal muscle [209,240,241], and in Tet-Off transgenic pigs [221]. The reason for this mosaic expression pattern could be due to the gene dose effects, which led to the widespread expression in transgenic animals. Furthermore, unknown molecular mechanisms can result in the observed differential expression in transgenic pigs.

Nevertheless, when RNA and protein were isolated from kidney cell of rtTA-transgenic lines, a strong over-expression of rtTA could be detected on mRNA, as well on protein level, confirming that the CAG promoter has an ability to strongly express the desired gene.

4.3.2 rtTA-kidney cells from piglet 9894 electroporated with TRE-psRANKL show the best induction of psRANKL upon Doxycycline stimulation

To further ensure selection of the best founders, primary kidney cells from each animal were nucleofected with TRE-psRANKL expression cassettes, and *in vitro* Doxy stimulation experiments were performed. After nucleofection with the TRE-RANKL cassette, cells from the three founders showed low baseline expression, but a high induction of transgene expression after stimulation with Dox. These findings demonstrate reliable and reproducible

Dox-regulated transgene expression in cell cultures derived from specific rtTA-transgenic founders. Why in two of the cell line (pKC-9890, 9891-psRANKL) the induction could be seen on protein level but not on mRNA level, is not clear. Furthermore, the psRANKL mRNA and protein expression allowed no determination of a background expression signal of the transgene in OFF state, showing tight control of the Tet-On system. On the other hand, in two cell lines (pKC-9892- and -9895-psRANKL) the expression of psRANKL could not be demonstrated on mRNA and protein level in ON state, indicating that these lines probably didn't integrate the TRE-psRANKL vector and thus, weren't able to express psRANKL.
Taken together, the best performance showed the cell line derived from the rtTA-piglet number 9894 which was electroporated with TRE-psRANKL. Subsequently, this cell line was the donor for the next nuclear transfer and led to the birth of double transgenic piglets.

4.4 rtTA-psRANKL DOUBLE TRANSGENIC PIGS

4.4.1 Ear fibroblasts from rtTA-psRANKL double transgenic piglets express rtTA but don't induce psRANKL upon stimulation

To test if these *in vitro* findings predict inducible transgene expression *in vivo*, we cloned double transgenic pigs with TRE-psRANKL on the background of rtTA founder 9894. In comparison to the SCNT experiments for generation of the TA transgenic founders, the efficiency of SCNT after nucleofection with the TRE-controlled expression vectors and the survival of the resulting piglets were poor. This is most likely due to an accumulation of epigenetic abnormalities [242] after subsequent rounds of nucleofection, selection and SCNT and should be overcome if cells from rtTA transgenic animals propagated by sexual breeding are used. Nevertheless, from each combination a viable animal was obtained and inducible transgene expression could be tested *in vivo*.
After successful nuclear and embryo transfer, four transgenic piglets were created. As expected, all four piglets were transgenic for rtTA (data not shown), but only one was not transgenic for the psRANKL gene (piglet 9959). This may be due to the selection process of the donor cells of the culture after electroporation. Taken together, we were able to obtain three double rtTA-psRANKL transgenic animals.
Even though three of four animals were transgenic for psRANKL gene, none of transgenic cells did express psRANKL on mRNA and protein level in OFF or ON state. These findings can be related to the transcriptional repression of cellular genes that may be happing and it is often associated with a higher frequency of methylated cytosine residues in the 5´ flanking DNA, particularly at CpG sites [243,244] or to a low expression level [189]. Two decades

ago, Prosch [245] showed that methylation of CpG sites in the CMV promoter abolished transcription in transfected cells in a culture. Moreover, methylation was associated with the absence of a viral gene expression and it has been suggested that *de novo* methylation of foreign DNA represents a cellular defence mechanism against the transcription of foreign genes [246,247]. Furthermore, a negative correlation between methylation of the promoter and gene expression has been documented previously [248,249]. Along this line it is interesting that the CpG depleted CMV promoter constructs gave rise to a long term expression *in vivo* [250]. Furthermore, it is well known that genomic methylation and histone modifications undergo major changes during development [251].

Additionally, this data could be explained by the fact that the expression was analyzed in ear fibroblasts and perhaps an unknown mechanism in porcine ear fibroblasts exists, which doesn't allow the transcription and transduction of the regulated gene. Indeed, a study by Kong *et al.* [252] showed a methylation in ear fibroblasts of transgenic pigs when compared to kidney cells. Moreover, an increase in transgene promoter methylation was demonstrated during individual development of transgenic pigs and transgenic fibroblast cells [252].

Therefore, our data probably indicate that the *de novo* methylation in ear fibroblast may interfere with the proper expression of the tetracycline sensitive promoter. Since we did not investigate the silencing effect, we cannot conclude for sure if this is happening in our system. Thus, further studies are required to analyze the silencing mechanisms of the exogenous DNA in order to understand the repression of the expression.

4.4.2 The rtTA-psRANKL transgenic pig shows upregulation of resorption markers

The major *in vivo* objective was to determine whether the expression of transgene psRANKL contributes to the increased bone resorption in transgenic pig. For this purpose, two resorption biochemical markers, Cathepsin K and CTX were chosen. To analyze the psRANKL induction upon Doxycycline stimulation, the plasma RANKL levels were evaluated in rtTA-psRANKL transgenic and wild type pig.

To test the effect of Doxy dose on transgene expression *in vivo*, rtTA-psRANKL transgenic boar 9961 was treated with ascending doses of Doxy (6.3, 12.5, 25, and 50 mg/kg body weight per day for 10 days each), resulting in a more or less dose-dependent increase in plasma RANKL concentrations. This observation demonstrates the achievement of a tightly controlled Tet-On system in transgenic pigs. Additionally, RANKL upregulation has been proven in osteoporotic women [253] and the $OPG^{-/-}$ mice, which exhibit an aberrant bone

metabolism, characterized by accelerated bone resorption [104,105,254]. Because the serum concentration of RANKL is markedly elevated in these mice [255], the circulating RANKL may be directly involved in osteoclastogenesis.

Biochemical markers of resorption and formation that provide sensitive and dynamic indices of bone turnover may be a useful investigation tool for quantifying and monitoring the effects of anti-osteoporotic drugs [253]. Recent data suggest that some molecular markers could provide a more profound analysis of the changes of resorption and show parallel changes of osteoclast function and number. These markers include osteoclast specific enzymes, tartrate-resistant acid phosphatase 5b (TRACP5b) and Cathepsin K [256,257], which may reflect the number of osteoclast and the Cathepsin K generated degradation fragments of collagen type I (NTX and CTX), which reflect the function of the osteoclasts [258]. Recently, Meier *et al.* [259] conclude that serum Cathepsin K appeared to discriminate better between osteoporotic and non-osteoporotic women compared with other bone turnover marker like ALP. For these reasons, we have chosen Cathepsin K and CTX as a means of showing osteoporotic phenotype. Additionally, circadian rhythms are known to exist for bone turnover and bone mineral metabolism, thus the time point of the collection of the samples may be very relevant for interpretation of the results [260-265].

In transgenic pig an obvious difference of plasma Cathepsin K levels was detected, comparing a non-transgenic littermate control at day 10 for 0,5X doses and at day 3 for 1X doses. At those time points, it can be stated that transgenic pig does develop an induced osteoporotic phenotype. The discrepancy in the expression pattern is probably due to different time points of collection of the samples.

No significant differences in serum CTX levels could be determined in transgenic pig when compared to its non-transgenic littermate control animal at any time point investigated. The C-telopeptide fragments of type I collagen measured in the CrossLaps ELISA that we used, was generated in bone; however, it may also be generated by several tissues unrelated to bone, because of the widespread occurrence of type I collagen in the body [266], thus masking the obtained results. Furthermore, the altered temporal relationship of the CTX may reflect changes in the indirect effect of RANKL.

5. CONCLUSIONS

1. Newly cloned porcine sRANKL cDNA has a template of 842 bps and a protein of 280 amino acids with molecular weight of 31,7 kDa with predicted pI of 6,90.

2. Porcine and human sRANKL share high sequence homology.

3. Sequence analysis suggests that TNF domain is located at C-terminus of porcine sRANKL.

4. Lentiviral gene transfer successfully over-expressed porcine sRANKL at a high level and in soluble form from porcine fibroblasts.

5. Porcine osteoclasts can be generated by human sRANKL, showing typical osteoclastic morphology.

6. Porcine sRANKL can stimulate osteoclastogenesis *in vitro*.

7. Porcine sRANKL is inducible in cells after Doxycycline stimulation in a Tet-On system.

8. Double-transgenic fetuses generated by electroporation of the lentiviral construct express rtTA and induce low porcine sRANKL upon Doxycycline stimulation in ear fibroblasts.

9. rtTA-psRANKL piglets originated from double-transgenic fetus 3 were born dead and their cells do not induce porcine sRANKL.

10. rtTA-transgenic piglets express rtTA at high level.

11. Electroporation of rtTA-kidney cells with TRE-psRANKL vector showed the induction of porcine sRANKL upon Doxycycline stimulation.

12. The rtTA-kidney cells electroporated with TRE-psRANKL vector from piglet 9894 showed the best expressional and inductional pattern.

13. The rtTA-psRANKL cells from piglet 9894 were used for further re-cloning of transgenic animals.

14. Re-cloned rtTA-psRANKL transgenic piglets are expressing rtTA, but don't induce psRANKL upon stimulation in ear fibroblasts.

15. rtTA-psRANKL transgenic pig shows upregulation of resorption markers in blood.

16. rtTA-psRANKL transgenic pig shows induction of RANKL in plasma upon Doxycycline stimulation.

In conclusion, our results strongly suggested that extracellular domain of newly cloned porcine RANKL is able to mediate osteoclast formation and function. Moreover, until now there are no published data about a transgenic pig carrying the rtTA inducible gene expression system (Tet-On system). In mice several transgenic models with tetracycline-controllable gene expression were already established [210,267,268]. Therefore, we produced the first piglet that exhibited functional Tet-On and showed inducible expression of the desired gene. Future analysis will show what impact has a long time over-expression of psRANKL on piglet 9961. We can only assume that this piglet will develop an osteoporotic phenotype. Moreover, in 7-8 month old mice, over-expression of sRANKL led to a significant deprivation of the femur bone mineral density, due to promoted osteoclast formation [105]. Thus, our transgenic pig would be useful tool as a therapeutic model and for the clarification of further physiological and pathological functions of sRANKL *in vivo*.

6. SUMMARY

Introduction: Osteoporosis, a bone disease which is characterized by a reduction of bone mass, affects many people worldwide. RANKL (Receptor Activator of NF-κB Ligand) is a cytokine belonging to the TNF superfamily and is essential for osteoclast differentiation. During normal bone turnover RANKL stimulates the formation and activity of osteoclasts, by binding to its receptor RANK, which expressed on osteoclasts and their progenitors. These processes are disrupted by binding of RANKL to osteoprotegerin (OPG), a soluble decoy receptor. Unfortunately, many mouse disease models often do not faithfully mimic the relevant human conditions. Therefore, the swine has been used as a major mammalian model for human biology. The similarity in size, physiology, organ development and disease progression make the swine an ideal model. The aim of this study was to over-express sRANKL in order to obtain an osteoporotic large animal model. Thus, we designed a construct encoding the tetracycline transactivator under constitutive promoter and extracellular domain of porcine RANKL under tetracycline-regulated promoter using so called Tet-On system. Finally, the transgenic piglets were obtained.

Materials and methods: A lentiviral transfer of psRANKL cDNA into porcine cells was applied for over-expression of the psRANKL protein. Quantitative PCR, Western blot and immunocitochemistry were used to assess the over-expression. Giemsa, TRACP, Phalloidin and Concanavalin A stainings were used to characterize generated porcine osteoclasts. Calcifluor assay was used to estimate the extent of osteoclast resorption. Somatic cell nuclear transfer was used to generate transgenic animals. Genomic PCR, RT-PCR, Western blot and ELISAs were used to evaluate the expression pattern of the transgenic animals.

Results: The development of the first large animal model for osteoporosis is described in this thesis. To obtain this, porcine sRANKL was first cloned. Sequence analysis and functional characterization of the newly cloned porcine sRANKL suggested that the TNF-like core domain is essential for the induction of osteoclastogenesis. For gaining a controllable expression of porcine sRANKL, it was necessary to establish a Tet-On system to sidestep harmful effects of RANKL over-expression during the fetal development. The first attempt to integrate both genes, transactivator (rtTA) and psRANKL, in a single step of cell transfection was successful, i.e. the cells induced psRANKL after Doxycycline stimulation. Unfortunately, after nuclear transfer of the cells having the same construct, the expression analysis produced

no satisfying result. We obtained two transgenic fetuses, showing an expression of rtTA and low inducibility of porcine sRANKL. Therefore, the strategy was changed in favor to two rounds of transfection and nuclear transfer. First rtTA-transgenic piglets were established and screened for integration and expression. One of five rtTA-piglets (piglet 9894) showed the best expression and severed as donor for next cell transfection step. These rtTA-psRANKL cells were tested *in vitro* for their inducibility. After this the nuclear transfer and embryo transfer of the best candidate was performed and it resulted in 4 pregnancies which all finished to term. The only alive double transgenic piglet (9961) was raised and will be kept until adulthood to establish a founder line of rtTA-psRANKL transgenic pigs, as well as for first studies on inducibility of psRANKL over-expression. Stimulation of this pig with Doxycycline resulted in increased serum levels of porcine sRANKL. Furthermore, elevated psRANKL levels caused an increase in bone resorption markers. Serum levels of Cathepsin K were significantly upregulated, strongly suggesting that the newly cloned porcine sRANKL is also functionally active *in vivo*.

Conclusions: Cloning and sequencing of the extracellular domain of the porcine homologue of RANKL showed that it has a high similarity with human sRANKL. The porcine homologue has an important role in regulation of bone homeostasis and osteoclastic function. These findings show for the first time that porcine sRANKL is sufficient to induce osteoclasts *in vitro*. In addition, many physiological characteristics of the pigs are more similar to the humans than those of the mouse. Moreover, the large size of a pig model facilitates a plethora of physiological and molecular studies as well as therapeutic trials that cannot be performed in the small rodent models. Thus, the sRANKL transgenic pig is a novel animal model with clinical relevance for osteoporosis research and treatment. Furthermore, other constructs might be based on the existing Tet-On system, offering an inducible system for a broad variety of different transgenes. In addition the present work describes a functional Tet-On system in pigs for the first time.

7. ZUSAMMENFASSUNG

Einleitung: Osteoporose, eine Knochenkrankheit bei der sich die Knochendichte verringert, betrifft weltweit viele Menschen. RANKL (Rezeptor Aktivator des NF-κB Liganden), ein Zytokin, gehört zur TNF-Superfamilie und ist essentiell für die Differenzierung der Osteoklasten. Während des normalen Knochenumbaus stimuliert RANKL die Bildung und Aktivität von Osteoklasten indem es an seinen Rezeptor RANK bindet, welcher auf Osteoklasten und ihren Verläuferzellen exprimiert wird. Gestört werden diese Prozesse durch die Bindung von RANKL an Osteoprotegerin (OPG), einem löslichen Rezeptor. Mausmodelle spiegeln die relevanten menschlichen Bedingungen für Osteoporose nur unzureichend wieder. Aus diesem Grund ist das Schwein ein wichtiges Tiermodell für die Untersuchung der menschlichen Biologie, da die Ähnlichkeiten in der Größe, der Physiologie, der Organentwicklung und des Krankheitsverlaufs das Schwein zu einem idealen Säugetiermodell machen. Das Ziel dieser Studie war die regulierte Überexpression des sRANKL, um ein Großtiermodell zu erstellen. Deshalb haben wir ein Konstrukt entwickelt, welches über einen Tetracyclin-regulierten Promotor (sog. Tet-On System) den Tetracyclin-Transaktivator bindet. In diesem Komplex befinden sich außerdem ein konstruktiver Promotor und eine extrazelluläre Domäne des Schweine RANKL. Sclussendlich konnten transgenetische Schweine entwickelt werden.

Materialen und Methoden: Mit Hilfe des lentiviralen Gentransfers wurde die psRANKL-cDNA in Schweinezellen eingebracht, um eine Überexpression des psRANKL Proteins zu erreichen. Zur Überprüfung der Überexpression wurden verschiedene Verfahren wie quantitative PCR, Western Blot und Immunzytochemie verwendet. Speziell zur Charakterisierung der Schweine Osteoklasten wurden Giemsa, TRACP, Phalloidin und Concanavalin A Färbungen durchgeführt. Durch die Anwendung des Calcifluor Assays wurde der Osteoklastenabbau dokumentiert. Für die Generierung der transgenen Tiere wurde der Kerntransfer angewendet. Zur Überprüfung des Expressionsmusters der transgenen Tiere wurden verschiedene Methoden wie Southern Blot, RT-PCR, Western Blot und ELISA durchgeführt.

Ergebnisse: In dieser Arbeit wird die Erstellung des ersten genetischen Großtiermodells zur Untersuchung der Osteoporose beschrieben. Dafür wurde das porcine sRANKL von uns erstmals kloniert. Sequenzanalysen und die Funktionscharakterisierung des neu geklonten

psRANKL wiesen daraufhin, dass die TNF-ähnliche Kerndomäne eine wesentliche Rolle für die Induktion der Osteoklastogenese spielt. Um eine kontrollierbare Expression des psRANKL zu erreichen, war es notwendig ein Tet-On System zu etablieren, um gesundheitsschädlichen Nebenwirkungen in der Embryonalentwicklung zu vermeiden. Der Versuch beide Gene, Transaktivator (rtTA) und psRANKL, in einem einzigen Transfektionsschritt zu integrieren war im ersten Anlauf erfolgreich, d. h. die Zellen produzierten nach Stimulation mit Doxycyclin psRANKL. Jedoch ergab eine Expressionsanalyse von Zellen mit dem gleichen Konstrukt nach einem Kerntransfer keine zufriedenstellenden Ergebnisse. Wir erhielten drei transgene Feten mit rtTA Expression und allerdings niedriger sRANKL Induzierbarkeit. Deshalb wurden darufhin zwei Transfektionsschritte und zwei Kerntransfers durchgeführt. Nach der Geburt der ersten rtTA-transgenen Ferkel wurden diese auf die Genintegration und –expression überprüft. Es wurde ein von fünf rtTA-Ferkeln (Ferkel 9894) identfiziert, welches die beste Expression zeigte. Dieses Tier wurde als Donor für den nächsten Kern- und Embryotransfer verwendet. Zunächst wurden die rtTA-psRANKL Zellen *in vitro* auf ihre Induzierbarkeit getestet. Danach wurde der Kern- und Embryotransfer des besten Kandidaten durchgeführt, welches in 4 Schwangerschaften resultierte, die alle ausgetragen wurden. Das einzige lebende doppelt transgene Ferkel (9961) wurde aufgezogen, um eine Gründerlinie von rtTA-psRANKL transgenen Schweinen zu etablieren. Des Weiteren wurde die Induzierbarkeit von psRANKL Überexpression untersucht. Die Stimulierung dieses Schweins mit Doxycyklin resultierte in einem erhöhten Serumspiegel von psRANKL. Weiterhin sorgt der angestiegene psRANKL Spiegel für eine Erhöhung der Knochenresorptionsmarker. Die Werte von Cathepsin K und CTX waren signifikant erhöht, woraus geschlossen werden kann, dass das neu klonierte psRANKL auch in vivo funktionell ist.

Schlussfolgerung: Die Klonierung und die Sequenzierung der extrazellulären Domäne des Schweine RANKL zeigt eine große Ähnlichkeit mit dem humanen RANKL. Das Schweinehomolog spielt eine große Rolle in der Knochenhomeostase und der Osteoklastenfunktion. Diese Ergebnisse zeigen zum ersten Mal, das auch Schweine sRANKL in der Lage ist Osteoklasten *in vitro* zu induzieren. Darüber hinaus eröffnen diese Erkenntnisse die Möglichkeit für physiologische und molekularbiologische Studien, als auch therapeutische Untersuchungen, welche nicht im Nagerkleintiermodell durchgeführt werden können. Aus diesem Grund ist das sRANKL transgene Schwein ein neues, klinisch relevantes Tiermodell für die Osteoporose-Forschung und Behandlung. Zudem eröffnet das Tet-ON-

transgene Schwein die Möglichkeit andere regulierte Modelle mit unterschiedlichen Transgenen zu generieren. In der vorliegenden Studie wird außerdem das funktionierende Tet-On System im Schwein zu ersten Mal beschrieben.

8. LITERATURE

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9. LIST OF FIGURES

10. LIST OF ABBREVIATIONS

$1,23(OH)_2D_3$	1,23-dihydroxyvitmain D3
100 kbp M	100 kbp marker
293FT cells	Human embryonal kidney cell line (HEK)
ALP	Alkaline phosphatase
AMV	Avian Myeloblastosis Virus
BCA	Bicinchoninic acid
BGH	Bovine growth hormone
Bla	Blasticidin
BM	Bone marrow
BMD	Bone mineral density
BMU	Basic multicellular unit
bp	Base pair
BSA	Bovine serum albumin
BSP	Bone sialoprotein
C1NP	C-terminal type 1 procollagen
CAG	Chicken β-actin
CA-II	Carbonic anhydrase type II
CD59	Protectin
cDNA	Complemetary DNA
c-Fms	Colony stimulating factor 1 receptor
CMV	Cytomegalovirus
c-myc	V-myc myelocytomatosis viral oncogene homolog
CT	Calcitonin
CTX	Collagen type 1 cross-linked C-telopeptide
DAB	Diaminiobenzidene
DAF	Decay-accelerating factor
DAP12	DNAX-activating protein 12
DAPI	4,6-diamidino-2-phenylindole
DAR	DAP12-associated receptor
DEXA	Dual energy X-ray absorptiometry
DFG	Deutsche Forschungsgemeinschaft
D-MEM	Dulbeco's minimal esential media
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DNase	Desoxyribonuklease
dNTP	Deoxyribonucleotide triphosphate
Doxy	Doxycycline
DPD	Deoxypyridinoline
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay

ET	Embryo transfer
F-actin	Filamentous actin
FBS	Fetal bovine serum
FcRγ	Fc receprot common γ subunit
FDA	Federal drug administration
FSD	Functional secretory domain
g	Gravitational force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gbp	Giga base pair
GFP	Green fluorescent protein
HIV-I	Human immunodeficiency virus type I
HMSC	Human mesenchymal stem cells
hRANKL	Human RANKL
HSC	Hematopoietic stem cells
hsRANKL	Human soluble RANKL
ICSI	Intracytoplasmic sperm injection
IFNγ	Interferon gamma
Ig	Immunoglobulin
IGF-I	Insulin-like growth factor
IGFR	Insulin growth factors receptor
IL-1, 11	Interleukin 1, 11
IU	International unit
JNK	c-jun N-terminal kinase
KLF4	Krueppel-like Factor 4
LB	Luria Bertani
LTR	Long terminal repeats
M-CSF	Macrophage colony stimulating factor
MGP	Matrix Gla protein
min	Minute
mRNA	Messenger RNA
MSC	Mesenchymal stromal cells or mesenchymal stem cells
NCP	Non-collagenous protein
NEAA	Non essential amino acids
Neo	Neomycin
NF-κB	Nuclear factor κ of activated B-cells
NT	Nuclear transfer
NTX	Collagen type 1 cross-linked N-telopeptide
OCT4	Octamer binding transcription factor 4
ODF	Osteoclast Differentiation Factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
Opti-MEM	Modified eagle's minimum essential media
OSCAR	Osteoclast-associated receptor
P1NP	N-terminal type 1 procollagen
PBS	Phosphate buffered saline

pDNA	Plasmid DNA
pEF	Porcine ear fibroblast
Pen-Strep	Penicillin-streptomycin
PFA	Paraformaldehyde
pFF	Porcine fetal fibroblast
PGE2	Prostaglandin 2
PI	Propidium iodide
PI(3)K	Phosphoinositide 3-kinases
PIR-A	Paired immunoglobulin-like receptor A
pKC	Porcine kidney cell
pRANKL	Porcine RANKL
psRANKL	Porcine solube RANKL
PTH	Parathyroid hormone
pUC	Plasmid cloning vector
PVDF	Polyvinylidene Fluoride
PYD	Pyridinoline
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
RB	Ruffled border
RNA	Ribonucleic acid
RNase	Ribonucleic acid nuclease
rpm	Round per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
rtTA	Reverse Tetracycline-responsive transactivator
S.O.C	Super optimal broth with Catabolite repression
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulfate
shRANKL	Soluble human RANKL
SOX2	Sex determining region Y box 2
sRANKL	Soluble RANKL
Stbl3	Competent *E. coli* strain
SV40	Simian virus 40
TACE	TNFα converting enzyme
Tc	Tetracycline
Tet system	Tetracycline system
TetR	Tet repressor
TGF	Transforming growth factor
THD	TNF homology domain
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor α
TRAF2,5,6	TNF receptor-associated factor 2, 5, 6
TRANCE	TNF-related activation-induced cytokine
TRACP	Tartrate resistant acid phosphatase

TRACP5b	Tartrate-resistant acid phosphatase 5b
TRE	Tetracycline response element
tTA	Tetracycline-responsive transactivator
U	Unit
V-ATPase	Vacuolar-type H^+-Adenosintriphosphatase
VP16	Etoposide phosphate 16
WHO	World health organization
WT	Wild type
αMEM	Eagle's minimum essential medium

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APPENDIX

Supplementary tables

Supplementary table 1.: Nucleotide sequences of the primer sets used in this study.

Target gene		Sequence	PCR conditions (annealing temperature, cycle number) and band size
sRANKL	F	5´-GCACATCAGAGCAGAGAAAG-3´	55°C, 30x
	R	5´-TTTCCTCTCCAGACCGTAAC-3´	
rtTA	F	5´-GAAAGTGGGTCCGCGTACAG-3´	55°C, 30x, 335 bps
	R	5´-ATATCCAGAGCGCCGTAGGG-3´	
psRANKL	F	5´-CACTCCTGCTATGGGTACTG-3´	55°C, 30x, 411 bps
	R	5´-AACCGGATGGGATGTCTGTG-3´	
CA-II	F	5´-TCAACAACGGCCACTCTTTC-3	57°C, 26x, 225 bps
	R	5´-TGTGCAGCTTCTCCAAAGTC-3´	
GAPDH	F	5´-CAACTACATGGTTTACATGTTC-3´	55°C, 30x, 177 bps
	R	5´-GCCAGTGGACTCCACGAC-3´	

* F- forward, R- reverse

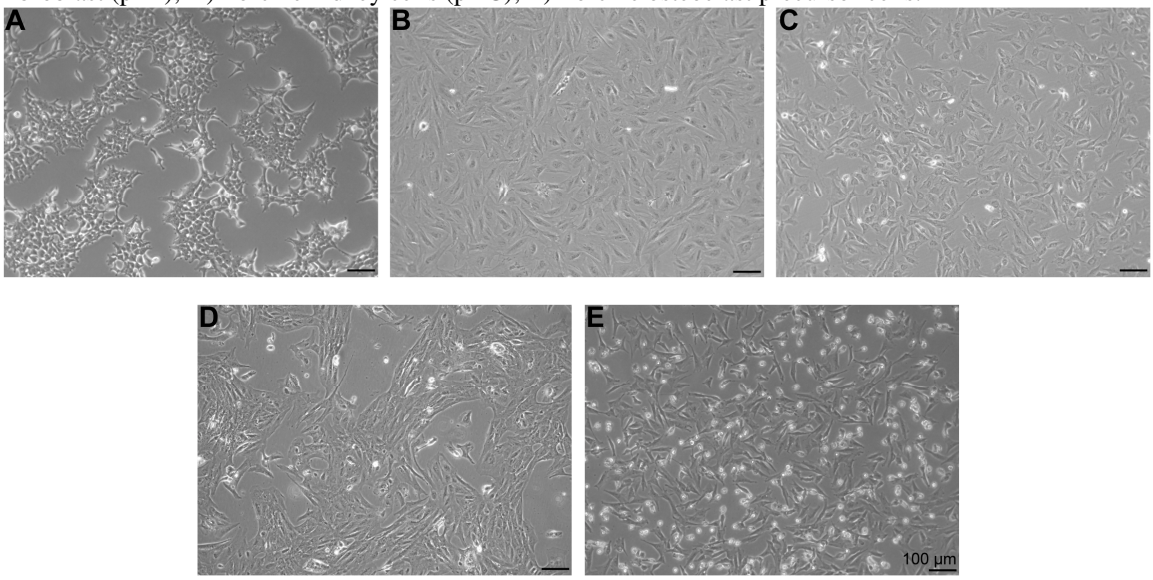
Supplementary table 2.: List of primary and secondary antibodies used in this study.

Name	Manufacturer	Host, affinity	Application	Dilutions
Primary antibodies				
Anti-Tet-repressor	Mobitec	Mouse, polyclonal	ICH	1: 50
TetR	Clontech	Mouse, polyclonal	WB	1: 500
Anti human sRANK Ligand	PeproTech	Rabbit, polyclonal	WB and ICH	1: 500 and 1:50
Secondary antibodies				
Anti- mouse AF488	Invitrogen	Donkey	ICH	1: 200
Anti-mouse biotynilated	Vector Labs	Goat, polyclonal	ICH	1:200
Anti-mouse HRP	Rockland	Rabbit	WB	1: 4000
Anti-rabbit HRP	Santa Cruz	Goat	WB	1: 4000
Anti-rabbit biotynilated	Vector Labs	Goat, polyclonal	ICH	1:200
Anti-rabbit AF488	Invitrogen	Goat	ICH	1: 500
Phalloidin	Invitrogen		ICH	1:20
Concanavalin A	Invitrogen		ICH	24 µg/ml

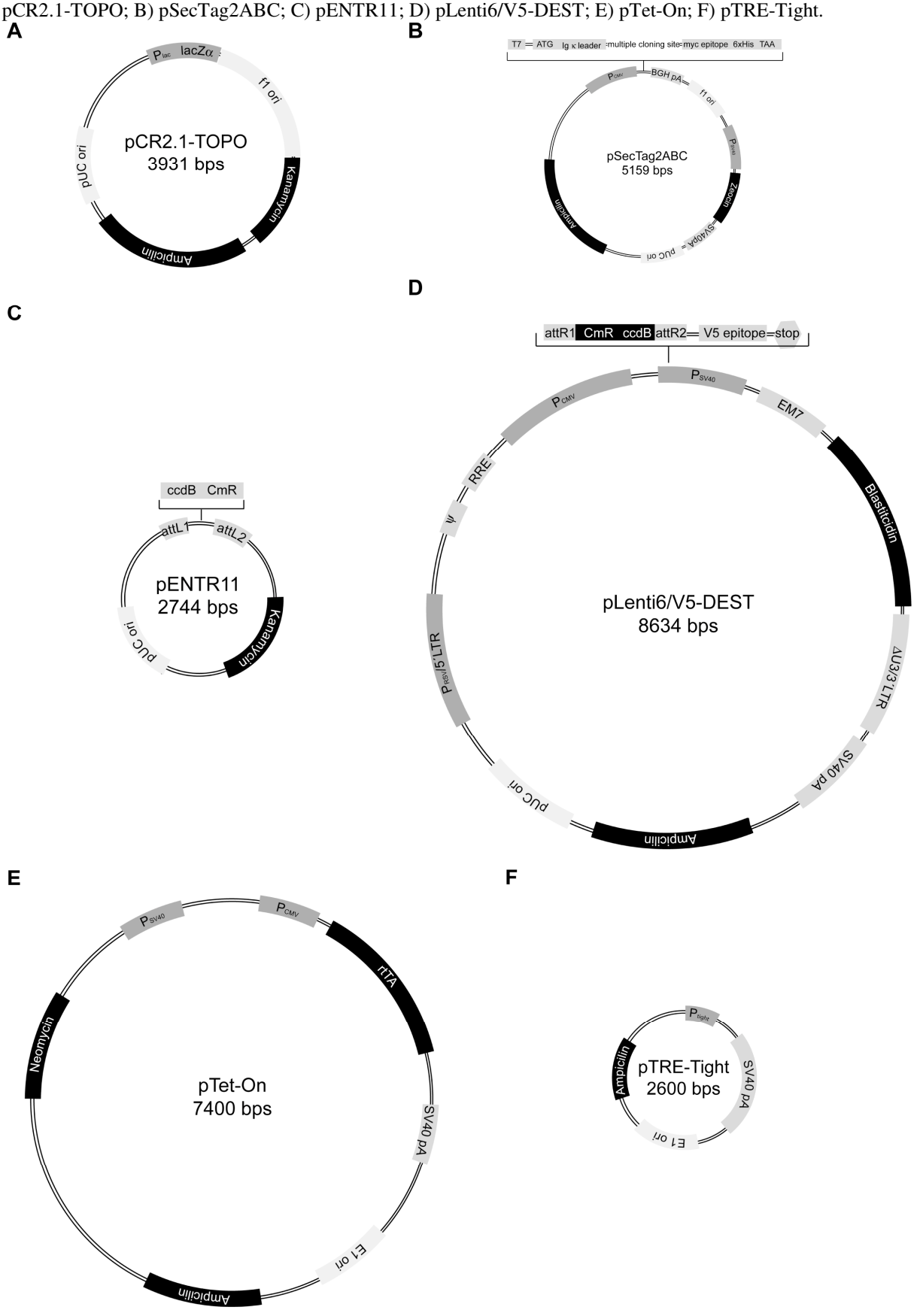
* WB – Western blotting, ICH - immunocitochemistry

Supplementary figures

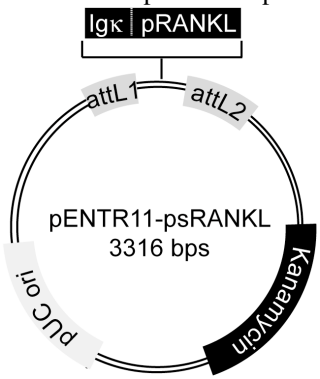
Supplementary figure 1.: Cells used in this study. A) 293FT; B) Porcine fetal fibroblast (pFF); C) Porcine ear fibroblast (pEF); D) Porcine kidney cells (pKC); E) Porcine osteoclast precursor cells.



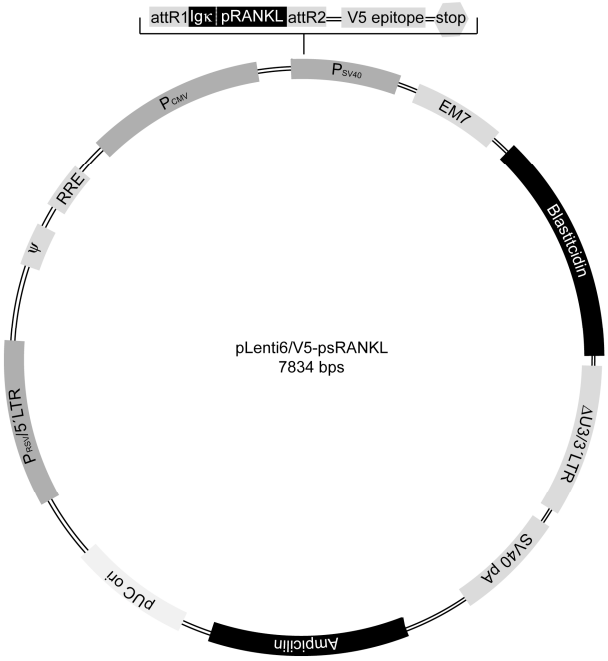
Supplementary figure 2.: Graphical representation of commercially available plasmid used in this study. A) pCR2.1-TOPO; B) pSecTag2ABC; C) pENTR11; D) pLenti6/V5-DEST; E) pTet-On; F) pTRE-Tight.



Supplementary figure 3.: Graphical representation of pENTR11-psRANKL plasmid.



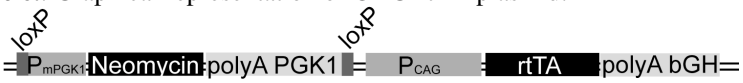
Supplementary figure 4.: Graphical representation of pLenti6/V5-psRANKL.



Supplementary figure 5.: Graphical representation of rtTA-psRANKL (Neo) plasmid.

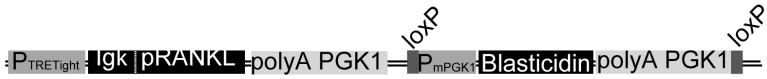
rtTA-psRANKL (Neo) (7793 bps)

Supplementary figure 6.: Graphical representation of CAG-rtTA plasmid.



pCAG-rtTA (4900 bps)

Supplementary figure 7.: Graphical representation of TRE-psRANKL plasmid.



TRE-psRANKL (3200 bps)

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DECLARATION

I, the undersigned, hereby declare that this dissertation entitled, "Regulated gene over-expression of Receptor Activator of NF-KB Ligand (RANKL) in a porcine animal model" is my own work and that all the sources I have used or quoted have been indicated or acknowledged by means of completed references.

München, 01.02.2012 _____

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PUBLICATIONS:

• **Tamara Radic**, Wolfgang Böcker, Veronika Schönitzer, Florian Haasters, Wolf Mutschler, Matthias Schieker. *Molecular Cloning and Functional Characterization of the Porcine Extracellular Domain of Receptor Activator of NF-KB Ligand (sRANKL).* (published in Gene, 16 Sep 2011)

• Nikolai Klymiuk, Wolfgang Böcker, Veronika Schönitzer, Andrea Bähr, **Tamara Radic**, Thomas Fröhlich, Annegret Wünsch, Barbara Keßler, Mayuko Kurome, Eleonore Schilling, Nadja Herbach, Rüdiger Wanke, Hiroshi Nagashima, Wolf Mutschler, Georg J. Arnold Reinhard Schwinzer, Matthias Schieker, Eckhard Wolf. *Inducible Transgene Expression for Tailored Biomedical Pig Models.* (published in FASEB Journal, 01 Dec 2011)

• Cvetan Popov, **Tamara Radic**, Florian Haasters, Wolf Christian Prall, Attila Aszodi, Donald Gullberg, Matthias Schieker and Denitsa Docheva. *The adhesion, spreading, migration and survival of bone marrow-derived human mesenchymal stem cells on collagen I are regulated by integrins α2β1 and α11β1.* (published in Cell Death and Disease, 28 Jul 2011)

• Theresa Schwarz, Simon Leicht, **Tamara Radic**, Iker Rodriguez, Patrick C. Hermann, Frank Berger, Jaimy Saif, Wolfgang Böcker, Joachim W. Ellwart, Alexandra Aicher and Christopher Heeschen. *Vascular incorporation of endothelial colony forming cells is essential for functional recovery of ischemic tissue following cell therapy.* (published in Arteriosclerosis, Thrombosis, and Vascular Biology, 22 Dec 2011).

POSTER PRESENTATION:

• Wolfgang Böcker, **Tamara Radic**, Eleonore Schilling, Nikolai Klymiuk, Wolf Mutschler, Eckhart Wolf, Matthias Schieker. *Over-expression of Receptor Activator of NF-KB Ligand (RANKL) to study metaphysial fracture healing in an osteoporotic large animal model.* Poster presented at 13. Chirurgische Forschungstage, Sep 2009

• Manfred Gödel, Julia Diegmann, **Tamara Radic**, Matha Merrow and Till Roenneberg. *Genomic and Proteomic Characterization of a New Neurospora Crassa Clock Mutant*. Poster presented at Gordon conference, May 2007