TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Biotechnologie der Nutztiere


RNA Interference in livestock

Knockdown of the porcine whey protein

Beta-Lactoglobulin

and the tumor suppressor protein p53


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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.


Vorsitzender:  Univ.-Prof. Dr. Dr. h.c. J. Bauer


Prüfer der Dissertation:  1. Univ.-Prof. A. Schnieke, Ph.D.
2. Univ.-Prof. Dr. M. Klingenspor


Die Dissertation wurde am 09.12.2009 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 19.04.2010 angenommen.

**Für meine Eltern**

"Two things are necessary for our work:

inexhaustible stamina and

the readiness to throw away something

in which one has invested a lot of time and work."

*Albert Einstein*

"Nothing shocks me. I'm a scientist."

*Indiana Jones*

# Abstract

RNA interference (RNAi) is a mechanism for sequence-specific gene silencing. It is initiated by short double stranded RNAs within a cell, leading to degradation of homologous mRNAs. This results in a reduction of the corresponding protein within the cell, mimicking loss-of-function mutations. RNAi is a routine technology in mice but application in large animals is not yet well established. In this work, RNAi mediated knockdown in pigs should be evaluated as an alternative to gene knockout by gene targeting.

Two target genes, the porcine milk whey protein Beta-Lactoglobulin (BLG) and the porcine tumor suppressor protein p53 were downregulated by RNAi. Moreover, two widely used RNAi vector systems, based on short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs), were compared.

The porcine milk whey protein Beta-Lactoglobulin (BLG) was chosen as a first target. The function of this protein is unknown and the bovine BLG protein is a major factor involved in cows' milk allergy and intolerance in children. ShRNAs were designed for the knockdown of porcine BLG and in preliminary experiments, two sequences leading to efficient downregulation were identified. These RNAi sequences were then also inserted into an artificial miRNA expression vector with different promoters (CMV, PGK, ovine BLG promoter). Cell lines expressing recombinant porcine BLG were established for the evaluation of knockdown efficiencies and the most promising shRNA and artificial miRNA construct used for the generation of RNAi transgenic porcine fetuses by somatic cell nuclear transfer. The obtained fetuses were transgenic for artificial miRNA constructs. However analysis of the RNAi transgenic fetuses by small RNA northern blot did not show detectable expression of the artificial miRNA.

The porcine tumor suppressor protein p53 was chosen because of its importance in cancer biology. Knockdown of p53 might serve as an alternative to gene knockout for the creation of an animal model for Li-Fraumeni syndrome, an inherited human cancer syndrome. ShRNAs and artificial miRNAs were designed against similar sequence positions of porcine p53 mRNA. The knockdown of porcine p53 was evaluated in a variety of assays and not all sequences showed the same knockdown potential. The most reliable prediction of the *in vivo* knockdown level is most likely provided by the downregulation of endogenous p53 in primary porcine cells. In these cells, two shRNAs and two artificial miRNAs showed up to 85% knockdown of endogenous p53. It was shown that shRNA and artificial miRNA were equally effective, depending on the target sequence. Additionally, the downregulation of porcine p53 by one artificial miRNA resulted in an enhanced resistance to the chemotherapeutic drug doxorubicin indicating a functional knockdown of porcine p53.

# Zusammenfassung

RNA Interferenz (RNAi) ist ein Mechanismus zur sequenz-spezifischen Stilllegung von Genen. Er wird durch kurze doppelsträngige RNAs in Zellen ausgelöst und führt zum Abbau von homologen mRNAs. Dadurch wird ein Effekt ähnlicher einer *loss-of-function* Mutation erzielt. Die RNAi Technologie ist in Nutztieren bisher kaum etabliert. Im Rahmen dieser Arbeit sollte ermittelt werden, ob RNAi vermittelte Herabregulierung von Genen als Alternative zu *gene knockout* mittels gezielter Genmodifikation in Schweinen dienen kann.

Zu diesem Zweck wurden zwei porzine Zielgene, das Molkeprotein Beta-Lactoglobulin (BLG) und das Tumorsuppressor Protein p53, ausgewählt. Zudem wurden zwei Vektorsysteme für RNAi, *short hairpin* RNAs (shRNAs) und künstliche mikroRNAs (miRNAs), verglichen.

Das porzine Molkeprotein Beta-Lactoglobulin wurde als erstes Zielgen ausgewählt. Die Funktion dieses Proteins ist unbekannt und bovines BLG ist einer der Auslöser für Kuhmilchallergie und -intoleranz bei Kindern. Für erste Untersuchungen wurden shRNAs gegen porzines BLG konstruiert und zwei Sequenzen mit effektiver Herabregulierung identifiziert. Diese Sequenzen wurden dann auch in einen Expressionsvektor für künstliche miRNAs mit unterschiedlichen Promotoren (CMV, PGK, oviner BLG Promotor) eingefügt. Für die Beurteilung der *knockdown* Effizienzen der Konstrukte wurden Zelllinien mit Expression von rekombinantem BLG etabliert. Die Vektoren mit der höchsten Effizienz wurden verwendet um durch somatischen Kerntransfer transgene Schweine zu erzeugen. Die erhaltenen Föten trugen künstliche miRNA Konstrukte, allerdings konnte mittels Northern Blot keine Expression von miRNAs gegen BLG nachgewiesen werden.

Das Tumorsuppressor Protein p53 wurde als Zielgen ausgewählt, da es eine wichtige Rolle in der Krebsentstehung spielt. Es sollte ermittelt werden, ob eine Herabregulierung von p53 als Alternative zu einem *gene knockout* für die Erstellung eines Großtier-Modells für eine menschliche Erbkrankheit, das Li-Fraumeni-Syndrom, dienen kann. Zu diesem Zweck wurden shRNAs und künstliche miRNAs gegen ähnliche Sequenzpositionen der p53 mRNA ausgewählt. Diese Sequenzen wurden in verschiedenen Testsystemen untersucht und nicht alle Sequenzen zeigten in diesen Untersuchungen das gleiche Potential. Die verlässlichste Aussage bezüglich eines *in vivo* Effekts ist sehr wahrscheinlich die Herabregulierung von endogenem porzinem p53 in Primärzellen. In diesen Zellen konnten je zwei shRNAs und künstliche miRNAs die Menge an p53 Protein unabhängig vom verwendeten Vektorsystem um bis zu 85% reduzieren. Zudem führte die Herabregulierung von p53 mit einer künstlichen miRNA Sequenz zu einer erhöhten Resistenz gegenüber dem Chemotherapeutikum Doxorubicin. Dies deutet auf einen funktionalen *knockdown* von p53 hin.

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# 1    Introduction

The goal of the work described in this thesis was to explore, whether the RNA interference technology can be used for the downregulation of gene expression in pigs. This introduction presents a brief overview of transgene technologies with an emphasis on pigs, including random transgenesis and targeted modification of endogenous genes by gene targeting. The advantages and disadvantages of RNAi compared to gene targeting, especially in livestock, are considered followed by an overview of the RNAi mechanism. Finally a brief description of the experimental target genes, which have relevance in nutritional research and functional genomics (Beta-Lactoglobulin) or in biomedicine (Tumor suppressor protein p53) will be provided.

## 1.1    Genetically modified animals in science

A transgenic animal is defined as an animal which has foreign DNA incorporated into its germline after experimental introduction of DNA (Palmiter and Brister, 1985). A wider definition includes the modification of the endogenous genomic sequence of an animal (Melo *et al.,* 2007). A variety of species have been used to generate transgenic animals for a wide range of applications in agriculture, medicine and industry. Transgenic animals, in particular mice, are valuable tools for scientific applications. Although the mouse remains the most important model for studying gene function and dysfunction, livestock species have gained relevance for research over the last years. This can be attributed to the fact that in particular pigs resemble humans more closely in size, organ development, physiology and are genetically closer to humans than mice (Lunney, 2007; Wernersson *et al.,* 2005). Large animals can be used to study the function of genes, as disease models, to develop new therapeutic drugs or to produce recombinant proteins. In addition genetically modified pigs might be used to provide organs for xenotransplantation in the following years.

The following section will focus on the generation of transgenic pigs by a variety of methods, including pronuclear microinjection of DNA, sperm-mediated gene transfer, retroviral transgenesis and genetic manipulation of cells in tissue culture followed by somatic cell nuclear transfer (SCNT).

### DNA microinjection

For many years, the most common technique to produce transgenic pigs was microinjection of purified DNA into the male pronucleus of a fertilized oocyte. This method was first successfully used in mice and five years later, the first transgenic pigs were born after pronuclear microinjection (Gordon *et al.,* 1980; Hammer *et al.,* 1985). Since then various transgenic pigs have been generated by this method. Some examples are pigs expressing human growth hormone, the human complement regulatory protein CD59 and miniature pigs

expressing the minipig huntingtin gene (Hirabayashi *et al.,* 2001; Niemann *et al.,* 2001; Uchida *et al.,* 2001). The percentage of transgenesis at birth ranges from 5 to 11%. The DNA microinjection procedure is outlined in Figure 1. Although pronuclear microinjection is one of the main methods used to generate transgenic pigs, it has several disadvantages. The efficiency of transgenic livestock per injected zygote is generally under 1%. Moreover, the integration site of the recombinant transgene or its copy number into the genome cannot be controlled. All these factors lead to variable gene expression and random integration of the foreign DNA can also result in insertional mutagenesis (Robl *et al.,* 2007).

**Sperm-mediated gene transfer**

An alternative approach to introduce foreign DNA into animal genomes is sperm-mediated gene transfer (SMGT). This method utilizes the ability of sperm cells to transport foreign DNA into the oocyte (Brackett *et al.* 1971; Francolini *et al.,* 1993). It was successfully applied in mice where transgenic animals were produced after *in vitro* fertilization and transmission of the introduced DNA to the F1 generation was observed (Lavitrano *et al.,* 1989). The technique was also used to produce transgenic pigs expressing human decay-accelerating factor (hDAF) or multi-transgenic pigs that express three different fluorescent proteins. Furthermore pigs expressing enhanced green fluorescent protein (EGFP) from an episomal vector have also been generated by SMGT (Lavitrano *et al.,* 2006; Manzini *et al.,* 2006) and transgenic efficiencies of up to 80% of born pigs have been reported (Lavitrano *et al.,* 2006). Nevertheless, SMGT suffers from low reproducibility between different laboratories and in different species and is therefore not widely used (Robl *et al.,* 2007).

In addition to sperm-mediated gene transfer, intracytoplasmatic sperm injection mediated gene transfer (ICSI-GT) provides an alternative method to produce transgenic animals. The method was established by Perry *et al.* (1999) and transgenic mice generated after incubation of sperm with plasmid DNA followed by injection of the sperm into the cytoplasm of an oocyte. Moreover, ICSI-GT has also been successfully applied to produce transgenic mice with large 176-200 kb bacterial artificial chromosome constructs (Osada *et al.,* 2005). In 2006, Kurome *et al.* reported a transgenic porcine fetus and the birth of a transgenic piglet expressing human albumin and EGFP after ICSI-GT. The transgenic efficiency per treated oocyte was low (0.3%) but the rate of transgenic fetuses or piglets of all fetuses/piglets reached 5.7% (Kurome *et al.,* 2006). Therefore the efficiencies are comparable to those achieved by DNA microinjection.

**Retro- and lentiviral transgenesis**

More recently, transgenic pigs have been generated by viral transgenesis with retro- or lentiviral vectors. Retrovirus mediated transgenesis was first demonstrated in mice (Jaenisch, 1976) and in 2001 Cabot *et al.* reported the generation of piglets with EGFP

expression from a Moloney murine leukemia virus (MMLV) based vector after injection of retroviral particles into the perivitelline space of porcine oocytes. However, expression of genes from retroviral constructs is often epigenetically inactivated during embryo development or after birth and therefore impairs the use of retroviral vectors to create transgenic animals (Chan *et al.,* 1998; Pfeifer, 2004).

Human immunodeficiency virus (HIV-1) based lentiviral vectors offer an alternative for generating transgenic animals since they are not silenced during embryogenesis and can also infect non dividing cells (Pfeifer, 2004; Robl *et al.,* 2007). Lentiviral transgenesis was established after injection of concentrated lentiviral supernatant under the *zona pellucida* of mouse zygotes (Lois *et al.,* 2002). Shortly after, transgenic pigs expressing EGFP in all tissues under the control of the ubiquitous PGK or skin-specific from a construct containing the keratin K14 promoter were obtained after injection of high titer ($10^9$ to $10^{10}$ infectious units per ml) HIV-1 based lentiviral vectors into the perivitelline space of zygotes. Analysis showed that at birth, 70% of the animals were transgenic and 65% expressed the transgene (Hofmann *et al.,* 2003). Furthermore a lentiviral vector derived from the equine infectious anemia virus (EIAV) showed efficient transgenesis in pig. 92% of the born animals were transgenic and 85% showed expression of EGFP (Whitelaw *et al.,* 2004). Lentiviral injection into the perivitelline space is outlined in Figure 1. Despite the high efficiency of generating transgenic animals, lentiviral transgenesis has some drawbacks. First, high viral titers are necessary to generate transgenic animals which is not easily achieved (Al Yacoub *et al.,* 2007), second, the capacity for foreign DNA in lentiviral vectors is limited to about 10 kb (Sinn *et al.,* 2005) and third, random integration of the provirus can lead to disruption of endogenous genes (Robl *et al.,* 2007).
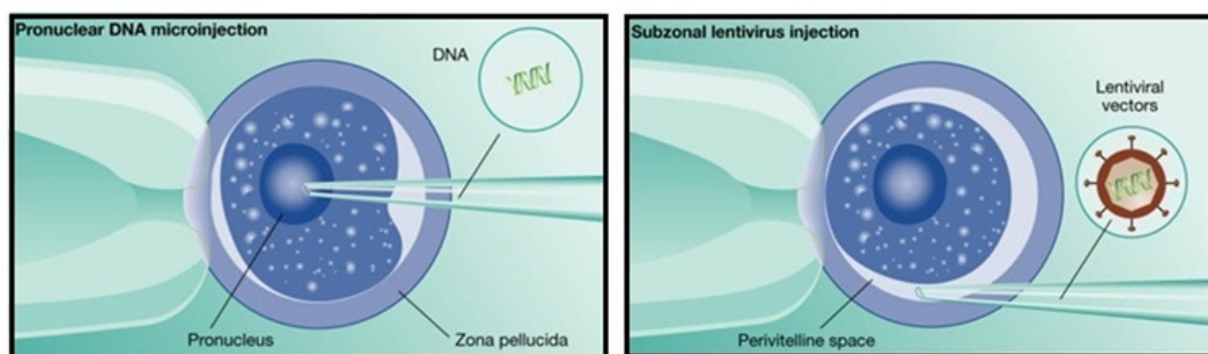


**Figure 1: Pronuclear DNA microinjection and subzonal lentivirus injection (Fässler, 2004).** For pronuclear microinjection, purified DNA is injected into one pronucleus of a fertilized oocyte. Subzonal lentivirus injections are performed by injecting high titer lentiviral vectors in the perivitelline space of a zygote.

**Somatic cell nuclear transfer**

Another widely used method to generate transgenic livestock animals employs the modification of somatic cells *in vitro* followed by somatic cell nuclear transfer (SCNT). While DNA microinjection, sperm-mediated DNA transfer and lentiviral vectors only allow addition of genes after random integration, *in vitro* manipulation of cells followed by SCNT can also be used to precisely modify the mammalian genome by gene targeting.

Three years after the first report of mammals cloned from somatic cells (Wilmut *et al.,* 1997), several groups reported successful cloning of pigs by SCNT (Polejaeva *et al.,* 2000; Onishi *et al.,* 2000; Betthauser *et al.,* 2000). Onishi *et al.* (2000) microinjected porcine fetal fibroblast nuclei into enucleated oocytes, Betthauser *et al.* (2000) fused porcine fetal fibroblasts and Polejaeva *et al.* (2000) granulosa cells with enucleated oocytes. In addition to fetal fibroblasts and granulosa cells, other cell types, such as preadipocytes and adult fibroblasts have also been used to generate cloned pigs and efficient SCNT with porcine adult somatic stem cells could be shown *in vitro* for bone marrow derived mesenchymal stem cells (Tomii *et al.,* 2005; Tomii *et al.,* 2009; Brunetti *et al.,* 2008; Colleoni *et al.,* 2005; Bosch *et al.,* 2006; Kumar *et al.,* 2007; Jin *et al.,* 2007).

SCNT can be applied to clone animals but also to generate genetically modified animals. The first transgenic animals born after genetic manipulation of cells *in vitro* followed by SCNT were sheep expressing human factor IX (Schnieke *et al.,* 1997). In the following years, several genetically modified pigs generated by SCNT, expressing for example fluorescent proteins or human erythropoietin, have been reported (Hyun *et al.,* 2003; Kurome *et al.,* 2006; Brunetti *et al.,* 2008; Lee *et al.,* 2005; Cho *et al.,* 2009; Umeyama *et al.,* 2009).

Manipulation of cells followed by SCNT can be used to precisely modify the cell genome by gene targeting. This approach uses homologous recombination (HR) to replace genomic sequences in cells with an exogenous DNA sequence, leading to knockout, knockin or precise mutations of endogenous genes (Thomas *et al.*, 1986). In mice, embryonic stem (ES) cells allow the transfer of a modified genotype into an animal by creating chimeric mice after injection of ES cells into blastocysts (Evans and Kaufman, 1981; Martin, 1981; Bradley *et al.,* 1984; Capecchi, 2005) and gene targeting by HR in ES cells has become a routine procedure. In addition, the complete genome sequence of the mouse is known, facilitating the generation of gene targeting vectors. Due to the fact that in livestock species, definitive ES cells have not yet been isolated (Talbot and Blomberg, 2008), somatic cells are used for gene targeting followed by SCNT. The procedure for creating gene-targeted mice from ES cells and gene-targeted livestock from somatic cells is illustrated in Figure 2.
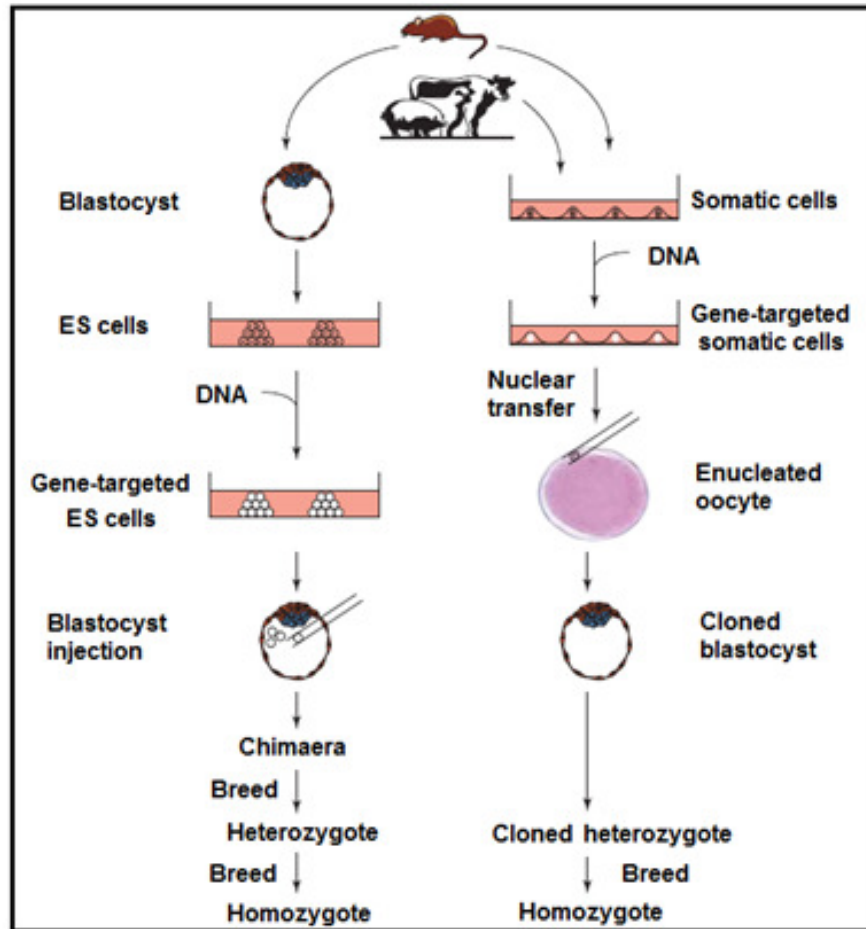
**Figure 2: Comparison of gene-targeting in mice and livestock (adapted, from Denning and Priddle, 2003).** Left: Generation of gene-targeted mice via transfection of ES cells followed by blastocyst injection. The resulting chimeric animals are bred to homozygosity. Right: Generation of gene-targeted mice and livestock by gene-targeting in somatic cells followed by somatic cell nuclear transfer. Heterozygous animals are bred to homozygotes.

Gene targeting by HR in somatic cells is less efficient than in ES cells. A direct comparison showed that one in six G418 resistant colonies in murine ES cells and one in hundred colonies in murine myoblasts were correctly targeted (Arbonés *et al.,* 1994). Moreover, it has been shown that in sheep fibroblasts the efficiency of HR is about two orders of magnitude lower than in murine ES cells (Piedrahita, 2000; Wang and Zhou, 2003).

This low rate of HR in somatic cells hampers the routine application of gene targeting in livestock species and therefore techniques to augment correctly targeted cells have been developed. If the target gene is expressed, cells can be selected using vectors with promoter-less selectable markers. These constructs, so called promoter-trap vectors, can only confer resistance if they are integrated downstream of endogenous promoter sequences allowing an enrichment of targeted cells (reviewed in Robl *et al.,* 2007). However, this approach cannot be applied to genes not transcribed in a particular cell type. In this case, selectable markers with promoter sequences are used which results in resistant cells after random integration as well as gene targeting. Therefore an enrichment of correctly targeted

cells is not possible, resulting in large numbers of cell clones which have to be analyzed. In a direct comparison in porcine fetal fibroblasts, the efficiency of targeting the alpha1,3-galactosyltransferase was 2.3% with and 0.07% without promoter-trap (Thomson *et al.,* 2003).

Modification of somatic cells is followed by SCNT to generate gene-targeted animals. The first gene-targeted animals born were sheep (McCreath *et al.,* 2000) and in pigs, gene targeting has been successful for the knockout of alpha1,3-galactosyltransferase and the disruption and mutation of the cystic fibrosis transmembrane conductance receptor (CFTR) (Lai *et al.,* 2002; Dai *et al.,* 2002; Rogers *et al.,* 2008). The low number of gene targeted pigs shows that it is still not a routine technology.

A comparison of the methods to generate transgenic mice and pigs is presented in Table 1.

**Table 1: Comparison of transgenesis methods in mouse and pig**

| Transgenesis method | Mouse | Pig | Efficiency in pigs (transgenic animals per born animals) |
|---|---|---|---|
| DNA microinjection | + | + | 5 – 11 % |
| Sperm-mediated gene transfer | + | + | up to 80 % |
| ICSI mediated gene transfer | + | + | 6 % |
| Lentiviral transgenesis | + | + | 70 – 90 % |
| *In vitro* manipulation of ES cells, chimera | + | - | - |
| *In vitro* manipulation of primary cells, SCNT | + | + | 100 % |

**RNA Interference**

A widely used method for gene silencing in cells and animals is RNA Interference (RNAi), a mechanism initiated by double stranded RNA leading to sequence-specific downregulation of gene expression either by translational repression or degradation of the homologous mRNA. RNAi therefore represents an alternative to gene knockout. Moreover, stable gene knockdown can be achieved by introducing double stranded RNA expression constructs into cells which can then either be used for *in vitro* experiments or for generating RNAi transgenic animals after SCNT or in case of ES cells by chimera formation. In addition, the constructs can be introduced into fertilized oocytes by pronuclear microinjection or lentiviral transgenesis to generate RNAi transgenic animals.

In livestock, the method might offer an alternative to gene targeting, especially if the target gene is not expressed in somatic cells. Additionally, many livestock species have not yet been fully sequenced. This limits the creation of gene-targeting vectors since genomic sequence information is necessary for generating them. The advantage of RNAi is that only

the mRNA sequence needs to be available to produce knockdown constructs and this sequence is more rapidly obtained than large genomic sequences.

Knockdown of genes by RNAi can generate effects similar to loss-of-function mutations and might serve as a substitute for gene targeting. Furthermore it is generally not necessary to breed animals to homozygosity to obtain a specific phenotype, allowing analysis in the F0 generation (Kunath *et al.,* 2003). This reduces the time needed to generate animals with downregulation of gene expression, which is especially valuable in livestock species with long gestation times. A comparison between gene knockout by gene targeting and knockdown by RNAi in mice is illustrated in Figure 3.
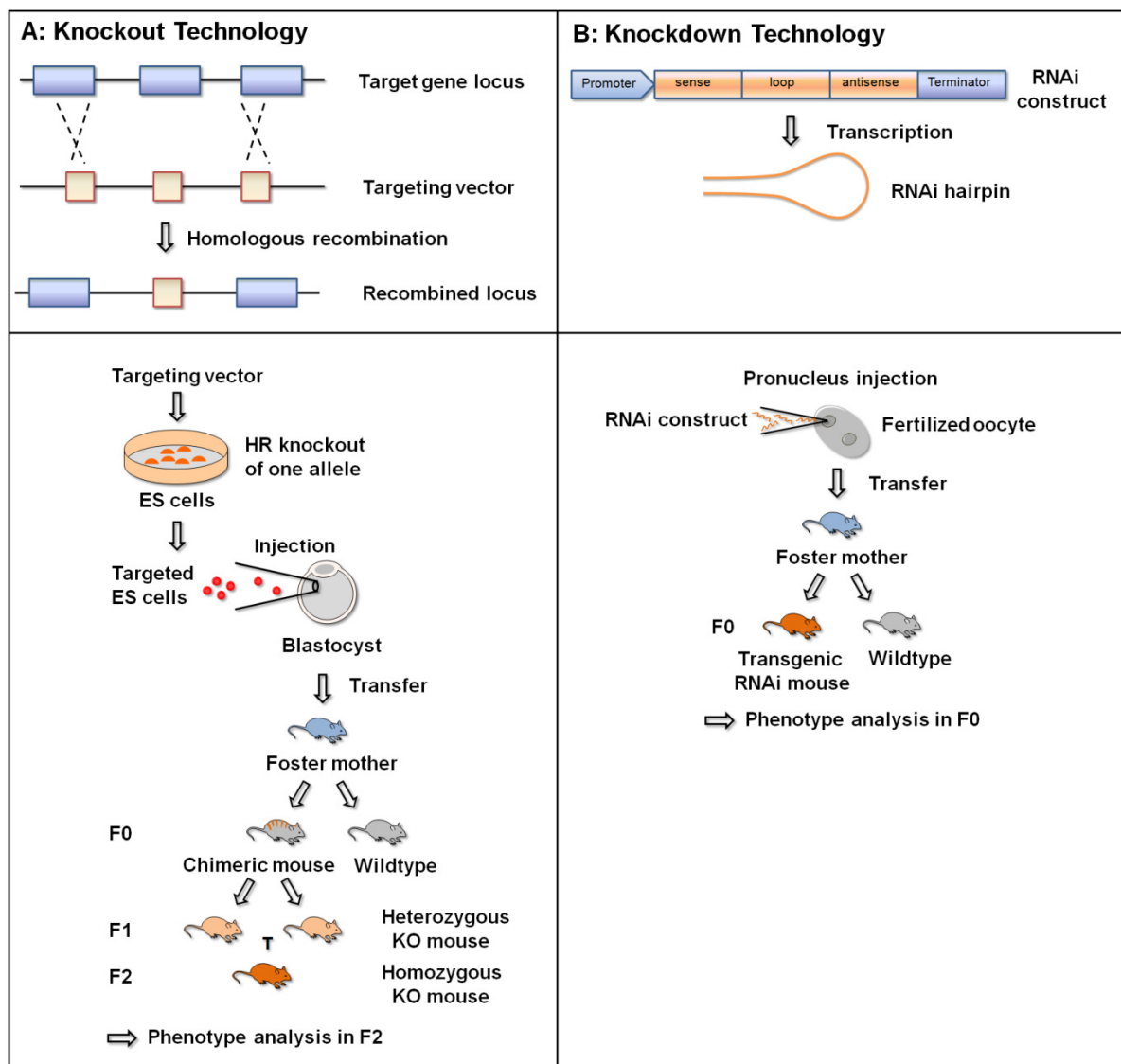


**Figure 3: Generation of knockout and knockdown mice (adapted, from Prawitt *et al.,* 2004).**  A: Knockout technology; B: Knockdown technology. Details see text.

In contrast to knockout animals produced by gene targeting, the reduction of gene expression by RNAi is not complete. This effect is a consequence of the copy number, the integration site and the knockdown potential of the chosen RNAi sequence in the animals

and allows different degrees of loss-of-function conditions, which can mimic hypomorphic mutations resembling human disease phenotypes (Rao and Wilkinson, 2006; Hemann *et al.,* 2003). Furthermore it is possible to downregulate gene expression in the whole animal or tissue/developmental stage specific. This is achieved using RNAi constructs based on microRNAs which can be expressed from RNA polymerase II promoters, allowing an appropriate promoter to be chosen to express the RNAi construct in a tissue of choice (Hasuwa *et al.,* 2002; Xia *et al.,* 2006; Rao *et al.,* 2006). Tissue or developmental stage specific gene-knockout approaches are more difficult to accomplish. Conditional gene targeting is based on site-specific recombinase systems such as Cre-loxP. To achieve tissue or developmental stage specific gene knockout, animals with the modified endogenous gene including the recombinase recognition sites have to be bred with animals expressing the recombinase from an appropriate promoter (Furuta and Behringer, 2005). Therefore, two different genetic modifications are necessary for tissue-specific gene knockout whereas one RNAi construct is sufficient. These are good reasons to apply the RNAi technology to livestock especially considering the lack of ES cells, difficulties regarding gene targeting and possible time savings.

At the time this project was started, only one report concerning RNAi in livestock species had been published. Golding *et al.* (2006) had produced one cloned goat fetus with knockdown of the prion protein (PrP). In the following years it was shown that RNAi is applicable in pigs by generating animals with downregulation of porcine endogenous retroviruses (PERVs) (Dieckhoff *et al.,* 2008; Ramsoondar *et al.,* 2009).

The following sections provide an outline of how RNAi downregulates gene expression, a more detailed description of applications of RNAi and background to the target genes chosen for this project.

## 1.2   RNA interference

This section describes the mechanism of RNA mediated gene silencing under endogenous and experimental conditions. Moreover, some examples of *in vitro* and *in vivo* applications will be given.

### 1.2.1  Overview of RNA mediated gene silencing

**Definition of RNA interference:**

RNA interference (RNAi) is a mechanism initiated by the presence of double stranded RNA within a cell leading to sequence-specific post-transcriptional gene silencing. Different types of double stranded RNA can downregulate the expression of genes by translational repression or degradation of the homologous mRNA.

**Short history of RNAi**

RNAi was first observed in plants and termed post-transcriptional gene silencing (PTGS) or co-suppression and in fungi it was named quelling but the exact mechanism was unknown (Napoli *et al.,* 1990; Baulcombe, 1996; Romano and Macino,1992). The phenomenon of RNAi in animals was first discovered by Fire and Mello in the nematode *Caenorhabditis elegans* (*C. elegans*) (Fire *et al.,* 1998) for which they were awarded the Nobel Prize in Physiology or Medicine in 2006. Fire and coworkers compared single stranded (ssRNA) and double stranded RNAs (dsRNA) for their ability to mediate gene silencing in *C. elegans*. After injection, only worms treated with dsRNA showed a phenotype identical to a loss-of-function mutation of the studied gene. To verify the effect, they performed similar experiments with three other genes. These experiments showed that dsRNA of 300 to 1000 basepairs (bp) was the trigger for gene specific silencing and the resulting phenotype resembled null-mutants. In addition the downregulation of endogenous mRNA complementary to one strand of the dsRNA could be shown. The phenomenon was referred to as "RNA-mediated interference" (RNAi) (Fire *et al.,* 1998).

In the following years the RNAi pathway was studied in more detail and in 2000, the central molecules of the RNAi pathway, the so-called short interfering RNAs (siRNAs), were discovered. These siRNAs with a size of 21 to 23 bp are generated from long exogenous and endogenous dsRNA in the cell by the endoribonuclease Dicer (Zamore *et al.,* 2000). They are the effector molecules of RNAi and lead to degradation of complementary mRNA (Chu and Rana, 2007).

The RNAi pathway is highly conserved and plays an important role in defending the genome from repetitive elements, transposons, viruses and aberrant transcripts by degrading the corresponding RNAs. The mechanism can also be used to downregulate gene expression by addition of artificial dsRNAs or siRNAs (Zamore, 2002).

RNAi presented a valuable tool for studying gene function but attempts to use dsRNA longer than 500 bp for RNAi in mammalian cells were only successful in mouse oocytes, early embryos, undifferentiated mouse ES cells and mouse embryonic carcinoma cells (Svoboda *et al.,* 2000; Wianny and Zernicka-Goetz, 2000; Yang *et al.,* 2001; Billy *et al.,* 2001). In other mammalian cells, long dsRNA caused activation of the interferon response resulting in general decrease of mRNA production instead of sequence-specific gene silencing (Caplen *et al.,* 2000; Elbashir *et al.,* 2001b). The interferon response was triggered by dsRNA longer than 30 bp, which led to an activation of the protein kinase PKR and 2',5'-oligoadenylate synthetase. This resulted in a stop of protein translation and a non sequence-specific degradation of mRNA (Stark *et al.,* 1998*;* Elbashir *et al.,* 2001a).

The success of RNAi as an efficient method to repress gene expression in mammalian cells emerged in 2001 when Elbashir *et al.* discovered that RNAi could be triggered by exogenous short instead of long dsRNAs. They showed that base-paired RNAs with a length of 21 to 23 bp and two 3' thymidine overhangs could mediate sequence-specific gene silencing in mammalian cells. The RNAs were named short interfering RNAs (siRNAs) and led to silencing of endogenous genes in mammalian cells without activating the interferon response pathway (Elbashir *et al.,* 2001a; Elbashir *et al.,* 2001b; Caplen *et al.,* 2001).

**Endogenous small RNAs - MicroRNAs:**

As already mentioned, the endogenous RNAi pathway is mainly a defense mechanism that protects the organism from exogenous and endogenous long dsRNA derived from viruses, repetitive elements, transposons and aberrant transcripts. Moreover, several types of genome encoded small dsRNAs with gene regulatory function have been found in a variety of organisms. One example is the so called microRNAs, originating from genomic pri-miRNA transcripts that form hairpin structures (Lagos-Quintana *et al.,* 2001). These precursors are processed into 19 to 25 bp long microRNAs (miRNAs) in the cell by the endoribonucleases Drosha and Dicer (Chu and Rana, 2007). The resulting miRNAs show homology to 3' untranslated regions (UTRs) of mRNAs and lead to translational inhibition or mRNA degradation (Lee *et al.,* 1993; Reinhart *et al.,* 2000). MiRNAs are involved in the regulation of gene expression in many developmental stages as well as tissues and about one third of all human genes are thought to be regulated by miRNAs (Lau *et al.,* 2001; Lagos-Quintana *et al.,* 2001; Lee and Ambros, 2001; O'Hara *et al.,* 2009). Generally miRNAs are not fully complementary to their cognate mRNA sequences and furthermore, miRNA stem loop precursors do not show perfect base-pairing within the hairpin region. In contrast to perfectly basepaired siRNAs, this results mainly in translational inhibition instead of mRNA cleavage (Pasquinelli, 2002; Agrawal *et al.,* 2003; Carrington and Ambros, 2003). Nevertheless, artificial experimental miRNAs can be designed to mediate mRNA cleavage similar to siRNAs. Since endogenous miRNA are generally expressed from RNA polymerase II promoters, the artificial miRNAs can be expressed ubiquitous or tissue-specific (McManus *et al.,* 2002; Gregory *et al.,* 2005).

### 1.2.2  Mechanisms of RNA interference mediated gene silencing

Sequence-specific gene silencing in animals mediated by double stranded can be divided into three steps. At first, long double stranded RNA (dsRNA) is converted to short interfering RNAs (siRNAs) by the enzyme Dicer in the cytosol. In the next step, the siRNA is unwound and one strand incorporated into the RNA-induced silencing complex (RISC). Finally, RISC complexed with the guide strand, mediates cleavage of the target mRNA (Siomi and Siomi, 2009; Carthew and Sontheimer, 2009).

More steps are involved in miRNA mediated gene silencing in animals. MiRNAs are transcribed as pri-miRNAs. Processing by Drosha/DGCR8 in the nucleus generates pre-miRNAs after flanking regions are removed. Then the pre-miRNA is exported from the nucleus. In the cytosol Dicer generates mature miRNAs and the guide strand is incorporated into the RISC. This complex, loaded with miRNAs, binds to target mRNAs and inhibits their translation or leads to their cleavage (Siomi and Siomi, 2009; Carthew and Sontheimer, 2009). An overview of the mechanism of siRNA and miRNA mediated gene silencing is shown in Figure 4.
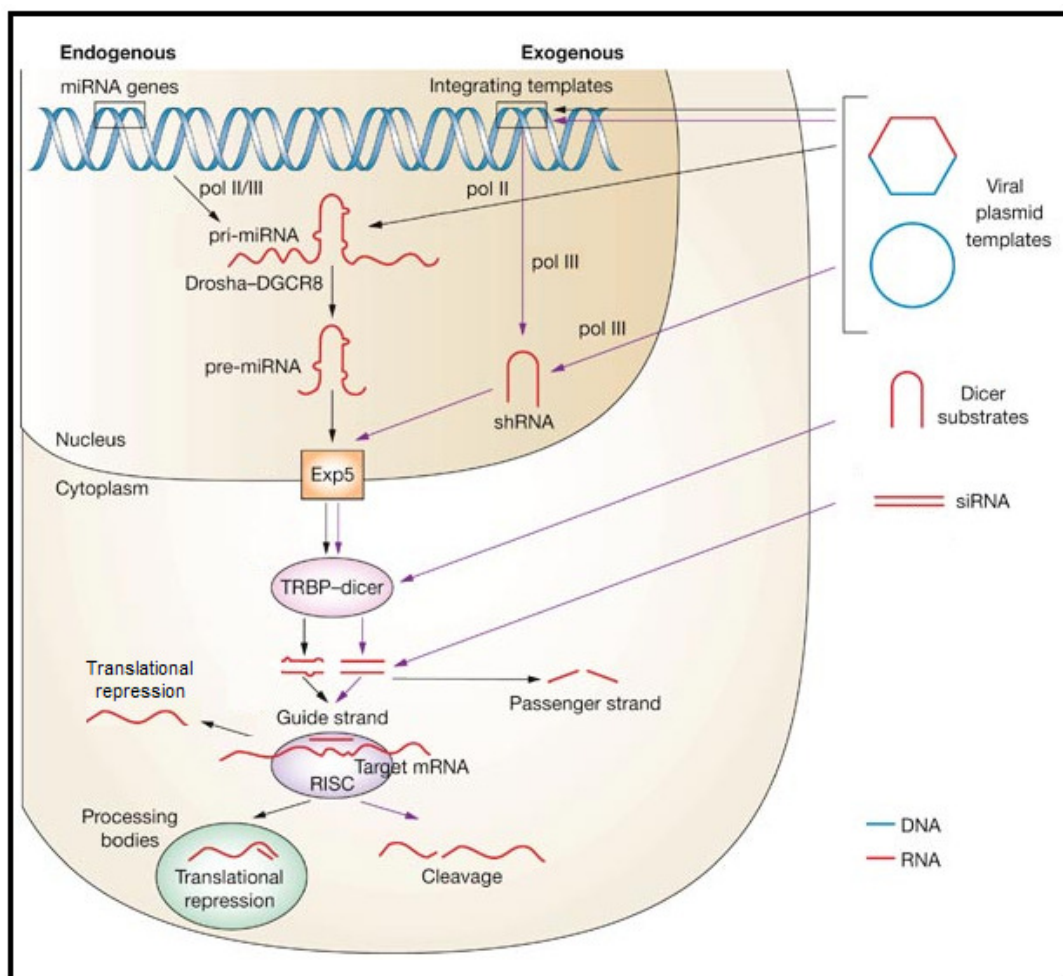


**Figure 4: Processing of and silencing by double stranded RNA in mammalian cells (adapted, from Gonzalez-Alegre and Paulson, 2007).** Different RNA effector molecules can mediate sequence-specific gene silencing. Among those are shRNAs, siRNAs, natural and artificial miRNAs. Although the generation of siRNAs and miRNAs differs, both molecule types are finally incorporated into RISC. The result is either sequence-specific degradation of the complementary mRNA after perfect base pairing between the short RNA and the mRNA or translational repression after imperfect base pairing.

### Biogenesis of small RNAs

Short interfering RNA (siRNAs) and microRNAs (miRNAs) are the central effector molecules of RNA mediated gene silencing. Several different precursors are processed into siRNAs or miRNAs.

**Short interfering RNAs:**

SiRNAs are either generated by Dicer cleavage of long endogenous or exogenous dsRNA, by introducing short hairpin RNAs (shRNAs) or are exogenously added to the cell (Bernstein *et al.,* 2001; Elbashir *et al.,* 2001a; Elbashir *et al.,* 2001b; Paddison *et al.,* 2002). Long dsRNA from natural or experimental sources is cleaved into siRNAs by the endoribonuclease Dicer in the cytosol after recognition of the 3' end of the dsRNA. The siRNAs with a size of 21 to 23 nt contain 5' phosphate groups, 3' hydroxyl groups and 3' overhangs of two nucleotides (Elbashir *et al.,* 2001a; Bernstein *et al.,* 2001; Zhang *et al.,* 2004; Macrae *et al.,* 2006). Short hairpin RNAs are artificial constructs resembling siRNAs where the two siRNA strands are connected by a loop structure. The shRNAs can be either added exogenously to the cells, transiently transcribed from plasmid DNA or transcribed after genomic integration of the construct (Paddison *et al.,* 2002; Yu *et al.,* 2002; Miyagishi and Taira, 2002; Paul *et al.,* 2002; Brummelkamp *et al.,* 2002). RNA polymerase III promoters are generally used for the expression of shRNAs. They are specific for the expression of small RNAs and usually have a defined transcriptional stop signal composed of four to five thymidine bases (Bogenhagen and Brown, 1981). Commonly used promoters are the murine or human U6 small nuclear RNA or the human H1 RNA promoter (Das *et al.,* 1988; Kunkel *et al.,* 1986; Myslinski *et al.,* 2001). After the shRNA is exported from the nucleus, the loop sequence of short hairpin RNAs is cleaved by Dicer, generating siRNAs (Yi *et al.,* 2003; Lund *et al.,* 2004; Vermeulen *et al.,* 2005). Exogenously added experimental siRNAs are not processed by Dicer because they are designed to resemble Dicer cleavage products (Provost *et al.,* 2002). The generation of siRNA is depicted in Figure 5.
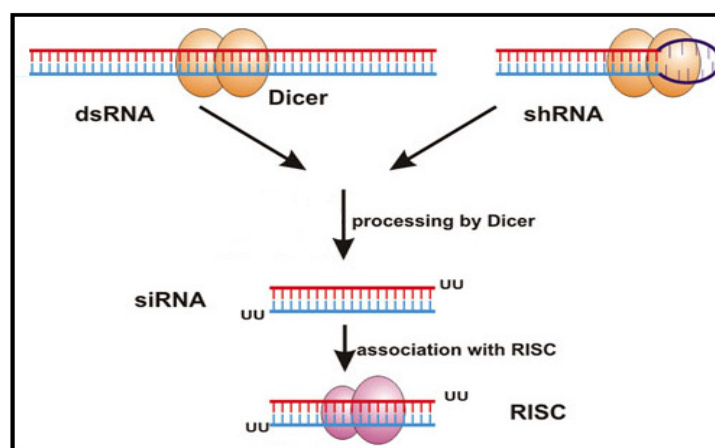


**Figure 5: Generation of siRNAs (adapted, from Rutz and Scheffold, 2004).** Long dsRNA or short hairpin RNAs (shRNA) are cleaved by Dicer into short interfering RNAs (siRNAs). Alternatively, siRNAs can be directly added to the cells.

**MicroRNAs:**

Another type of small RNAs is miRNAs. These can originate from genome encoded pri-miRNAs, after splicing from introns or from artificial miRNA expression cassettes. Natural genome encoded pri-miRNAs are generated mostly after transcription by RNA polymerase II. The precursors contain 5' caps and polyA tails (Lee *et al.,* 2004). As an alternative, artificial miRNA expression cassettes mimicking natural miRNA genes can be introduced (McManus *et al.,* 2002). These precursors comprise stem loop structures containing the mature miRNA and flanking regions (Lau *et al.,* 2001; Lee *et al.,* 2002). In animals, the RNAse III enzyme Drosha, complexed with its cofactor DGCR8, removes non-paired flanking regions from the pri-miRNA in the nucleus to generate stem-loop containing pre-miRNAs of about 65 to 70 nucleotides (Lee *et al.,* 2003; Gregory *et al.,* 2004; Denli *et al.,* 2004). Another mechanism for generating pre-miRNAs is splicing dependent. In this case the pre-miRNAs are located in introns, called mirtrons. They are spliced from the precursor RNA and do not require processing by Drosha (Berezikov *et al.,* 2007; Okamura *et al.,* 2007; Ruby *et al.,* 2007).

In animals pre-miRNAs are exported from the nucleus via exportin-5. Mature miRNAs are then generated by Dicer mediated removal of the loop sequence connecting the two miRNA strands (Yi *et al.,* 2003; Lund *et al.,* 2004; Bernstein *et al.,* 2001; Ketting *et al.,* 2001). The processing steps for the generation of miRNAs in animals are shown in Figure 6.
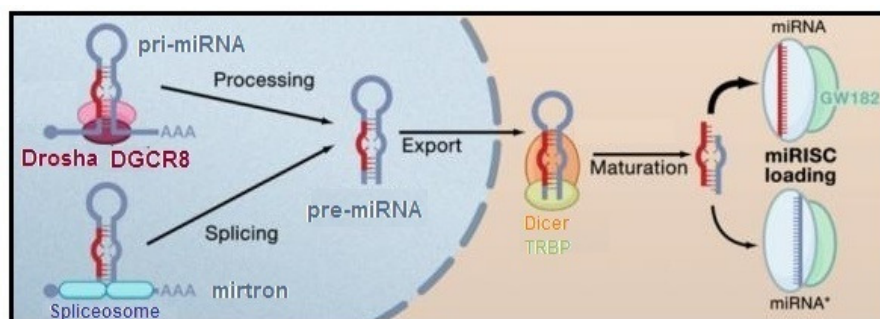


**Figure 6: Biogenesis of miRNAs in animals (Carthew and Sontheimer, 2009**). The flanking regions of pri-miRNAs are removed by Drosha/DGCR8 or the pre-miRNA is generated by splicing from mirtrons. The resulting pre-miRNAs are exported to the cytosol and a miRNA duplex is generated by Dicer. Thereafter miRNAs are assembled into RISC.

**Loading of siRNAs and miRNAs onto the RNA-induced silencing complex:**

To mediate silencing activity, the siRNAs or miRNAs generated by Dicer have to be loaded onto the effector complex of RNA mediated gene silencing. This complex, called RNA-induced silencing complex (RISC), leads to sequence-specific gene silencing (Hammond *et al.,* 2000).

In human cells, Dicer together with the double-strand RNA binding protein TRBP and Ago2 form the RISC-loading complex (RLC) which can bind to double stranded RNA. In this complex, dsRNA is cleaved into siRNAs by Dicer and TRBP and then transferred to Ago2

within the complex where the passenger strand is eliminated after Ago2 mediated cleavage (Gregory *et al.,* 2005; Matranga *et al.,* 2005). The mechanism responsible for distinguishing between the RNA strand used for gene silencing (guide strand or siRNA/miRNA) and the RNA strand that is degraded instead of being incorporated into RISC (passenger strand or siRNA*/miRNA*) is not fully understood.

One proposed model for siRNAs is based on the thermodynamic asymmetry of the small dsRNA. Preferential loading of the guide strand into the RISC is achieved by incorporating the strand with lower thermodynamic stability at its 5' end (Schwarz *et al.,* 2003; Khvorova *et al.,* 2003). In some cases, both strands are loaded onto RISC with similar frequencies (Hong *et al.,* 2008; Wei *et al.,* 2009).

For the loading of miRNAs onto RISC an additional mechanism has to be considered, because in most miRNA/miRNA* duplexes, the passenger strand is not cleaved by Ago2 but removed from the RISC. This might be due to the fact, that miRNA/miRNA* duplexes have internal bulges preventing cleavage (Matranga *et al.,* 2005). The proposed mechanism is based on unwinding of the miRNA/miRNA* complex by the P68 RNA helicase followed by the elimination of the passenger strand (Salzman *et al.,* 2007). A similar mechanism, based on unwinding and elimination, has to be considered for RISCs containing Argonaute proteins without endonuclease activity (Liu *et al.,* 2004; Meister *et al.,* 2004; Okamura *et al.,* 2004). The thermodynamic asymmetry rule for strand selection, previously described for siRNAs, has also been observed for miRNAs (Khvorova *et al.,* 2003). Another study showed tissue-dependent selection of the miRNA or miRNA* strand instead of incorporation of the strand with lower 5' thermostability (Ro *et al.,* 2007).

### Post-transcriptional silencing by siRNAs:

Messenger RNAs complementary to the incorporated siRNA are cleaved after the incorporation of siRNAs into RISC. The Argonaute protein Ago2 mediates cleavage of the cognate mRNA in a variety of species such as humans, mice and *D. melanogaster.* Due to this activity, the Ago2 protein has been termed Slicer (Meister *et al.,* 2004; Okamura *et al.,* 2004; Liu *et al.,* 2004). The cleavage site is located between nucleotide 10 and 11 from the 5' end of the siRNA in perfectly matched siRNA/mRNA duplexes (Elbashir *et al.,* 2001a). The mRNA is then degraded by cellular exonucleases (Orban and Izaurralde, 2005). Mismatches between siRNA and mRNA at these positions prevent mRNA cleavage. In these cases, silencing is still possible by translational inhibition or degradation of the mRNA similar to miRNA mediated mechanisms (Doench *et al.,* 2003).

**Post-transcriptional silencing by miRNAs:**

After the incorporation of endogenous miRNAs into RISC, gene silencing is achieved either by translational repression or mRNA degradation. In animals, endogenous miRNAs target the 3' UTR of mRNAs (Wightman *et al.,* 1993; Lee *et al.,* 1993; Reinhart *et al.,* 2000). Binding is mostly imperfect with bulges or mismatches in the central part of the miRNA/mRNA duplex, that inhibit mRNA cleavage. Binding of the miRNA to the mRNA between the second and the eighth nucleotide, the so called seed region, is mostly perfect (Pillai *et al.,* 2007). In case of perfect homology between miRNA and mRNA, the Ago2 containing RISC can directly cleave the mRNA (Gregory *et al.,* 2005). This mechanism is also relevant for artificial miRNAs designed to knockdown the expression of a specific gene (McManus *et al.,* 2002). In case of imperfect base-pairing, two different mechanisms can lead to gene silencing. Silencing is then either mediated by translational repression or by mRNA decay. These two main mechanisms are presented in Figure 7.
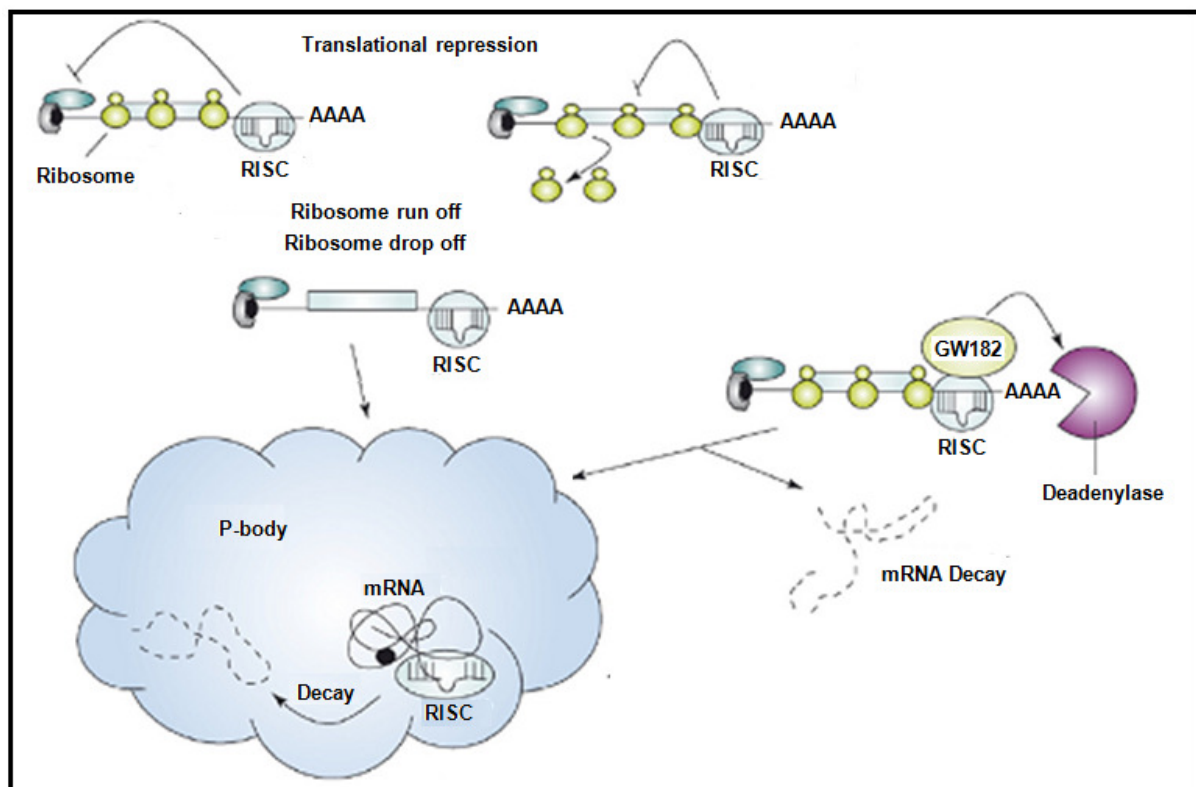


**Figure 7: Gene silencing mediated by endogenous miRNAs (adapted, from Pillai *et al.,* 2007).** 5' cap dependent translational repression at translation initiation, posttranslational repression and deadenylation of mRNA followed by mRNA degradation in cytoplasmatic processing bodies (P-bodies) are shown.

The most common mechanism is translational repression. This can take place either at the initiation step of translation, or after translation initiation. It has been suggested that miRNAs impair the 5' cap dependent initiation of translation by interfering with cap recognition (Humphreys *et al.,* 2005; Pillai *et al.,* 2005). Other studies have shown that the repression takes place after translation initiation, since miRNA targeted mRNA is associated with

ribosomes (Maroney *et al.,* 2006). A proposed mechanism is based on the observation that 5' cap independent repression occurs by rapid dissociation of ribosomes during translation (Petersen *et al.,* 2006). A recent study showed that the same mRNA can be silenced either at the initiation step of translation or thereafter, depending on the promoter sequence used to transcribe the mRNA (Kong *et al.,* 2008).

MiRNA mediated gene silencing by mRNA decay has been shown to take place in so-called cytoplasmatic processing bodies (P-bodies). Argonaute proteins complexed with miRNA and the corresponding mRNA accumulate in P-bodies in the cytosol of cells. There the Argonaute proteins can interact with mRNA decapping enzymes, the P-body protein GW182 and deadenylation complexes. GW182 promotes mRNA decay via deadenylation and decapping of the mRNA followed by mRNA degradation (Liu *et al.,* 2005a; Liu *et al.,* 2005b; Rehwinkel *et al.,* 2005; Behm-Ansmant *et al.,* 2006).

### 1.2.3   Applications of RNA mediated gene silencing

The technology of RNA mediated gene silencing can be used in a variety of areas, such as studying gene functions in low- or high-throughput applications for functional genomics, development of therapeutics in medicine, identifying genes involved in diseases, generating animal models for diseases and various other biotechnological applications.

SiRNAs and shRNAs can be directly employed to knockdown gene expression *in vitro* by transfecting cells and large scale genetic screens have been performed to identify gene functions. These screens have for example identified novel tumor suppressor genes, previously unknown modulators of p53 activity and novel components of the mammalian circadian clock (Westbrook *et al.,* 2005; Berns *et al.,* 2004; Maier *et al.,* 2009). Furthermore, RNA mediated gene silencing can also be applied *in vivo*. SiRNAs and shRNA expression plasmids have been introduced into mice via hydrodynamic tail vein injection resulting in transient gene silencing and moreover the administration of siRNAs has been shown to protect mice from fulminant hepatitis and liver fibrosis in mouse models for liver failure (Song *et al.,* 2003; Zender *et al.,* 2003).

ShRNA expression plasmids have been used to generate transgenic animals with long term gene silencing. Those animals were generated to study gene functions and to create disease models. Transgenic mice and rats with constitutive or inducible expression of shRNAs have been produced for example with ubiquitous knockdown of GFP (Hasuwa *et al.,* 2002) or a diabetes mellitus rat model in which a doxycyclin regulated shRNA downregulates the expression of the insulin receptor (Kotnik *et al.,* 2009). ShRNA mediated RNAi has also been applied in livestock to downregulate the expression of the prion protein in brain tissue of a

goat fetus (Golding *et al.,* 2006) and of porcine endogenous retroviruses (PERV) in pigs (Dieckhoff *et al.,* 2008; Ramsoondar *et al.,* 2009).

Not only shRNAs but also artificial miRNAs have also been described for ubiquitous or tissue-specific knockdown. Xia *et al.* (2006) utilized this approach to systemically downregulate the expression of the Mangan superoxide dismutase 2 (Sod2) in mice, creating a phenotype resembling Sod2 knockout mice. Rao *et al.* (2006) reported tissue-specific knockdown of the tumor-suppressor gene Wilms' tumor 1 (WT1) specifically in sertoli cells *in vivo* in mice with an artificial miRNA construct and identified previously unknown functions of WT1.

The treatment of diseases by RNAi has also been attempted and several siRNA therapeutics are being investigated in clinical trials. SiRNAs against Respiratory Syncytial Virus (RSV), Human immunodeficiency virus (HIV), Hepatitis B virus (HBV), acute kidney failure, diabetic macular edema, pachyonychia congenita and neovascular age-related macular degeneration (AMD) are currently in phase I to III clinical trials (López-Fraga *et al.,* 2008; Whitehead *et al.,* 2009).

## 1.3 Target genes and proteins for RNA interference

To assess the novel technology in pigs, two different target genes were selected for RNAi mediated downregulation in this work. These targets were the porcine whey protein Beta-Lactoglobulin (BLG) and the porcine tumor suppressor protein p53.

Several reasons led to the selection of porcine BLG as a target gene. First of all, porcine BLG is a whey protein with unknown function and therefore, the knockdown of BLG would provide valuable information, especially because gene knockout of BLG by gene targeting has not yet been achieved. Second, it is only expressed in the lactating mammary gland and it is therefore unlikely that a knockdown of BLG would harm the animal. Moreover, since BLG is a whey protein, sample acquisition can easily be performed over a period of time without sacrificing the animal. Another reason for the choice of this target gene was, that bovine BLG is a major cause of milk allergy and intolerance in humans. Therefore the downregulation of porcine BLG could serve as a model for reducing the amount of bovine BLG in milk which would be of importance and benefit to human health.

The porcine tumor suppressor p53 was chosen because of its importance in biomedicine and cancer development as it is mutated in about 50% of human cancers. The protein is expressed in primary cells of pigs and knockdown effects can be analyzed *in vitro* before the cells are employed to generate transgenic pigs. TP53 knockout pigs by gene targeting would be important for cancer research but, as previously described, the targeted gene knockout has several disadvantages in livestock species such as the time period required to create

homozygous animals displaying a cancer phenotype. Therefore RNAi mediated knockdown could serve as a faster alternative to establish large animal models for cancer research. More detailed information on both target genes will be given in the following sections.

### 1.3.1  Beta-Lactoglobulin (BLG)

BLG is a milk whey protein with unknown function. It is the major protein component in the whey fraction in ruminants with a concentration of 2 to 4 g/l in cow's milk (Kontopidis *et al.*, 2004; Farrell *et al.*, 2004). Several non-ruminants such as pigs, horses, dogs, manatees, dolphins and cats express BLG (Kessler and Brew, 1970; Jones and Kalan, 1971; Godovac-Zimmermann *et al.*, 1985; Pervaiz and Brew, 1986; Halliday *et al.*, 1990). However, BLG is not present in the milk of rodents, lagomorphs or humans (Monti *et al.*, 1989). Furthermore it is one of the main allergens involved in cow's milk allergy or intolerance (Wal, 1998a). The following section will provide information regarding the protein structure of porcine and bovine BLG, suggested biological functions of the proteins, *in vitro* cell culture systems to evaluate protein function and also outline the benefit of producing pigs and cows with RNAi mediated gene silencing of BLG.

**<u>BLG structure:</u>**

The mature bovine BLG protein is composed of 162 amino acids. It is derived from 178 amino acids precursor form and has a molecular weight of 18.4 kDa. Bovine and porcine BLG show about 80% homology at the mRNA and 67% at the amino acid level (Gallagher *et al.,* 1997). The porcine BLG gene has been mapped close to the telomeric region of chromosome 1 (Ballester *et al.*, 2005). The mature porcine protein consists of 160 amino acids with an apparent molecular weight of 18.5 kDa originating from a precursor protein with 178 amino acids (Mercier *et al.*, 1980). At physiological pH, bovine BLG forms dimers which tend to separate into monomers at acidic pH (Sakurai and Goto, 2002). In contrast, porcine BLG exists predominantly as a monomer at neutral pH and forms dimers at pH 3 (Pérez and Calvo, 1995; Ugolini *et al.,* 2001; Hoedemaeker *et al.,* 2002). The bovine protein contains two disulfide bridges and a free cysteine residue, whereas the porcine protein has no free cysteine groups, all four cysteine residues are joined in disulfide bonds (Sawyer and Kontopidis, 2000; Kessler and Brew, 1970).

BLG is a member of the lipocalin protein family. It shows the characteristic lipocalin central core cavity (calyx) which is formed by eight anti-parallel β-strands connected to a ninth β-strand by one α-helix. The calyx seems to be the main ligand-binding site of bovine BLG. It can bind hydrophobic molecules, such as 12-bromododecanoic acid, vitamin $D_3$, retinol, retinoic acid and palmitate *in vitro* whereas *in vivo* only binding of fatty acids, mainly palmitic, oleic and myristic acid, has been observed in the native protein (Qin *et al.,* 1998; Yang *et al.,* 2008; Kontopidis *et al.,* 2002; Wu *et al.,* 1999; Kontopidis *et al.*, 2004, Pérez *et al.,* 1989). In

addition, a secondary binding site for vitamin $D_3$ has been identified (Papiz *et al.,* 1986; Kontopidis *et al.*, 2004, Yang *et al.,* 2008, Farrell *et al.,* 2004). The central calyx of porcine BLG is empty after extracting the protein at native conditions and porcine BLG cannot bind palmitic acid *in vitro* at any pH which seems to be due to a lack of positively charged residues at the top of the calyx (Ugolini *et al.,* 2001). These differences indicate diverse functional properties of bovine and porcine BLG. The structure of the porcine and bovine BLG protein is presented in Figure 8.
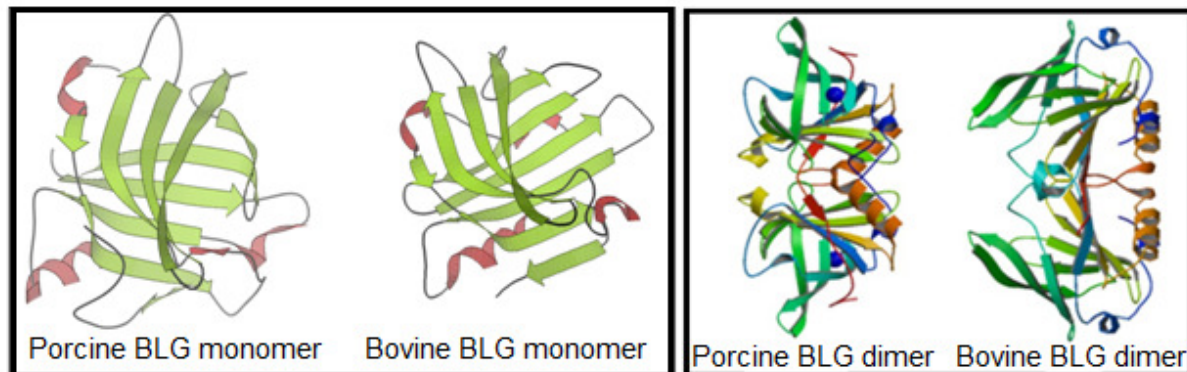


**Figure 8: Porcine and bovine BLG monomer (left) and dimer (right) protein structure (1EXS/1b8e).** BLG folds into a beta-barrel structure. Arrow: beta-strand, helix: alpha-helix, line: loops, blue spheres: sodium ion.

### Proposed biological functions:

There have been many suggestions regarding possible biological functions of BLG other than being an amino acid source for the nourishment of the offspring. Bovine BLG is mainly considered to be a transport protein. The lack of ligand binding *in vivo* and *in vitro* at neutral pH suggests that, in contrast to bovine BLG, a transport function for porcine BLG is not likely (Ugolini *et al.,* 2001).

A relationship between possible transport functions and pH dependent structural changes of the protein has been suggested. At pH 8, the central core cavity is accessible due to the conformation of the protein. At pH 6, the cavity is closed by the α-helix and the subsequent β-strand. This indicates a physiological function in binding of small molecules and protecting them from acidic pH in the stomach (McKenzie and Sawyer, 1967; Uhrinová *et al.,* 2000).

Similarities in the 3D structure of BLG and human plasma retinol-binding protein have led to the proposal, that BLG has a role in retinol transport. However the similarity at the amino acid level is limited. BLG-retinol complexes can bind to purified microvilli from one-week old calf intestine *in vitro*, but not from 6 month old animals (Papiz *et al.,* 1986). Thus Papiz *et al.* (1986) suggested that there might be specific receptors for the BLG-retinol complex in the small intestine of newborn calves important for retinol uptake. Kushibiki *et al.* (2001) also proposed that bovine BLG enhances retinol uptake in the intestine of calves. Calves fed with milk supplemented with retinol and bovine BLG showed elevated plasma retinol

concentrations compared to control calves. However, the BLG-retinol complex has never been detected *in vivo*, therefore such a role seems unlikely (Pérez and Calvo, 1995).

Another attempt to identify a function of BLG was made by Pérez *et al.* (1992). They demonstrated that bovine BLG can increase the activity of ruminant pregastric lipase *in vitro*. This enzyme is important for the hydrolysis of milk fat globules but is inhibited by free fatty acids. Therefore the increased activity of pregastric lipase seems to depend on the ability of BLG to bind these free fatty acids. Because of these results, it has been suggested, that BLG facilitates the digestion of milk fat in newborn calves (Pérez *et al.,* 1992). In addition, Kushibiki *et al.* (2001) showed that calves fed with milk enhanced with bovine BLG and milk fat, showed significantly higher plasma concentrations of triglycerides than the control group. They concluded that BLG is important for the digestion and/or intestinal absorption of fatty acids and triglycerides in newborn calves. Nevertheless, BLG from non-ruminant species does not bind fatty acids and is therefore not likely to play an essential role in this process in other animals (Pérez *et al.,* 1992).

To study possible functions of BLG in pigs, Sutton and Alston-Mills (2006) tried to determine its effect on intestinal morphology and development in newborn piglets. They compared piglets raised with pig milk and piglets fed bovine colostrum powder with and without bovine BLG. No effect on DNA concentration in the intestine, gut maturation on enzymatic level and intestinal morphology was found between pigs fed with or without BLG. These findings lead to the conclusion that BLG does not play a role in intestinal development and morphology in pigs and its function remains elusive.

### Milk allergy and intolerance:

Bovine BLG is a major factor for cow's milk intolerance and cow's milk allergy (CMI/CMA) both of which mainly affect children. CMA or CMI is diagnosed in about 2 to 3% of children younger than 2 years in northern Europe and is characterized by skin reactions, gastrointestinal and respiratory allergy symptoms (Wal, 1998a; Kagan, 2003). Like many other animal allergens, bovine BLG is a member of the lipocalin protein family (Mäntyjärvi *et al.,* 2000). The acid stability of bovine BLG originating from the stable lipocalin 3D structure confers resistance to acid hydrolysis and protease digest. This is the reason why a certain percentage of BLG remains intact during milk processing or gastric digestion, retaining its allergenicity (Wal, 1998b; Sélo *et al.,* 1998; D'Alfonso *et al.,* 2005). It has been shown that BLG can readily pass through the small intestine epithelium into the *lamina propria* of mice and cause anaphylaxis after ingestion (Roth-Walter *et al.,* 2008). Whether ingested BLG is transferred to the milk of nursing mothers is controversial (Jakobsson *et al.,* 1985; Monti *et al.,* 1989; Conti *et al.,* 2000). Although after the cleavage of the disulfide bridges in bovine BLG the lipocalin 3D structure is not retained, the immunogenicity is not eliminated since the

relevant epitopes are distributed over the whole protein. Some hydrolytic peptides are even more immunogenic than intact BLG (Sélo *et al.,* 1998). Due to the stability against acid hydrolysis and the allergenic potential of BLG peptides after cleavage of the protein, elimination of BLG from bovine milk by processing is not easily achieved. Therefore it would be beneficial to produce milk with lower BLG content.

**Elimination of BLG from milk in transgenic animals:**

As described above, possible biological functions of the bovine protein have been suggested but none have been proved definitively. The function of porcine BLG remains unknown. This has motivated several attempts to generate BLG knockout animals by gene targeting. Only one report has been published so far concerning a knockout for bovine BLG. Lin *et al.* (2009) analyzed the DNA methylation changes in cells derived from a BLG knockout fetus. The fetus died at the age of 3 months *in utero* and showed abnormal methylation patterns which might have led to the death of the fetus. The knockout itself has not been published and no further details were given. In sheep, targeted elimination of BLG has been attempted for several years without success since enrichment for gene targeting with promoter-trap vectors has not been possible for BLG. This is due to the fact, that no cells with long-term *in vitro* expression of BLG are available. Targeting vectors without promoter trap did not lead to successful knockout of another milk protein, ovine ß-casein, either (Schnieke, personal communication; Thomson *et al.,* 2003). Therefore an alternative approach to eliminate BLG expression is necessary. Downregulation of BLG expression by RNAi would allow functional studies of the protein and in addition could serve as a model system for establishing RNAi in livestock. The cow is clearly the most important dairy species, but because of the small litter size, the long gestation period (around 283 d) and the late sexual maturity of cows (9-10 months), they are not the most suitable animal in which to establish the RNAi technology (Barnett, 2007). Once it has been shown that milk composition can be effectively changed by RNAi in other species, the method could then be transferred to cows. Since mice and other small laboratory animals do not express BLG (Monti *et al.*, 1989), the pig seems to be a suitable animal for RNAi mediated knockdown of BLG expression. Pigs have an average litter size of 10.5 piglets per pregnancy, reach sexual maturity at 5-6 months and have an average gestation period of 115 d (Barnett, 2007). Since the protein is only expressed in the lactating mammary gland it is unlikely that a lack of BLG would cause major health problems in transgenic pigs. The ease of sample acquisition by obtaining milk from knockdown pigs over a period of time is an additional advantage for establishing the RNAi technology in livestock with a milk protein. Since porcine and bovine BLG show 80% similarity at the mRNA level, knockdown of porcine BLG could serve as a model for downregulation of BLG in the cow (Gallagher *et al.,* 1997).

**Cell culture to evaluate function of BLG:**

Before RNAi mediated downregulation of BLG expression is attempted *in vivo*, it would be helpful if preliminary data could be obtained by using *in vitro* tissue culture. Several *in vitro* cell culture systems for the expression of milk proteins mimicking mammary gland function have been described.

Immortalized mammary epithelial cell (MEC) lines have been generated from livestock species such as cow, sheep, goat and pig after spontaneous immortalization or transfection with SV40 large-T antigen (Huynh *et al.,* 1991; Zavizion *et al.,* 1996; Ilan *et al.,* 1998; Pantschenko *et al.,* 2000; Sun *et al.,* 2006). These cell lines preserved certain functional properties of mammary gland cells *in vivo*. A bovine MEC line (MAC-T) can produce alpha- and beta-casein after induction with prolactin when cultured on collagen gels (Huynh *et al.,* 1991). Another bovine MEC line (BME-UV) only produces low levels of casein (Zavizion *et al.,* 1996) and BLG expression has not been examined in MAC-T and BME-UV cell lines. The spontaneously immortalized porcine MEC line SI-PMEC is reported to produce alpha-lactalbumin, beta-casein and BLG in three dimensional cultures in Matrigel after lactogenic hormone induction (Sun *et al.,* 2006) and could be useful to test RNAi mediated knockdown of BLG.

Besides the use of immortalized MEC lines, few groups have studied milk gene expression in primary cell cultures. Bovine primary MEC can synthesize lactose, alpha $s_1$-casein, beta-casein, alpha-lactalbumin, lactoferrin and BLG after induction with lactogenic hormones when cultured on collagen gels (Baumrucker *et al.,* 1988; Talhouk *et al.,* 1993; Cifrian *et al.,* 1994). The protein secretion of porcine primary MEC has been studied by Kumura *et al.* (2001) and Sun *et al.* (2005). Secretion of beta-casein, lactoferrin, alpha-lactalbumin and BLG could be detected after culturing them on floating collagen gels or extracellular basement membrane (Matrigel) and addition of lactogenic hormones (Kumura *et al.,* 2001; Sun *et al.,* 2005). The expression of lactoferrin and BLG did not require hormonal induction with prolactin in the cells isolated by Kumura *et al.* (2001). Due to the ability to express milk proteins *in vitro* in cell culture, primary MEC and immortalized MEC lines are useful systems to elucidate the function of BLG, the application of RNAi and possibly to generate animals after SCNT of tested primary cells.

### 1.3.2  Tumor suppressor protein p53

The tumor suppressor protein p53 is involved in various cellular pathways. It was identified in 1979 (e.g. Lane and Crawford, 1979; Linzer and Levine 1979) during immunoprecipitation studies of the SV40 large T antigen. The function of p53 as a tumor suppressor was discovered by Finlay *et al.* and Eliyahu *et al.* in 1989. This section will briefly describe the

functions of p53. Moreover, information about mutation or deletion of the TP53 and gene-targeted knockout as well as knockdown animal models for p53 will be presented.

**Functions of p53:**

The p53 protein acts as a transcription factor and regulates the expression of many genes in response to stress signals. In normal cells, p53 has a relatively short half-life and is present in very low levels due to the activity of the ubiquitin ligase MDM2. Stress signals lead to stabilization and accumulation of p53 in the nucleus. These stress signals include activation of oncogenes, DNA damage, ribonucleotide depletion, loss of survival signals and hypoxia. Activation of p53 can lead to growth arrest, DNA repair, senescence, differentiation or apoptosis (see Figure 9) (Vousden and Lu, 2002; Shu *et al.*, 2007).

Because of all of these functions, p53 has been termed ‚guardian of the genome' (Lane, 1992). Deletions or inactivating mutations in TP53 therefore lead to a loss of its regulatory function. In response to stress signals, inactivated p53 cannot bind to p53 response elements of target genes. This leads to a reduced rate of apoptosis, growth arrest and senescence (Vousden and Lu, 2002). A comparison of mutant and wildtype p53 stress response is outlined in Figure 9.
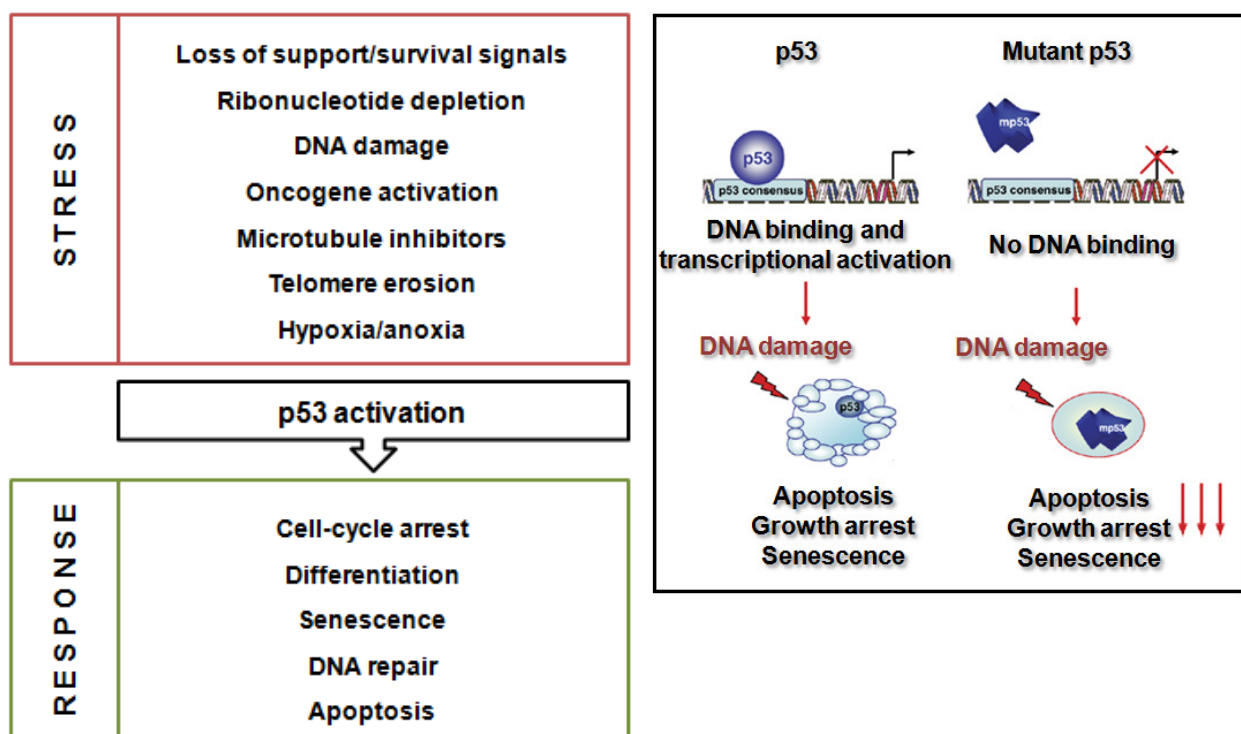


**Figure 9: Activation of p53 by stress signals and p53 mediated response (left) (adapted, from Vousden and Lu, 2002) and comparison of wildtype and mutant p53 (adapted, from Strano *et al.,* 2007).** Left: p53 is activated in cells by stress signals, including activation of oncogenes, DNA damage, ribonucleotide depletion, loss of survival signals, hypoxia and many more. In response to stress signals, p53 is stabilized and accumulates in the nucleus initiating DNA repair, differentiation, cell-cycle arrest, apoptosis or senescence. Right: The DNA binding ability of wildtype p53 and mutant p53 are different. In contrast to wildtype p53, the mutant form cannot bind to p53 response elements, leading to reduced apoptosis, lower levels of growth arrest and reduced senescence.

**Structure:**

Human p53 consists of 393 amino acids. It contains three main domains, the N-terminal transactivation domain, a central DNA binding domain and the C-terminal domain (Shu *et al.,* 2007) which are presented in Figure 10. The N-terminal domain is important for the transcriptional activation of p53 target genes and the interaction with the p53 inhibitory protein MDM2 (Fields and Jang, 1990; Chen *et al.,* 1995). The central core domain represents the DNA-binding domain of p53 and is therefore important for the recognition of p53 target promoters (Foord *et al.,* 1993). The C-terminal domain contains a tetramerization motif necessary for the formation of the active transcription factor (Stürzbecher *et al.,* 1992; Wang *et al.,* 1994).
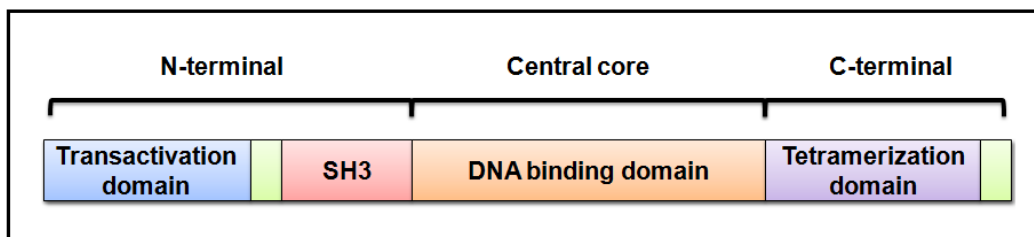


**Figure 10: Domains of the p53 protein (adapted, from Bode and Dong, 2004).** The N-terminal transactivation and proline rich SH3 domain (blue and pink), the central DNA binding domain (orange) and the C-terminal domain containing the tetramerization domain (purple) are shown. Details see text.

The porcine TP53 gene is located on chromosome 12 (Rettenberger *et al.,* 1993). It encodes a 386 amino acid protein. Porcine TP53 shows 87% homology at the nucleotide and 88% homology at the amino acid level to human TP53 (Burr *et al.,* 1999; UniGene Ssc.15917). The DNA-binding region is highly conserved. Due to the short lifespan of pigs for commercial use, no information about spontaneous and hereditary mutations in the TP53 gene and cancer development is available (Burr *et al.,* 1999).

**p53 and cancer:**

The TP53 gene is mutated in about 50% of all human tumors. The mutations mainly include point mutations and chromosomal deletion of TP53 (Greenblatt *et al.,* 1994; Soussi *et al.,* 2000). In addition a gene amplification or over-expression of the p53 ubiquitin ligase MDM2 can lead to a reduced level of p53 in cells due to accelerated ubiquitinylation of p53 (Momand *et al.,* 1998). This leads to altered p53 molecules that accumulate in the cell or to a loss of p53 protein and therefore loss of p53 function (Chari *et al.,* 2009).

Inherited TP53 mutations are found in patients with Li-Fraumeni syndrome (LFS). This multiple cancer syndrome is caused by a heterozygous mutation in TP53 and results in predisposition to multiple primary tumors and a high rate of multiple cancers usually before the age of 50. Cancers include adrenocortical carcinoma, sarcomas, leukemia, breast cancer and brain tumors. Analysis of tumor samples revealed that the second allele of TP53 is often

affected by another mutation or chromosomal deletion. This results in tumor cells with a lack of p53 function (Malkin *et al.,* 1990; Evans and Lozano, 1997).

**Animal models for Li-Fraumeni syndrome:**

Mouse models with heterozygous and homozygous deletion of TP53 have been produced to investigate the role of p53 in tumor development and as models for LFS. Mice with a homozygous knockout of TP53 are viable and develop various tumors before the age of 6 months. Even heterozygous mice are prone to tumor development with tumors occurring after 9 months of age. The tumors are mainly lymphomas and sarcomas. These knockout mice show that the absence of the protein is sufficient for tumor development and that the survival rate of TP53 knockout mice is decreased compared to wildtype animals due to tumor formation (Donehower *et al.,* 1992; Jacks *et al.,* 1994). The survival rates of wildtype and knockout mice are presented in Figure 11.
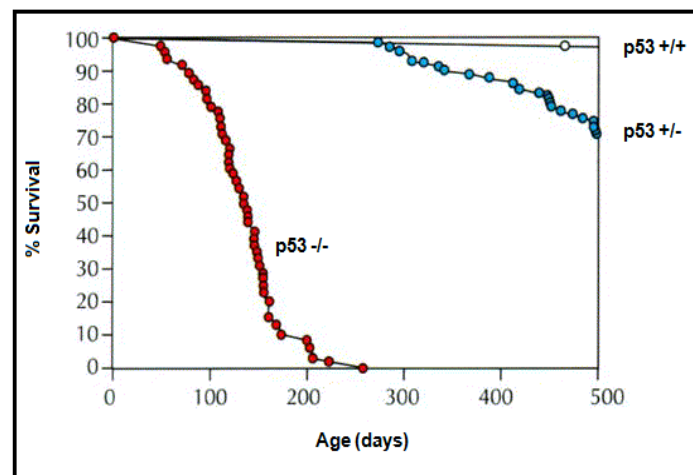


**Figure 11: Effect of p53 deletion on the survival rate of mice (adapted, from Jacks *et al.,* 1994).** Wildtype mice (white) survived more than 500 days (d). Heterozygous animals (blue) showed tumor formation from 9 months onwards. After 500 d 28% of the animals had died or had to be sacrificed. Homozygous knockout mice (red) had all died or been sacrificed by 250 d and 84% of them showed tumor formation.

RNAi offers an alternative method to produce animals or cells with a reduced amount of p53. Hemann *et al.* (2003) used shRNAs to knockdown p53 in hematopoietic stem cells from Eµ-myc mice. These mice express the c-myc oncogene from the immunoglobulin heavy-chain enhancer and develop lymphomas. Hematopoietic stem cells with p53 knockdown were introduced in lethally irradiated mice. Depending on the *in vitro* knockdown efficiency of the construct, mice either showed no difference in survival compared to control mice or they developed lymphomas and died within 95 days proving that the depletion of p53 accelerated tumor development *in vivo*. The experiment and the survival rates are depicted in Figure 12.
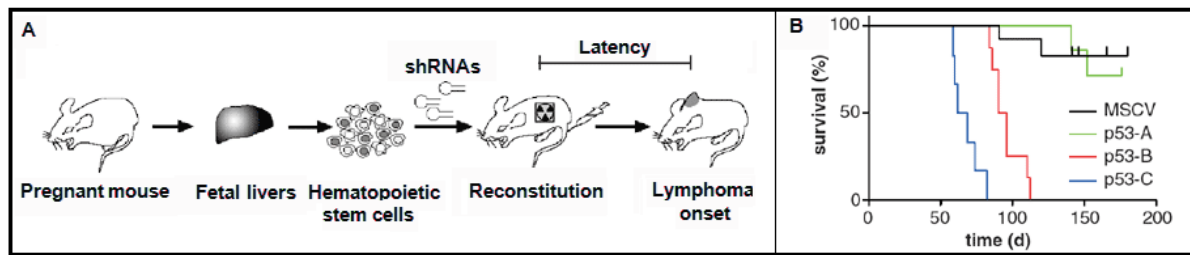
**Figure 12: p53 knockdown by shRNAs in Eµ-Myc mice (adapted, from Hemann *et al.,* 2003).** A: Diagram of the experimental procedure B: Kaplan-Meier curve showing survival rates and lifespan in relation to the construct used (p53-A: minimal p53 knockdown; p53-B: moderate p53 knockdown; p53-C: highly efficient knockdown construct).

A similar experiment with artificial miRNAs instead of shRNAs was performed by Dickins *et al.* (2005) where mice developed lymphomas that even showed resistance to chemo-therapeutic treatment with adriamycin, also known as doxorubicin, *in vivo*.

**The pig as an animal model for cancer research:**

Since pigs are genetically closer to humans than mice, have a similar physiology, size and organ development to humans, they might be suited to serve as large animal models for cancer research (Wernersson *et al.,* 2005; Lunney, 2007). Genetically modified pigs with RNAi mediated knockdown of p53 would be useful to study cancer development, improve detection methods for early stage cancers, develop new anti-tumor treatments and increase tumor progression in pigs with precise genetic modifications of oncogenes or tumor suppressor genes.

Our laboratory has successfully generated pigs with a heterozygous mutation in the tumor suppressor gene adenomatous polyposis coli (APC) resembling one of the most common mutations in human familial adenomatous polyposis (FAP), an inherited colorectal cancer syndrome. In FAP patients, tumors only arise after a series of activating mutations in oncogenes such as K-Ras and inactivating mutations in tumor suppressor genes such as SMAD4 and TP53 (Fodde, 2002). Therefore RNAi mediated downregulation of p53 combined with the targeted APC mutation might accelerate tumor formation and increase tumor progression.

## 1.4 Aim

The aim of this project was to establish RNAi technology in livestock. For this purpose, two target genes for RNAi mediated downregulation were chosen. The first target was the porcine whey protein Beta-Lactoglobulin (BLG), a lactating mammary gland specific protein not expressed in other tissues. The knockdown of BLG might elucidate the function of this protein and serve as a test whether RNAi mediated knockdown can be used to modify the composition of milk in livestock. The second target, the porcine tumor suppressor protein p53, is a ubiquitously expressed protein important in cancer biology. In case of p53, the aim was to evaluate whether RNAi mediated knockdown could be used as an alternative to gene

knockout by gene targeting to create a porcine animal model for human Li-Fraumeni syndrome.

Efficient sequences for the downregulation of these two target genes had to be designed and the knockdown efficiency had to be evaluated in several independent systems such as mammalian cell lines and primary cells in a variety of assays. In addition, shRNAs and artificial miRNA for the downregulation of gene expression had to be compared to determine if one of the construct types is more suitable for the generation of RNAi transgenic pigs.

# 2   Material and methods

## 2.1   Materials

### 2.1.1   Equipment

| | |
|---|---|
| +4 °C fridge | Beko Technologies, Dresden, Germany |
| -20 °C freezer | Liebherr International, Bulle, Switzerland |
| -80 °C ultra low temperature freezer | Thermo Electron, Dreieich, Germany |
| AxioCAM MRc camera | Zeiss AG, Oberkochen, Germany |
| Axiovert 25 microscope | Zeiss AG, Oberkochen, Germany |
| Bio Imaging System Gene Genius | Syngene, Cambridge, United Kingdom |
| BioPhotometer | Eppendorf, Hamburg, Germany |
| Centrifuge 1-15 | Sigma, Osterode, Germany |
| Centrifuge 3-16 | Sigma, Osterode, Germany |
| Centrifuge 4K15C | Sigma, Osterode, Germany |
| Centrifuge 5810 | Eppendorf, Hamburg, Germany |
| Centrifuge Minispin | Eppendorf, Hamburg, Germany |
| Digital Graphic Printer UP-D895MD | Syngene, Cambridge, United Kingdom |
| Electrophoresis chamber PerfectBlue mini ExW | Peqlab, Erlangen, Germany |
| Electrophoresis chamber HE 33 Mini Submarine Unit | GE Healthcare, Munich, Germany |
| Electrophoresis chamber Classic CSSU1214 | Thermo Electron, Dreieich, Germany |
| Electrophoresis chamber Classic CSSU78 | Thermo Electron, Dreieich, Germany |
| Electrophoresis Power Supply EPS 301 | Amersham Bioscience |
| Electroporator Multiporator | Eppendorf, Hamburg, Germany |
| Handy Step Multipipette | Brand, Wertheim, Germany |
| Ice maker | Eurfrigor, Lainate, Italy |
| Ika MS2 Minishaker | RCO Janke & Kunkel, Staufen, Germany |
| Ika-Combimag | RCO Janke & Kunkel, Staufen, Germany |
| Improved Neubauer chamber | Brand, Wertheim, Germany |
| Incubator | Binder GmbH, Tuttlingen, Germany |
| Laminar Flow Hood HERAsafe Type HSP | Heraeus Instruments, Munich, Germany |

| | |
|---|---|
| Luminometer GloMAX 20/20 | Turner Biosystems, Mannheim, Germany |
| Microwave NN-E202W | Panasonic |
| Mini-PROTEAN 3 Cell | BioRad, Munich, Germany |
| Mr. Frosty freezing device | Nalgene, Rochester, U.S.A. |
| Orbital shaker | Thermo Electron, Dreieich, Germany |
| PCR cycler DNA Engine DYAD | MJ Research, Watertown, USA |
| pH meter Cyberscan 510 | Eutech Instruments, Singapore, Singapore |
| Pipette BioHit m10, m100, m1000 | BioHit, Rosbach v.d. Höhe, Germany |
| Pipette Pipetman P20, P200, P1000 | Gilson, Bad Camberg, Germany |
| Pipette Pipetman Ultra U2 | Gilson, Bad Camberg, Germany |
| Pipetus reddot | Hirschmann, Eberstadt, Germany |
| Power supply EC 105 | Thermo Electron, Dreieich, Germany |
| Power supply EPS 301 | GE Healthcare, Munich, Germany |
| Pure water system Astacus | membraPure, Bodenheim, Germany |
| Rainin Pipet-Lite (2, 20, 200 and 1000 µl) | Mettler Toledo, Giessen, Germany |
| Scale 440-33N | Kern, Balingen, Germany |
| Scale APX-1502 | Denver Instruments, Göttingen, Germany |
| Steri-Cycle $CO_2$ incubator | Thermo Electron, Dreieich, Germany |
| Trans-Blot SD Semi-dry Transfer Cell | BioRad, Munich, Germany |
| Vortex Genie 2 | Scientific Industries, New York, USA |
| Vortex Mixer | VELP Scientifica, Usmate, Italy |
| Waterbath | Thermo Electron, Dreieich, Germany |

### 2.1.2  Consumables

| | |
|---|---|
| 0.5 ml, 1.5 ml and 2.0 ml reaction tubes | Brand, Wertheim, Germany |
| 14 ml Round bottom tube with ventilation lid | Becton, Dickinson & Company, Sparks, USA |
| 15 and 50 ml centrifugation tubes | Corning, New York, USA |
| Cell culture flasks (25, 75, 150 cm²) | Corning, New York, USA |
| Cell culture plates (6-, 12-, 24-, 48-well) | Corning, New York, USA |
| Cryopreservation tube | Corning, New York, USA |
| Disposable inoculating loop (10 µl) | Cole-Parmer Instrument, Illinois, USA |

Electroporation cuvettes (2 and 4 mm)         Peqlab, Erlangen, Germany

Filter Stericup and Steritop (0.22 µm)         Millipore Corporation, Billerica, USA

Glass pasteur pipettes                         Brand, Wertheim, Germany

Glassware (bottles, flasks)                    Marienfeld, Lauda-Königshofen, Germany

Hybond-N$^+$ nylon transfer membrane           Amersham Bioscience, Freiburg, Germany

Immobilon-P PVDF transfer membrane             Millipore Corporation, Billerica, USA

Petri dish (10 cm)                             Brand, Wertheim, Germany

Photometer cuvettes UVette                     Eppendorf, Hamburg, Germany

Pipette tips with filter (2, 20, 200, 1000 µl) Mettler Toledo, Giessen, Germany

Pipette tips without filter (2, 20, 200, 1000 µl) Brand, Wertheim, Germany

Rotilabo Blotting paper (1 mm)                 Roth, Karlsruhe, Germany

Sterile Filter, 0.22 µm                        Sartorius AG, Göttingen, Germany

Sterile plastic pipettes (1, 2, 5, 10, 25 ml)  Corning, New York, USA

### 2.1.3  Chemicals

1,4-Dithiothreitol (DTT)                       Sigma, Steinheim, Germany

2-Propanol                                     Roth, Karslruhe, Germany

3-Isobutyl-1-methylxanthin (IBMX)              Sigma, Steinheim, Germany

Acetic acid                                    Fluka, Seelze, Germany

Ammonium persulfate (APS)                      Carl Roth GmbH, Karlsruhe, Germany

Ascorbic acid                                  Sigma, Steinheim, Germany

Beta-glycerol phosphate                        Sigma, Steinheim, Germany

Biozym LE Agarose                              Biozym, Oldendorf, Germany

Bovine Serum Albumin (BSA), pH 7.0             PAA, Pasching, Austria

Bromophenol blue                               Sigma, Steinheim, Germany

Chloroform                                     Sigma, Steinheim, Germany

D-Glucose                                      Sigma, Steinheim, Germany

Dimethylsulfoxid (DMSO)                        Sigma, Steinheim, Germany

Ethanol                                        Riedel-de-Haen, Seelze, Germany

Ethidium bromide solution                      Fluka, Seelze, Germany

Ethylenediaminetetraacetic acid (EDTA)         Sigma, Steinheim, Germany

Formaldehyde                                   Sigma, Steinheim, Germany

| | |
|---|---|
| Formamide (deionized) | Sigma, Steinheim, Germany |
| GenAgarose L.E | Genaxxon, Biberach, Germany |
| Glycerol | Sigma, Steinheim, Germany |
| Glycine | Roth, Karlsruhe, Germany |
| Hydrochloric acid | Sigma, Steinheim, Germany |
| Indomethacin | Sigma, Steinheim, Germany |
| Maleic acid | Sigma, Steinheim, Germany |
| Methanol | Roth, Karlsruhe, Germany |
| Oil Red O | Sigma, Steinheim, Germany |
| Rotiphorese Gel 40 (19:1) | Roth, Karlsruhe, Germany |
| Rotiphorese Gel 40 (29:1) | Roth, Karlsruhe, Germany |
| Silver nitrate | Sigma, Steinheim, Germany |
| Sodium acetate | Roth, Karlsruhe, Germany |
| Sodium chloride | Sigma, Steinheim, Germany |
| Sodium citrate tribasic dihydrate | Sigma, Steinheim, Germany |
| Sodium hydroxide | Fluka, Seelze, Germany |
| Sodium thiosulfate | Sigma, Steinheim, Germany |
| Sodiumdodecylsulfate (SDS) | Sigma, Steinheim, Germany |
| Sucrose | Sigma, Steinheim, Germany |
| TEMED | Roth, Karlsruhe, Germany |
| Trizma base | Sigma, Steinheim, Germany |
| Trizma HCl | Sigma, Steinheim, Germany |
| Tween 20 | Sigma, Steinheim, Germany |

### 2.1.4  Kits

| | |
|---|---|
| Calcium CPC Liquicolor Kit | Stanbio Laboratories, Boeme, USA |
| Dual-Glo Luciferase Assay | Promega, Mannheim, Germany |
| GenElute Mammalian Genomic DNA Miniprep Kit | Sigma, Steinheim, Germany |
| High Pure RNA Isolation Kit | Roche Diagnostics, Mannheim, Germany |
| NucleoBond Xtra Maxi | Macherey-Nagel, Düren, Germany |
| NucleoBond Xtra Midi | Macherey-Nagel, Düren, Germany |

NucleoSpin Plasmid Quick Pure                    Macherey-Nagel, Düren, Germany

Wizard SV Gel and PCR Clean-Up System            Promega, Madison, USA

### 2.1.5 Reverse Transcriptase and polymerases

GoTaq DNA Polymerase                             Promega, Mannheim, Germany

MolTaq DNA Polymerase                            Molzym, Bremen, Germany

OmniTaq DNA Polymerase                           Omni Life Science, Hamburg, Germany

Phusion High-Fidelity DNA Polymerase             New England Biolabs, Frankfurt, Germany

Platinum Taq DNA Polymerase                      Invitrogen, Karlsruhe, Germany

SuperScript III Reverse Transcriptase            Invitrogen, Karlsruhe, Germany

SuperScript One-Step RT-PCR System with          Invitrogen, Karlsruhe, Germany
Platinum *Taq* DNA Polymerase

SuperScript One-Step RT-PCR System with          Invitrogen, Karlsruhe, Germany
Platinum *Taq* High Fidelity

### 2.1.6 Enzymes for molecular cloning

Calf Intestine Phosphatase (CIP)                 New England Biolabs, Frankfurt, Germany

DNA Polymerase I Large (Klenow) Fragment         New England Biolabs, Frankfurt, Germany

Proteinase K                                     Sigma, Steinheim, Germany

Restriction endonuclease BglII                   Fermentas, St. Leon-Rot, Germany

Restriction endonucleases                        New England Biolabs, Frankfurt, Germany

RNAse A solution                                 Sigma, Steinheim, Germany

T4 DNA Ligase                                    New England Biolabs, Frankfurt, Germany

### 2.1.7 Strains of *Escherichia coli*

**Table 2: Strains and genotype of *Escherichia coli* (*E. coli*)**

| Strain | Genotype | Source |
|--------|----------|--------|
| DH10B | F⁻ endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ⁻ | Invitrogen |
| ER2925 | ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2 | New England Biolabs |

## 2.1.8  Media and antibiotics for bacterial culture

Difco LB Agar, Miller                          Becton Dickinson and Company, Sparks, USA

Difco Luria Broth Base                     Becton Dickinson and Company, Sparks, USA

| Antibiotic | Supplier | Stock solution | Working concentration |
|---|---|---|---|
| Ampicillin | Sigma, Steinheim, Germany | 100 mg/ml | 100 µg/ml |
| Kanamycin | Sigma, Steinheim, Germany | 30 mg/ml | 30 µg/ml |
| Spectinomycin | Fluka, Seezle, Germany | 10 mg/ml | 50 µg/ml |

## 2.1.9  Mammalian cell lines

| Cell line | Organism | Description | Source |
|---|---|---|---|
| CHO-K1 | Chinese hamster | Ovary | Dr. A. Schnieke |
| HEK293 | Human | Embryonic kidney | Dr. A. Schnieke |
| MiaPaca2 | Human | Pancreatic cancer | Dr. D. Saur |
| MPC | Mouse | Pancreatic cancer, p53 deficient | Dr. D. Saur |
| HEK293FT | Human | Embryonic kidney, SV40 large T antigen | Invitrogen |
| HT1080 | Human | Fibrosarcoma | Dr. A. Schnieke |

## 2.1.10 Media and additives for mammalian cell culture

Advanced DMEM                             GIBCO, Paisley, UK

Amphotericin B                              PAA, Pasching, Austria

Blasticidin S                                 Cayla-Invivogen, Toulouse, France

Chicken serum                              PAA, Pasching, Austria

DMEM (4.5 g/l glucose)                 PAA, Pasching, Austria

DMEM/F12                                    PAA, Pasching, Austria

D-PBS                                           PAA, Pasching, Austria

Fetal calf serum, ES qualified (FCS)   PAA, Pasching, Austria

G418                                            PAA, Pasching, Austria

GlutaMAX                                      GIBCO, Paisley, UK

Hanks Balanced Salt Solution         Biochrom AG, Berlin, Germany

Heparin                                         Sigma, Steinheim, Germany

ITS+1                                           Sigma, Steinheim, Germany

Lymphocyte separation medium      PAA, Pasching, Austria

Non essential amino acids             PAA, Pasching, Austria

| | |
|---|---|
| Penicillin/Streptomycin | PAA, Pasching, Austria |
| Porcine serum | PAA, Pasching, Austria |
| Quantum 263 | PAA, Pasching, Austria |
| Quantum 286 | PAA, Pasching, Austria |
| Sodium pyruvate solution | PAA, Pasching, Austria |
| Defined Lipid Supplement | GIBCO, Paisley, UK |
| Lecithin | Fluka, Seezle, Germany |
| Cholesterol, water soluble | Sigma, Steinheim, Germany |

## 2.1.11 Enzymes, hormones and growth factors for cell culture

| | |
|---|---|
| Accutase | PAA, Pasching, Austria |
| Basic fibroblast growth factor (bFGF, FGF-2) | Genaxxon, Ulm, Germany |
| Bovine insulin | Sigma, Steinheim, Germany |
| Collagenase Typ I A | Sigma, Steinheim, Germany |
| Collagenase Typ III | Sigma, Steinheim, Germany |
| Dispase | Becton, Dickinson & Company, Sparks, USA |
| Hyaluronidase | Genaxxon, Ulm, Germany |
| Ovine prolactin | Genaxxon, Ulm, Germany |
| Transforming growth factor beta (TGF-β1) | AbD Serotec, Düsseldorf, Germany |
| Trypsin EDTA | PAA, Pasching, Austria |

## 2.1.12 Miscellaneous reagents

| | |
|---|---|
| Cytoskeleton Advanced Protein Assay Reagent | tebu-bio, Offenbach, Germany |
| Complete mini protease inhibitor | Roche Diagnostics, Mannheim, Germany, |
| Novagen Cytobuster Lysis Buffer | Merck, Nottingham, United Kingdom |
| Passive Lysis Buffer | Promega, Mannheim, Germany |
| Trizol | Invitrogen, Karlsruhe, Germany |
| Hypoosmolar Buffer | Eppendorf, Hamburg, Germany |
| Nanofectin | PAA, Pasching, Austria |
| Calcium Phosphate Transfection Kit | Invitrogen, Karlsruhe, Germany |
| AGFA Developer G 150 for X-Ray film | Röntgen Bender, Baden-Baden, Germany |
| AGFA Fixer G 354 for X-Ray film | Röntgen Bender, Baden-Baden, Germany |
| AGFA Cronex 5 X-Ray film | Röntgen Bender, Baden-Baden, Germany |

## 2.1.13 Oligonucleotides

Oligonucleotides for DNA sequencing

| Oligonucleotide name | Sequence 5' – 3' |
| --- | --- |
| psiCHECK Seq f | aggaggacgctccagatgaa |
| IMG-800 Seq f | aatacgtgacgtagaaagta |
| IMG-800 Seq r | tgatatccgcagatctcc |
| miRNA Seq f | tcccaagctggctagttaag |
| miRNA Seq r | ctctagatcaaccactttgt |
| pLenti-U6 f | atagtaggaggcttggtagg |
| pLenti-U6 r | ccggtagaattcgaagcttg |
| T7 | taatacgactcactataggg |
| SP6 | tatttaggtgacactatag |

Oligonucleotides for shRNA vector constructs directed against porcine BLG

Underlined: Overhangs for cloning; **Bold**: loop region

| Oligonucleotide | Sequence 5' – 3' |
| --- | --- |
| BLGsh219 for. | tcgagggcgtatgtggaggggctg**gagtactg**cagcccctccacatacgccttttt |
| BLGsh219 rev. | ctagaaaaaaggcgtatgtggaggggctg**cagtactc**cagcccctccacatacgccc |
| BLGsh279 for. | tcgaggcgggagaatgacaagtgt**gagtactg**acacttgtcattctcccgctttttt |
| BLGsh279 rev. | ctagaaaaaagcgggagaatgacaagtgt**cagtactc**acacttgtcattctcccgcc |
| BLGsh334 for. | tcgagcctgccgtgttcaagatca**gagtactg**tgatcttgaacacggcaggtttttt |
| BLGsh334 rev. | ctagaaaaaacctgccgtgttcaagatca**cagtactc**tgatcttgaacacggcaggc |
| BLGsh369 for. | tcgagccagctcttcctgctggac**gagtactg**gtccagcaggaagagctggtttttt |
| BLGsh369 rev. | ctagaaaaaaccagctcttcctgctggac**cagtactc**gtccagcaggaagagctggc |
| BLGsh380 for. | tcgagtgctggacactgactacga**gagtactg**tcgtagtcagtgtccagcatttttt |
| BLGsh380 rev. | ctagaaaaaatgctggacactgactacga**cagtactc**tcgtagtcagtgtccagcac |
| BLGsh489 for. | tcgagccagatcagggagaaattt**gagtactg**aaatttctccctgatctggttttttt |
| BLGsh489 rev. | ctagaaaaaaccagatcagggagaaattt**cagtactc**aaatttctccctgatctggc |

Oligonucleotides for miRNA vector constructs directed against porcine BLG

<u>Underlined</u>: Overhangs for cloning; **Bold**: loop region

| Oligonucleotide | Sequence 5' – 3' |
| --- | --- |
| BLGmi334 for. | <u>tgctg</u>attgatcttgaacacggcagggt**tttggccactgactg**accctgccgttcaagatcaat |
| BLGmi334 rev. | <u>cctg</u>attgatcttgaacggcagggt**cagtcagtggccaaa**accctgccgtgttcaagatcaat<u>c</u> |
| BLGmi490 for. | <u>tgctg</u>ttcaaatttctccctgatctggt**tttggccactgactg**accagatcagagaaatttgaa |
| BLGmi490 rev. | <u>cctg</u>ttcaaatttctctgatctggtcagt**cagtggccaaa**accagatcagggagaaatttgaa<u>c</u> |

Oligonucleotides for shRNA vector constructs directed against porcine TP53

<u>Underlined</u>: Overhangs for cloning; **Bold**: loop region

| Oligonucleotide | Sequence 5' – 3' |
| --- | --- |
| TP53sh312 for. | <u>tcgag</u>gggttcctgcattctggaaca**gagtactg**tgttccagaatgcaggaaccctt<u>tttt</u> |
| TP53sh312 rev. | <u>ctaga</u>aaaaagggttcctgcattctggaaca**cagtactc**tgttccagaatgcaggaaccc<u>c</u> |
| TP53sh787 for. | <u>tcgag</u>gctttgaggtgcgtgtttgtg**gagtactg**cacaaacacgcacctcaaagctt<u>tttt</u> |
| TP53sh787 rev. | <u>ctaga</u>aaaaagctttgaggtgcgtgtttgtg**cagtactc**cacaaacacgccctcaaagc<u>c</u> |
| TP53sh944 for. | <u>tcgag</u>gccactggatggcgagtattt**gagtactg**aaatactcgccatccagtggctt<u>tttt</u> |
| TP53sh944 rev. | <u>ctaga</u>aaaaagccactggatggcgagtattt**cagtactc**aaatactcgccatccagtggc<u>c</u> |

Oligonucleotides for miRNA vector constructs directed against TP53

<u>Underlined</u>: Overhangs for cloning; **Bold**: loop region

| Oligonucleotide | Sequence 5' – 3' |
| --- | --- |
| TP53mi304 for. | <u>tgctg</u>aatgcaggaaccctagacggagt**tttggccactg**actgactccgtctagttcctgcatt |
| TP53mi304 rev. | <u>cctg</u>aatgcaggaactagacggagtcagt**cagtggccaaa**actccgtctagggttcctgcatt<u>c</u> |
| TP53mi785 for. | <u>tgctg</u>caaacacgcacctcaaagctggt**tttggccactgactg**accagctttggtgcgtgtttg |
| TP53mi785 rev. | <u>cctg</u>caaacacgcaccaaagctggtcagt**cagtggccaaa**accagctttgaggtgcgtgtttg<u>c</u> |
| TP53mi943 for. | <u>tgctg</u>aatactcgccatccagtggctgt**tttggccactgactg**acagccactgtggcgagtatt |
| TP53mi943 rev. | <u>cctg</u>aatactcgccacagtggctgtcagt**cagtggccaaa**acagccactggatggcgagtatt<u>c</u> |

Oligonucleotides for shRNA and miRNA control constructs

Underlined: Overhangs for cloning; **Bold**: loop region

| Oligonucleotide | Sequence 5' – 3' |
|---|---|
| shnc forward | <u>tcga</u>tcagtcacgttaatggtcgtttt**caagag**aaacgaccattaacgtgactgatttt |
| shnc reverse | <u>ctaga</u>aaaatcagtcacgttaatggtcgttt**ctcttg**aaaacgaccattaacgtgactga |
| minc forward | <u>tgctg</u>aaatgtactgcgcgtggagacgt**tttggccactgactg**acgtctccacgcagtacattt |
| minc reverse | <u>cctg</u>aaatgtactgcgtggagacgt**cagtcagtggccaaa**acgtctccacgcgcagtacattt<u>c</u> |
| shLuc1 forward | <u>tcga</u>gggctcgctgcaagcaaatgaa**gagtactg**ttcatttgcttgcagcgagcctttt<u>t</u> |
| shLuc1 reverse | <u>ctaga</u>aaaaggctcgctgcaagcaaatgaa**cagtactc**ttcatttgcttgcagcgagccc<u>c</u> |
| miLuc1 forward | <u>tgctg</u>ttcatttgcttgcagcgagccgt**tttggccactgactg**acggctcgctaagcaaatgaa |
| miLuc1 reverse | <u>cctg</u>ttcatttgcttagcgagccgt**cagtcagtggccaaa**acggctcgctgcaagcaaatgaa<u>c</u> |
| miLuc2 forward | <u>tgctg</u>tgaaggagtccagcacgttcagt**tttggccactgactg**actgaacgtgggactccttca |
| miLuc2 reverse | <u>cctg</u>tgaaggagtcccacgtt**cagtcagtcagtggccaaa**actgaacgtgctggactccttca<u>c</u> |

Oligonucleotides for circularization of the vector pcDNA6.2-GW/miR (pCMV-miRNA)

Underlined: Overhangs for cloning; **Bold**: SmaI restriction site; *Italics*: BsaI restriction site

| Oligonucleotide | Sequence 5' – 3' |
|---|---|
| BsaI oligonucleotide f | <u>tgctt</u>*gagacc*atatatatat**cccggg**atatatatat*ggtctc*a |
| BsaI oligonucleotide r | <u>cctg</u>t*gagacc*atatatatat**cccggg**atatatatat*ggtctc*a |

DIG-labeled LNA probes for miRNA Northern Blot

| Oligonucleotide | Sequence 5' – 3' |
|---|---|
| BLGmi490 probe | cagatcagggagaaatttgaa |
| U6 snRNA probe | cacgaatttgcgtgtcatcctt |

## 2.1.14 Plasmids

**Table 3: Plasmids used for transfection or cloning (schematic diagrams see section 8.2)**

| Plasmid | Description | Source |
|---|---|---|
| pcDNA6.2-GW/miR (pCMV-miRNA) | Mammalian artificial miRNA construct with CMV promoter | Invitrogen |
| pcDNA-BLG | Mammalian expression construct for porcine BLG based on pcDNA3.1 (Invitrogen) | Dr. H. Wieland |

| pJet1.2-blunt | Cloning vector | Fermentas |
|---|---|---|
| pLenti4s | Lentiviral plasmid derived from pLenti4/V5-DEST (Invitrogen) after removal of the CMV promoter, the *aat*R1/2 sites, the chloramphenicol resistance, the ccdB gene and the V5 epitope | Dr. H. Wieland |
| pLenti6/TR-woodchuck | Lentiviral plasmid based on pLenti6/TR (Invitrogen) with an insertion of the woodchuck hepatitis virus enhancer element (WPRE) | Dr. H. Wieland |
| pLP/VSVG | Lentiviral packaging plasmid containing the Vesicular Stomatitis Virus envelope G glycoprotein | Invitrogen |
| pLP1 | Lentiviral packaging plasmid containing HIV *gag* and *pol* coding sequences | Invitrogen |
| pLP2 | Lentiviral packaging plasmid containing *rev* coding sequences | Invitrogen |
| pMIX-FIX | Mammalian expression plasmid with ovine BLG promoter based on pMIX (A. Schnieke) without the Factor IX DNA sequence | M.Sc. A. Saalfrank |
| pPGK-EGFP1 | Mammalian EGFP expression plasmid with PGK promoter | Dr. H. Wieland |
| pRGC-Luc | Mammalian expression vector with minimal CMV promoter containing 17 p53 response elements | Kind gift of Prof. Dr. Schneider |
| psiCHECK2 | RNA interference test vector with *Renilla* and firefly luciferase | Promega |
| pSL1180 Amersham | Superpolylinker cloning plasmid | Amersham |
| pSuppressorNeo (IMG-800) | Mammalian shRNA vector with U6 promoter | Imgenex |

## 2.1.15 Computer software

| | |
|---|---|
| Axiovision 3.1 | Zeiss AG, Oberkochen, Germany |
| Basic local alignment search tool (BLAST) | http://www.ncbi.nlm.nih.gov/blast/Blast.cgi |
| GeneSnap 6.01 | Syngene, Cambridge, United Kingdom |
| Image J | NIH, USA |
| Microsoft Excel 2003/2007 | Microsoft, Unterschleißheim, Germany |
| Primer 3 | Whitehead Institute, http://frodo.wi.mit.edu |

| | |
|---|---|
| VectorNTI 10 | Invitrogen, Karlsruhe, Germany |
| Block-iT RNAi Designer | https://rnaidesigner.invitrogen.com |
| ClustalW | http://www.ebi.ac.uk/Tools/clustalw2 |

## 2.2  Methods

### 2.2.1  Microbiological methods

Liquid and solid growth media were produced with distilled water (dH$_2$O). Media and glassware were sterilized by autoclaving for 20 min at 121°C before use. Additives were filter-sterilized (0.22 μm pore size). Work was carried out in a class II laminar airflow cabinet under sterile conditions.

#### 2.2.1.1  Storage of E. coli

For short term storage (up to 2 weeks) *E. coli* plates and liquid cultures were kept at 4°C. Long term storage was performed by inoculating 5 ml liquid LB medium with a single colony of *E. coli* followed by overnight (O/N) incubation at 37°C. 500 μl of the *E. coli* liquid culture were mixed with 500 μl sterile 99% glycerol and frozen at -80°C in cryopreservation tubes.

#### 2.2.1.2  Inoculation and growth of E. coli

For small scale plasmid preparation, 5 ml of Luria Broth Base in a 14 ml culture tube with ventilation lid were inoculated with a single colony of *E. coli* from agar plates. Antibiotics were added to obtain a final concentration of 100 μg/ml Ampicillin, 30 μg/ml Kanamycin or 50 μg/ml Spectinomycin, depending on the antibiotic resistance gene encoded on the plasmid. The cultures were then incubated O/N at 37°C in an orbital shaker with agitation at 230 rpm.

Cultures for the isolation of larger amounts of plasmid DNA were prepared by setting up a 5 ml pre-culture according to the method described above. In the exponential growth phase, 1 ml of the starter culture was transferred to an Erlenmeyer flask with 50 ml (Midiprep) or 300 ml (Maxiprep) Luria Broth Base containing the appropriate antibiotics. The culture was incubated O/N at 37°C on an orbital shaker with agitation at 230 rpm.

In addition to liquid cultures, *E. coli* was also cultured on LB agar plates. Bacteria were plated on LB agar after transformation by electroporation, from liquid cultures or from cryopreserved stock. Plates were incubated O/N at 37°C.

#### 2.2.1.3  Transformation of electrocompetent E. coli

50 μl of electrocompetent cells were thawed on ice. 2 μl of a ligation reaction mixture or 1 ng of purified plasmid was added and the mixture transferred into precooled 2 mm electroporation cuvettes. Cells were pulsed once at 2500 V for 5 ms in an Eppendorf Multiporator. 500 μl of prewarmed Luria Broth Base were added immediately, the bacteria transferred to a 14 ml culture tube and grown for 30 min at 37°C with agitation at 230 rpm. Afterwards 50 to 200 μl of the *E. coli* suspension were plated onto LB agar plates and incubated O/N at 37°C.

### 2.2.2  Molecular biological methods

All reactions and solutions were prepared with sterile double distilled $H_2O$ (dd$H_2O$). Polymerase chain reactions (PCR) and reverse transcriptase PCRs (RT-PCR) were assembled with filter tips to avoid sample cross-contamination.

### 2.2.2.1  Isolation of plasmid DNA from E. coli

<u>Small scale isolation of plasmid DNA (Miniprep):</u>

Small scale isolation (Miniprep) of DNA from *E. coli* cultures was routinely performed by the alkaline lysis method. Briefly, 2 ml of a 5 ml O/N culture were pelleted for 1 min at 16000 x g in a tabletop centrifuge. The pellet was resuspended in 100 µl Resuspension Solution (2 mM sucrose, 10 mM EDTA, 25 mM Trizma-HCl, pH 8.0) and lysed for 5 min with 200 µl Lysis Solution (0.2 M NaOH, 1% (w/v) SDS). The lysis reaction was stopped by addition of 200 µl Neutralisation Solution (3 M sodium acetate, pH 4.8) followed by incubation for 20 min at -20°C. The suspension was centrifuged for 10 min at 16000 x g and the supernatant transferred to a new reaction tube. The DNA was precipitated with 1 ml 95% ethanol and centrifuged at 16000 x g to pellet the DNA. The supernatant was discarded and the DNA pellet washed with 80% ethanol and air-dried. The DNA was dissolved in 50 µl dd$H_2O$ containing 20 µg/ml RNAse A to remove RNA contamination.

For sequencing, small scale DNA isolation was performed with the NucleoSpin Plasmid QuickPure kit according to manufacturer's instructions to obtain pure plasmid DNA.

<u>Large scale isolation of plasmid DNA (Midi-/Maxiprep):</u>

Midi- and Maxipreparations of plasmid DNA were performed with the NucleoBond Xtra Midi/Maxi kit according to manufacturer's instructions from 50 or 300 ml overnight cultures.

### 2.2.2.2  Polymerase Chain Reaction (PCR)

PCR was used to amplify fragments from plasmid DNA or genomic DNA for cloning purposes, to screen stable cell clones for the presence of exogenous DNA, to determine the sex of porcine fetuses and to evaluate the genetic sequence of the porcine Ryanodine receptor.

Amplification of the PGK-EGFP-IRES cassette from the plasmid pPGK-EGFP-IRES-EGFP1 was performed with primers IRESPGKEGFP forward and reverse with MolTaq DNA Polymerase from 200 ng plasmid DNA. (<u>underlined</u>: overhang; **bold**: EcoRV restriction site)

| IRESPGKEGFP forward | <u>actg</u>**gatatc**atcatatgccaagtacg | 2300 bp |
|---|---|---|
| IRESPGKEGFP reverse | <u>actg</u>**gatatc**gttgtggccatattatc | |

| Reagent | Final concentration |
|---------|---------------------|
| IRESPGKEGFP f | 2 µM |
| IRESPGKEGFP r | 2 µM |
| MolTaq | 5 U |
| 10 x reaction buffer | 1 x |
| dNTPs | 200 µM per dNTP |
| ddH$_2$O | up to 50 µl |

| | | | |
|---|---|---|---|
| | Initial denaturation | 94 °C | 2 min |
| x 30 | Denaturation | 94 °C | 30 s |
| | Annealing | 53 °C | 30 s |
| | Elongation | 72 °C | 2 min |
| | Final elongation | 72 °C | 5 min |

Amplification of a short fragment of the miRNA cassette in stable porcine adipose tissue derived mesenchymal stem cells was performed after transfection with pPGK-BLGmi490 and pBLG-BLGmi490 from 6 µl Accutase samples with primers miRNA cassette f/r and OmniTaq DNA Polymerase.

| miRNA cassette forward | gatcctggaggcttgctgaa | 264 bp |
|------------------------|----------------------|--------|
| miRNA cassette reverse | ggttccttccggtattgtct | |

| Reagent | Final concentration |
|---------|---------------------|
| miRNA cassette f | 500 nM |
| miRNA cassette r | 500 nM |
| OmniTaq Polymerase | 1.25 U |
| 10 x Reaction buffer C | 1 x |
| dNTPs | 200 µM per dNTP |
| ddH$_2$O | up to 50 µl |

| | | | |
|---|---|---|---|
| | Initial denaturation | 94 °C | 5 min |
| x 35 | Denaturation | 94 °C | 30 s |
| | Annealing | 58 °C | 30 s |
| | Elongation | 72 °C | 45 s |
| | Final elongation | 72 °C | 5 min |

Amplification of a long fragment of the miRNA cassette in stable porcine adipose tissue derived mesenchymal stem cells was performed after transfection with pPGK-BLGmi490 with 6 µl Accutase samples with primers PGK-mi_F/_R and GoTaq DNA Polymerase. The same PCR reaction was used to screen porcine fetuses for the miRNA cassette from 100 ng genomic DNA.

| PGK-mi_F | cattccacatccaccggtag | 720 bp |
|----------|----------------------|--------|
| PGK-mi_R | ggttccttccggtattgtct | |

| Reagent | Final concentration |  | Initial denaturation | 95 °C | 2 min |
|---|---|---|---|---|---|
| PGK-mi_F | 500 nM |  | Denaturation | 95 °C | 30 s |
| PGK-mi_R | 500 nM | x 35 | Annealing | 58 °C | 30 s |
| GoTaq Polymerase | 1.25 U |  | Elongation | 72 °C | 1 min |
| 10 x reaction buffer | 1 x |  |  |  |  |
| dNTPs | 200 µM per dNTP |  | Final elongation | 72 °C | 5 min |
| ddH₂O | up to 50 µl |  |  |  |  |

Amplification of a long fragment of the miRNA cassette in stable porcine adipose tissue derived mesenchymal stem cells was performed after transfection with pBLG-BLGmi490 and for screening of porcine transgenic fetuses from 100 ng genomic DNA with primers BLG-mi_F/_R and GoTaq DNA Polymerase.

| BLG-mi_F | agcatggcatcctagagtgt | 2732 bp |
|---|---|---|
| BLG-mi_R | ggttccttccggtattgtct |  |

| Reagent | Final concentration |  | Initial denaturation | 95 °C | 2 min |
|---|---|---|---|---|---|
| BLG-mi_F | 500 nM |  | Denaturation | 95 °C | 30 s |
| BLG-mi_R | 500 nM | x 35 | Annealing | 58 °C | 30 s |
| GoTaq Polymerase | 1.25 U |  | Elongation | 72 °C | 3 min |
| 10 x reaction buffer | 1 x |  |  |  |  |
| dNTPs | 200 µM per dNTP |  | Final elongation | 72 °C | 5 min |
| ddH₂O | up to 50 µl |  |  |  |  |

Amplification of the shRNA cassette in stable porcine adipose tissue derived mesenchymal stem cells was performed after transfection with pLenti-BLGsh489 from 6 µl Accutase samples with primers shRNA cassette f/r and GoTaq DNA Polymerase. The same PCR configuration was used to screen porcine fetuses for the integration of the shRNA cassette from 100 ng genomic DNA.

| shRNA cassette f | gctcgcttcggcagcacata | 290 bp |
|---|---|---|
| shRNA cassette r | cctaccggtggatgtggaat |  |

| Reagent | Final concentration | | | |
|---|---|---|---|---|
| shRNA cassette f | 500 nM | | | |
| shRNA cassette r | 500 nM | | | |
| GoTaq Polymerase | 1.25 U | | | |
| 10 x reaction buffer | 1 x | | | |
| dNTPs | 200 µM per dNTP | | | |
| ddH₂O | up to 50 µl | | | |

| | | |
|---|---|---|
| Initial denaturation | 95°C | 2 min |
| Denaturation | 95°C | 30 s |
| x 35 Annealing | 58°C | 30 s |
| Elongation | 72°C | 45 s |
| Final elongation | 72°C | 5 min |

For the sex determination of porcine fetuses, genomic DNA was prepared and 200 ng used for the PCR reaction. The SRYB5' and SRYB3' primers amplify a Y chromosome specific region, the sex-determining region Y *Sry* (Pomp *et al.,* 1995). The primers 5'ZFX+Y and 3'ZFX+Y amplify a region on the X and Y chromosome, the testis-determining factor ZFY and ZFX (Aasen and Medrano, 1990). From male fetuses both regions can be obtained by PCR, female fetuses only show the specific product of 5'ZFX+Y and 3'ZFX+Y.

| SRYB5' | tgaacgctttcattgtgtggtc | 163 bp |
|---|---|---|
| SRYB3' | gccagtcgtctctgtgcctcct | |
| 5'ZFX+Y | ataatcacatggagagccacaagc | 445 bp |
| 3'ZFX+Y | gcacttctttggtatctgagaaag | |

| Reagent | Final concentration | | | |
|---|---|---|---|---|
| SRYB5' | 200 nM | | | |
| SRYB3' | 200 nM | | | |
| 5'ZFX+Y | 200 nM | | | |
| 3'ZFX+Y | 200 nM | | | |
| dNTPs | 200 µM per dNTP | | | |
| 10 x reaction Buffer | 1 x | | | |
| GoTaq Polymerase | 1.25 U | | | |
| ddH₂O | up to 50 µl | | | |

| | | |
|---|---|---|
| Initial denaturation | 95°C | 5 min |
| Denaturation | 95°C | 30 s |
| x 40 Annealing | 58°C | 30 s |
| Elongation | 72°C | 45 s |
| Final elongation | 72°C | 5 min |

PCR was used to examine the cells used for somatic cell nuclear transfer for a possible mutation in the Ryanodine receptor 1 (RYR1) related to porcine malignant hyperthermia. 200 ng genomic DNA were used per PCR reaction.

| RYR1_forward | gttccctgtgtgtgtgtgtgtgcaat | 118 bp |
|---|---|---|
| RYR1_reverse | ctggtgacatagttgatgaggtttg | |

| Reagent | Final concentration |
|---------|---------------------|
| RYR1_forward | 500 nM |
| RYR1_reverse | 500 nM |
| dNTPs | 200 µM per dNTP |
| 10 x reaction Buffer | 1 x |
| GoTaq Polymerase | 1.25 U |
| ddH$_2$O | up to 50 µl |

| | | | |
|---|---|---|---|
| | Initial denaturation | 95 ℃ | 5 min |
| x 35 | Denaturation | 95 ℃ | 30 s |
| | Annealing | 58 ℃ | 30 s |
| | Elongation | 72 ℃ | 30 s |
| | Final elongation | 72 ℃ | 5 min |

### 2.2.2.3  Restriction enzyme digestion of DNA

For analytical digests up to 1 µg DNA was digested with 3 U restriction enzyme, for preparative digests 5 to 15 µg DNA were digested with 3 U restriction enzyme per µg DNA according to manufacturer's instructions.

### 2.2.2.4  Conversion of overhanging DNA ends to blunt ends (Klenow treatment)

DNA Polymerase I, Large (Klenow) Fragment was used to convert overhanging DNA ends to blunt ends. 3' overhangs were removed and 5' overhangs were filled in according to manufacturer's instructions.

### 2.2.2.5  Dephosphorylation of plasmid DNA

Calf Intestine Phosphatase (CIP) was used according to manufacturer's instructions to remove 5' and 3' phosphate groups to prevent self-ligation of plasmid DNA.

### 2.2.2.6  Agarose gel-electrophoresis of DNA

Agarose gel-electrophoresis was used to separate DNA fragments according to size after restriction digestion, to isolate DNA fragments and to estimate the concentration of DNA preparations. DNA fragments were separated in agarose gels according to size in an electric field in TAE (40 mM Trizma base, 20 mM acetic acid, 1 mM EDTA, pH 8.0) or TBE buffer (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.0). 1.5% TAE/TBE agarose gels were used for fragments up to 1 kb, fragments larger than 1 kb were separated on 1% TAE/TBE agarose gels. To assess the success of oligonucleotide annealing, 3% TAE agarose gels were used. All gels contained 0.1 µg/ml ethidium bromide to visualize the DNA by UV light at 254 nm excitation wavelength in the Gene Genius Bioimaging System. Samples were mixed with 5 x gel loading buffer (50% (v/v) glycerol, 10 mM EDTA, 0.1% (w/v) SDS, traces of bromophenol blue) before loading onto the gel. To determine the size and estimate the concentration of the DNA fragments, 100 bp or 1 kb DNA ladder from NEB was used. Gels were run at 80 to 120 V for 30 to 90 min depending on the fragment sizes.

### 2.2.2.7  DNA extraction from agarose gels

DNA was extracted from TAE agarose gels by cutting the DNA from the gel with a clean scalpel after minimal UV exposure. The isolation was performed with the Wizard SV Gel and PCR Clean-Up System according to manufacturer's instructions and the DNA eluted in 30 µl of nuclease free water.

### 2.2.2.8  Precipitation of DNA with sodium chloride and ethanol

To precipitate DNA for purification or for concentrating, the DNA solution was mixed with 0.1 volumes of 3 M NaCl and 2 volumes of ice cold 100% ethanol. After O/N incubation at -20°C the sample was centrifuged for 10 min at 16000 x g to pellet the DNA. The supernatant was discarded and the DNA washed with 70% ethanol, centrifuged 5 min at 16000 x g and air dried. The DNA was either resuspended in ddH$_2$O or Tris-low EDTA (10 mM Trizma-HCl, 0.1 mM EDTA) under sterile conditions.

### 2.2.2.9  Determination of DNA concentration

DNA concentration was either measured using an Eppendorf BioPhotometer according to manufacturer's instruction or digested DNA was analyzed on TAE/TBE agarose gels and compared with DNA of known concentration.

### 2.2.2.10      Annealing of single stranded DNA oligonucleotides

Single stranded oligonucleotides for vector circularization, short hairpin RNA and artificial microRNA cloning were annealed before insertion into plasmid vectors. Oligonucleotides were dissolved in ddH$_2$O at a concentration of 1 µg/µl. Then, 1 µg of the sense and reverse oligonucleotide were mixed, 1 x annealing buffer (10 mM Trizma-HCl, pH 8.5, 0.1 M NaCl, 1 mM EDTA) added and volume made up to 20 µl with ddH$_2$O. The mixture was incubated in a PCR Cycler at 95°C for 10 min and then allowed to cool to room temperature in the PCR Cycler over a period of 3 h. Annealed double stranded oligonucleotides were used directly for cloning or stored at 4°C. The annealing efficiency was evaluated by separating 2 µl of the mixture on a 3% TAE agarose gel.

### 2.2.2.11      Ligation of DNA fragments

Ligation of DNA fragments was performed in a total volume of 20 µl according to manufacturer's instructions. A molar vector to insert ratio of 1:3 or 1:5, starting with 50 ng vector DNA was used. Ligations were either incubated for 1 h at RT or 4°C O/N before electroporation into bacteria. Ligation of DNA fragments into pGEM-T easy was performed according to manufacturer's instructions. Annealed oligonucleotides were ligated in 20 µl reaction containing 2 µl of the annealing mixture and 100 ng of vector DNA at 4°C O/N.

## 2.2.2.12    DNA sequencing

DNA sequencing was either performed by Entelechon GmbH (Regensburg, Germany), 4BaseLabs (Ulm, Germany) or according to the BigDye Terminator v1.1 Cycle Sequencing Kit protocol. 100 to 200 ng plasmid DNA were mixed with 2 µl of BigDye Reaction mix, 2 µl BigDye Sequencing Buffer, 2.5 pmol sequencing primer and filled up to 10 µl with nuclease free $H_2O$.

|  | Initial denaturation | 96°C | 15 s |
|---|---|---|---|
|  | Denaturation | 96°C | 10 s |
| x 35 | Annealing | 60°C | 4 s |
|  | Extension | 60°C | 4 min |
|  | Cooling | 8°C | forever |

Further processing of the sequencing samples was performed by Dr. K. Flisikowski (Chair of Animal Breeding, TUM).

## 2.2.2.13    Isolation of RNA

Isolation of RNA from cell culture samples:

Trizol was used for the isolation of RNA from mammalian cells. Up to $1 \times 10^6$ cells were dissolved in 1 ml Trizol and incubated for 5 min at RT. The samples were transferred to 2 ml Eppendorf tubes and either immediately processed or stored at -80°C. 200 µl chloroform per ml Trizol were added, mixed by shaking for 15 s then incubated at room temperature for 2 min. This was followed by centrifugation at 4°C for 15 min at 12000 x g. The clear aqueous phase was transferred to a new 2 ml reaction tube, mixed with 1 ml of ice cold 100% isopropanol, incubated for 10 min at room temperature and centrifuged at 4°C for 10 min at 12000 x g to pellet the RNA. The supernatant was removed and the pellet washed with 1 ml of 70% ethanol, followed by centrifugation at 4°C for 5 min at 7500 x g. The supernatant was removed, the pellet air dried and dissolved in 50 µl of nuclease free water at 55°C for 10 min. The RNA was stored at -80°. To remove contaminating DNA, isolated RNA was treated with the Turbo DNAfree kit according to manufacturer's instructions.

Isolation of RNA from porcine milk:

As a positive control for RT-PCR reactions, RNA was isolated from cells present in porcine milk. Approximately 30 ml of milk were obtained manually from lactating sows at the animal research station in Thalhausen, Germany. The milk was aliquoted in 2 x 15 ml reaction tubes and centrifuged for 15 min at 5000 x g. The fat layer and the milk were removed and cells

resuspended in a total of 1 ml Trizol. Subsequent isolation steps were identical with the RNA isolation from cell culture samples.

### 2.2.2.14    Determination of RNA concentration

RNA concentration was measured using an Eppendorf BioPhotometer according to manufacturer's instructions.

### 2.2.2.15    Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to detect the expression of various genes and for cloning purposes. In general, all RT-PCRs were performed with the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase. In addition to the RT-PCR reaction, water controls (with Superscript III RT/Platinum *Taq* DNA Polymerase, without template RNA) and controls to detect DNA contamination (without Superscript III RT, with Platinum *Taq* DNA polymerase, with template RNA) were performed. RT-PCR was generally performed from 200 ng total RNA.

To confirm expression of porcine BLG in mammary epithelial cells, RNA isolated from milk or in stable cell clones expressing BLG, one-step RT-PCR with primers BLG forward/reverse was performed.

| BLG forward | tgcagccatgaggtgtc | 624 bp |
|---|---|---|
| BLG reverse | ctcctgattccgctgat | |

To confirm expression of porcine β-casein in porcine mammary epithelial cells and porcine milk, one-step RT-PCR with primers b-Casein f/r was used.

| b-Casein f | ctcaatgcatctggtgagac | 424 bp |
|---|---|---|
| b-Casein r | gaatctggtgcatcagagac | |

Internal controls were also used to evaluate the RNA quality. Either β-actin of porcine glyceraldehyde-3-phosphate dehydrogenase (pGAPDH) RNA was amplified. The β-actin primers and competimer (QuantumRNA Beta-actin Internal Standard) were included in gene specific RT-PCR reactions (2 µl primer pair, 2 µl competimer). Porcine GAPDH was amplified separately in a standard one-step RT-PCR using the primers pGAPDH short f/r.

| pGAPDH short f | aaggtcggagtgaacggatt | 444 bp |
|---|---|---|
| pGAPDH short r | acaggaggcattgctgatga | |
| Actin f | sequence unknown (Ambion) | 298 bp |
| Actin r | | |

The general RT-PCR reaction and program is shown below.

| Reagent | Final concentration |
|---------|---------------------|
| 2 x Reaction Mix | 1 x |
| Superscript III RT/Platinum *Taq* | 1 µl |
| Primer f | 200 - 500 nM |
| Primer r | 200 - 500 nM |
| ddH$_2$O | up to 50 µl |

| | | | |
|---|---|---|---|
| | Reverse transcription | 55 °C | 30 min |
| | Initial denaturation | 94 °C | 2 min |
| x 35 | Denaturation | 94 °C | 30 s |
| | Annealing | 55 °C | 30 s |
| | Elongation | 72 °C | 30-60 s |
| | Final elongation | 72 °C | 5 min |

Amplification of the porcine p53 cDNA for cloning purposes was performed with the SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity with gene specific primers Ex2_1F and Ex11_2R using 1 µg of total RNA.

| Ex2_1F | gcaatggaggagtcgcagt | |
|--------|---------------------|---|
| Ex11_R | agggacttcaaaaggggatg | 1198 bp |

| Reagent | Final concentration |
|---------|---------------------|
| 2 x Reaction Mix | 1 x |
| Superscript III RT/Platinum *Taq* High Fidelity | 1 µl |
| Primer Ex2_1F | 200 nM |
| Primer Ex11_2R | 200 nM |
| ddH$_2$O | up to 50 µl |

| | | | |
|---|---|---|---|
| | Reverse transcription | 55 °C | 30 min |
| | Initial denaturation | 94 °C | 2 min |
| x 30 | Denaturation | 94 °C | 30 s |
| | Annealing | 55 °C | 30 s |
| | Elongation | 72 °C | 1.5 min |
| | Final elongation | 72 °C | 5 min |

### 2.2.2.16    Small RNA northern blot analysis

To detect the expression artificial miRNA against porcine BLG, small RNA northern blot was used. Probes for the detection of mature miRNA were ordered as DIG-labeled LNA oligonucleotides.

Sample preparation, quality evaluation and electrophoresis of RNA:

RNA was isolated from a 75% confluent T150 cell culture flask with 4 to 6 ml TRIZOL, isolated and dissolved in 30-50 µl ddH$_2$O per ml TRIZOL. To check the quality of the RNA preparation, 5 µg RNA were concentrated or diluted to 5 µl end volume and mixed with 5 µl FDE loading buffer (deionized formamide, 10 mM EDTA pH 8.0, 1.86 mM xylene cyanol, 1.5

mM bromophenol blue). The RNA was denatured for 10 min at 65°C and then cooled on ice. It was separated on a 1.2% TBE agarose gel (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.0). For small RNA northern blot analysis, 15% denaturing polyacrylamide gels were prepared. Per gel, 2.5 g Urea was dissolved in 0.5 ml 10 x TBE buffer (0.9 M Trizma Base, 0.9 M boric acid, 20 mM EDTA, pH 8.0), 1.88 ml Rotiphorese Gel 40 (19:1) and filled up to 5 ml with ddH$_2$O. 30 µl of 10% APS Solution (10% (w/v) ammonium persulfate in ddH$_2$O) and 2 µl TEMED were added to initiate gel polymerization. The minigels were pre-run in 5 x TBE buffer at 300 V for 60 min. 10-20 µg of total RNA were concentrated to volume of 10 µl in a SpeedVac. 10 µl of FLS loading buffer (deionized formamide, 10 mM EDTA pH 8.0, traces of xylene cyanol and bromophenol blue) was added, the samples denatured for 20 min at 65°C, cooled on ice and loaded onto the pre-run polyacrylamide gel. Samples were run in 5 x TBE buffer at 100 V for 20 min to ensure uniform entry of the samples into the gel, followed by 200 V until the bromophenol blue front reached the bottom of the gel. The gel was stained for 10 min with TBE buffer containing 0.1 µg/ml ethidium bromide and rinsed for 10 min in TBE buffer to visualize the RNA.

Transfer of RNA to membrane:

The gel, thick blotting paper and the Hybond N+ membrane were transferred into 0.5 x TBE buffer for 5 min to equilibrate. The RNA was electroblotted onto the Hybond N+ membrane for 1 h at 250 mA. The damp membrane was removed, rinsed with 2 x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) and the RNA fixed to the membrane by UV crosslinking (1 min, 254 nm) followed by baking for 45 min at 80°C.

Detection of RNA:

The dry membrane was transferred into a roller bottle and prehybridized with DIG Easy Hyb for 1 h at the 50°C. The LNA probe to detect the mature miRNA against BLG was heated to 95°C for 1 min, cooled on ice and diluted in prewarmed DIG Easy Hyb buffer (50°C) at a concentration of 4 pmol per ml and the blot hybridized at 50°C O/N. The membrane was washed 2 x 10 min in Low Stringency Buffer (2 x SSC, 0.1% SDS) at 50°C followed by 1 x 30 min incubation in High Stringency Buffer (0.5 x SSC, 0.1% SDS) at 50°C. After washing the membrane for 5 min in Washing Buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% TWEEN 20, pH 7.5), non-specific binding sites on the membrane were blocked with Blocking Solution (0.1 M maleic acid, 0.15 M NaCl, 1 x blocking reagent, pH 7.5) for 60 min at RT. Anti-DIG antibody was added to the membrane in a 1:10000 dilution in Blocking Solution for 30 min at RT followed by 2 x 15 min incubation in Washing Buffer. The membrane was equilibrated in Detection Buffer (0.1 M Trizma-HCl, 0.1 M NaCl, pH 9.5) for 2 min at RT. CDP-Star was diluted 1:100 in Detection Buffer and added to the membrane. X-Ray film was exposed to the

membrane, incubated in Developer Solution and Fixation Solution to visualize the chemiluminescent reaction.

For reprobing the membrane with the U6 snRNA LNA probe, the wet membrane was washed after chemiluminescent detection for 1 x 10 min in Washing Buffer and then prehybridized as described above. The U6 snRNA probe was diluted in prewarmed (50 °C) DIG Easy Hyb buffer at a concentration of 0.2 pmol per ml. The rest of the procedure was performed as described above.

### 2.2.3  Methods in mammalian cell culture

Tissue culture work was performed in a laboratory with biological safety level S2 in a class II laminar airflow cabinet under sterile conditions. Materials were either autoclaved for 20 min at 121 °C or single packed plastic disposables were used. Solutions and solvents prepared outside the laminar flow hood were filter sterilized (0.22 µm) prior to use. Cell culture of established cell lines and primary cells was generally conducted without antibiotics or antimycotics. The morphology of the cells was regularly inspected under an inverted microscope.

#### 2.2.3.1  Culture of mammalian cell lines and primary porcine cells

In general, cells were cultured in a cell culture incubator at 37 °C, in a 5% $CO_2$ humidified atmosphere in cell culture flasks with ventilation lids. The growth medium was replaced every 2 to 3 days with fresh medium prewarmed to 37 °C. If necessary, selection reagents (Blasticidin or G418) or antibiotics for the first days after isolation of primary cells (Penicillin, Streptomycin or Amphotericin B) were added. Cells were subcultured before they formed a confluent monolayer. The medium was removed and the cells were washed once with D-PBS. The cells were then detached by incubation at 37 °C with 1 to 6 ml Accutase, depending on the size of the culture flask. The cell suspension was either directly transferred into a new culture flask with prewarmed growth medium or the cells were centrifuged at 324 x g for 5 minutes, resuspended in growth medium and then used for experiments or subculturing. Growth media, subculturing ratios and concentration of selection antibiotics used are shown in Table 4.

**Table 4: Growth media, selection antibiotics concentration and subculturing ratios of mammalian cells**

| Mammalian cell | Growth medium | Selection antibiotics concentration | Subculturing ratio |
|---|---|---|---|
| ADMSC | MSC medium | Blasticidin: 8 µg/ml | 1:10 |
| CHO-K1 | Quantum 263 medium | G418: 375 µg/ml<br>Blasticidin: 7 µg/ml | 1:30 |
| HEK293 | DMEMplus medium | G418: 600 µg/ml;<br>Blasticidin: 6 µg/ml | 1:10 |
| HEK293FT | DMEMplus2 medium | --- | 1:10 |
| HT1080 | DMEMplus medium | Blasticidin: 3 µg/ml | 1:10 |
| MiaPaCa2 | DMEMplus medium | --- | 1:10 |
| MPC | DMEMplus medium | --- | 1:30 |
| BMMSC | MSC medium | Blasticidin: 8 µg/ml | 1:10 |
| PMEC | MEC medium | --- | 1:20 |
| poFF | poFF medium | --- | 1:10 |

MEC medium:

DMEM/F12
10 µg/ml Insulin
1 µg/ml Hydrocortisone
10% (v/v) FCS

poFF medium:

DMEM (4.5 g/l glucose)
 2 mM GlutaMAX
1 x Non essential amino acids
1mM Sodium pyruvate
10% (v/v) FCS

DMEM-lipid medium:

DMEM (4.5 g/l glucose)
2 mM GlutaMAX
1 x Non essential amino acids
1 mM Sodium pyruvate
10% (v/v) FCS
10 mM Lecithin
1 x Defined Lipid Supplement
10 nM Cholesterol

MSC starvation medium:

Advanced DMEM
2 mM GlutaMAX
1 x Non essential amino acids
0.5% (v/v) FCS

MSC medium:

Advanced DMEM
2 mM GlutaMAX
1 x Non essential amino acids
10% (v/v) FCS
5 ng/ml bFGF

DMEMplus2 medium:

DMEM (4.5 g/l glucose)
2 mM GlutaMAX
1 x Non essential amino acids
1 mM Sodium pyruvate
10% (v/v) FCS
0.5 mg/ml G418

DMEMplus medium:

DMEM (4.5 g/l glucose)
2 mM GlutaMAX
1 x Non essential amino acids
10% (v/v) FCS

Cryopreservation medium:

70% (v/v) Growth medium
20% (v/v) FCS
10% (v/v) DMSO

Virus production medium:

Advanced DMEM
2% FCS
10 mM Lecithin
1 x Defined Lipid Supplement
10 nM Cholesterol

### 2.2.3.2  Cryopreservation and thawing of mammalian cell lines

Cells were detached from the culture vessel. After centrifugation the cells were resuspended in sterile filtered Cryopreservation medium and frozen at -80°C in a 'Mr. Frosty' freezing device to ensure gradual cooling of -1°C per minute. After a minimum of 2 days at -80°C, cells were stored in the vapor phase above liquid nitrogen.

Cell were thawed by incubation in a 37°C water bath, then mixed with 10 ml of prewarmed culture medium and centrifuged at 324 x g for 5 min. Afterwards, the supernatant was removed, the cell pellet resuspended in culture medium and plated on suitable culture plates.

### 2.2.3.3  Cell counting

Cell counting was performed with an improved Neubauer counting chamber by applying 10 µl of diluted cell suspension. After counting four large squares the cell number per ml was determined according to the following formula:

number of cells per ml = number of cells in 4 large squares / 4 x dilution factor x $10^4$

### 2.2.3.4  Transfection of mammalian cell lines

Different transfection methods were used. CHO-K1, HEK293 and MPC cells were transfected by Nanofection, BMMSCs and ADMSCs by electroporation.

Nanofection:

Nanofection was usually performed in one well of a six well plate. 2 x $10^5$ cells were seeded the day before transfection. They were transfected with 3 µg of plasmid DNA and 9.6 µl of Nanofectin according to the manufacturer's instructions. The transfection mixture was replaced with growth medium after 4 h incubation.

Electroporation:

Porcine BMMSCs and ADMSCs were transfected by electroporation. Cells were detached from the culture flask and counted. 1 to 2 x $10^5$ cells were collected by centrifugation for 5 min at     324 x g and resuspended in 800 µl prewarmed hypoosmolar buffer containing 6 to 8 µg plasmid DNA. After 5 min incubation at RT, cells were electroporated in an Eppendorf Multiporator with one pulse at 1200 V for 85 µs. The cells were allowed to recover for 5 min at RT and transferred to medium containing T75 flasks.

### 2.2.3.5  Generation of stable genetically modified cell pools and lines

To generate stable cell pools, transfected cells were passaged into culture dishes with medium containing selection reagents 48 h after transfection. The medium was changed every second day until no viable cells could be detected in the non-transfected control (10 to 20 days).

For the generation of cell clones, cells were passaged 48 h after transfection at low density into 150 mm culture dishes containing growth medium supplemented with selection reagent. Resistant colonies usually formed after 10 to 15 d. The medium was removed from the culture dish and the cells washed with D-PBS. Filter disks treated with Accutase were placed on the colonies and incubated for 2 min. The filter disks were then transferred into single wells of 12 well plates. The cell clones were expanded and frozen.

BMMSC and ADMSC clones after transfection with pLenti-BLGsh489, pPGK-BLGmi490 and pBLG-BLGmi490 were screened for the transgene with PCR. For this purpose cells were either directly frozen in Accutase for PCR from 6 well plates or genomic DNA was purified using the GenElute Mammalian Genomic DNA Miniprep. 6 µl of Accutase cell suspension or 100 ng purified genomic DNA per PCR were used.

### 2.2.3.6 Lentivirus production, titer determination and transduction

<u>Transfection:</u>

HEK293FT cells were used to produce lentiviral particles. $6 \times 10^6$ cells were plated per 100 mm dish in triplicates in DMEMplus2 medium without G418. After 6 h the growth medium was replaced with 10 ml DMEM-lipid medium. 24 h later the medium was replaced with 5 ml Virus production medium and after an additional 3 h cells were transfected. A mixture of equal amounts of the three packaging plasmids pLP1, pLP2, pLP/VSVG and the lentiviral plasmids in a 1:0.75 ratio was used. Transfection was performed using the calcium phosphate precipitate method according to the manufacturer's manual. 25 µM chloroquine-diphosphate was added to enhance transfection efficiency. After incubation for 5 h the medium was replaced with 5 ml Virus production medium without chloroquine-diphosphate. All incubation steps were performed at 37 °C, in a 5% $CO_2$ humidified atmosphere.

<u>Harvesting of lentivirus:</u>

The supernatant of the cells containing infectious lentiviral particles was harvested 48 h after transfection. The virus stock was cooled on ice for 15 min followed by centrifugation for 15 min at 1000 x g to remove cell debris. The supernatant was aliquoted and frozen at -80 °C.

<u>Determination of infectious viral titer:</u>

On the day of transduction, $4 \times 10^5$ HT1080 cells were plated per well in a 6 well plate. After the cells had attached to the culture dish, virus containing medium was added. Polybrene was included at a concentration of 6 µg/ml to enhance transduction efficiency. Undiluted and diluted ($10^{-1}$ to $10^{-5}$) virus stock was added in a total volume of 1 ml per well. After 6 h the transduction mix was replaced with DMEMplus2 without G418. 48 h after transduction, cells were detached and seeded in 100 mm dishes with Blasticidin. The selection was performed for 14 d.

Crystal violet staining:

To determine colony forming units of transduced cells, crystal violet staining was applied. The cells were washed with D-PBS. Afterwards they were stained with crystal violet solution (0.5% (w/v) crystal violet, 20% (v/v) methanol in $ddH_2O$) for 5 min at RT. Surplus staining solution was removed by extensive rinsing with $ddH_2O$ and the colonies counted. The viral titer in transducing units per ml (TU/ml) was calculated according to the following formula:

No. of colonies per well x dilution factor = TU/ml

## 2.2.3.7 Isolation and culture of primary porcine mammary epithelial cells (PMEC)

Isolation of PMEC:

Mammary tissue from lactating and non-lactating sows was obtained from a local abattoir. The tissue was incubated in D-PBS containing 1 x Penicillin/Streptomycin, 50 µg/ml Gentamycin and 2.5 µg/ml Amphotericin B for 30 min at 37°C after cleaning the tissue surface with Barricidal and 80% ethanol. 1 $cm^3$ of mammary gland tissue was excised with sterile scissors and scalpel and finely minced. The minced tissue was transferred to a 50 ml Falcon tube filled with D-PBS containing 2.4 U/ml Dispase, 0.1% (w/v) Collagenase type III and 0.05% (w/v) Hyaluronidase. The tissue was incubated for 2 to 4 h at 37°C with occasional shaking. The digested cell suspension was filtered through a 100 µm cell strainer to remove undigested and connective tissue. The filtered cells were pelleted by centrifugation at 800 x g for 10 min, washed with D-PBS and centrifuged again. The cell pellet was resuspended in MEC medium containing 1 x Penicillin/Streptomycin and 2.5 µg/ml Amphotericin B and plated in T75 flasks. The medium was changed daily for seven days. On the eighth day, antibiotics/antimycotics were removed.

Culture of PMEC:

To remove contaminating fibroblasts, cells were treated at each passage with Accutase for 5 min. During this incubation, fibroblasts detached from the culture dish and were removed. Afterwards the PMEC were detached with Trypsin/EDTA solution for 10 to 15 min. The cells were passaged at 90% confluence.

Media for growth condition test:

| MCDB170EGF medium: | MEC medium: | Quantum 286 |
|---|---|---|
| MCDB170 | DMEM/F12 (with L-Glutamine) | |
| 35 µg/ml bovine pituitary extract | 10 µg/ml insulin | |
| 10 µg/ml insulin | 1 µg/ml hydrocortisone | |
| 1 µg/ml hydrocortisone | 10% (v/v) FCS | |
| 10 ng/ml rhEGF | | |

Modified Kumura medium:          MEC PS medium:

DMEM/F12 (with L-Glutamine)      DMEM/F12 (with L-Glutamine)
1 x ITS+1                        10 µg/ml insulin
1 µg/ml hydrocortisone          1 µg/ml hydrocortisone
                                10% (v/v) porcine serum

Three-dimensional culture of PMECs:

To induce milk protein expression, PMECs were cultured on top of or embedded in Matrigel and in medium supplemented with ovine prolactin. Matrigel is a basement membrane matrix supporting 3D growth of polarized cells. It is a soluble basement membrane extract from murine Engelbrecht Holm-Swarm (EHS) sarcoma model (reviewed in LeBleu *et al.,* 2007; Kleinman and Martin, 2005). Various culture conditions were used to induce milk protein expression as specified in the results section.

## 2.2.3.8 Isolation and culture of porcine bone marrow derived mesenchymal stem cells

Bone marrow derived mesenchymal stem (multipotent stromal) cells from pigs (BMMSC) were isolated from the femur and/or tibiae of 6 month old German landrace animals. Bones were transported in D-PBS and sprayed with Barricidal and 80% ethanol and cut open with a saw at both ends. Bone marrow was flushed from the bones with HBSS-heparin solution (50 mg/ml Heparin in Hanks Balanced Salt Solution (HBSS)) and collected in culture dishes. A maximum of 20 ml cell suspension was pipetted on top of 25 ml Lymphocyte separation medium and centrifuged for 20 min at 1200 x g to separate mononuclear cells from red blood cells and plasma. About 7 ml of the interphase, containing mononuclear cells, was extracted with a 10 ml pipette, mixed with 20 ml HBSS and centrifuged 5 min at 324 x g. The pellet was washed once with D-PBS and resuspended in MSC medium containing 1 x Penicillin/Streptomycin and 2.5 µg/ml Amphotericin B after centrifugation. The cells were plated in one T150 culture flask per bone. The medium was changed daily for two days. Thereafter, cells were incubated in medium without antibiotics/-mycotics for three days and the cells frozen on day 6. For subculturing the cells were passaged at 80% confluence.

## 2.2.3.9 Isolation and culture of porcine adipose tissue derived mesenchymal stem cells

Adipose tissue derived mesenchymal stem cells (ADMSC) were isolated from fat tissue from the neck of 6 month old German landrace (female) or German landrace x pietrain (male) pigs obtained from a local abattoir. The fat tissue was transported in D-PBS, disinfected with Barricidal and 80% ethanol and incubated for 30 min at 37°C in D-PBS containing 1 x Penicillin/Streptomycin and 2.5 µg/ml Amphotericin B. Then the fat tissue was aseptically

separated from skin and muscle tissue with a sterile scalpel and finely minced with sterile scissors. Up to 6 g fat was enzymatically digested in 10 ml sterile filtered Collagenase type I A solution (1 mg/ml in D-PBS) in a 37 °C waterbath with shaking every 5 min. The suspension was then filtered through a 100 μm cell strainer to remove remaining fat clumps and connective tissue. The cell suspension was mixed with an equal volume of MSC medium, centrifuged for 10 min at 1000 x g. The pellet was resuspended in MSC medium containing 1 x Penicillin/Streptomycin and 2.5 μg/ml Amphotericin B and plated in one T150 flask per 6 g fat. The medium was changed daily for two days. Thereafter the cells were cultured in MSC medium without antibiotic/-mycotics for three days and cells frozen on day 6. For subculturing the cells were passaged at 80% confluence.

### 2.2.3.10        Differentiation of ADMSC and BMMSC

Adipogenic differentiation:

$2 \times 10^5$ cells were plated in each well of a 6 well plate. 48 h later, Adipogenesis medium (Advanced DMEM, 1 x non essential amino acids, 2 mM GlutaMAX, 10% FCS, 50 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 1 x ITS+1, 100 μM indomethacin, 5 ng/ml bFGF) was added to three wells and three control wells were incubated with MSC medium. Medium was changed 2 to 3 times per week for 21 d. The cells were washed twice with D-PBS and fixed with 1 ml Fixation Solution (10% (v/v) formaldehyde in methanol) for 5 min. The Fixation Solution was removed and the cells washed 3 times with $dH_2O$. 1ml Oil Red O Solution (1.5 parts 0.3% Oil Red O in Isopropanol + 1 part $ddH_2O$) was added per well and stained for 30 min at RT. Excess Oil Red O was rinsed off with $ddH_2O$ and the results were documented. Lipid droplets in cells stained red.

Osteogenic differentiation:

$3 \times 10^4$ cells were plated in each well of two 6 well plates. 48 h after plating, Osteogenesis medium (Advanced DMEM, 1 x non essential amino acids, 2 mM GlutaMAX, 10% FCS, 10 mM ß-glycerol phosphate, 50 μg/ml ascorbic acid, 100 nM dexamethasone, 5 ng/ml bFGF) was added to 6 wells and MSC medium as a control on the remaining wells. The medium was changed 2 to 3 times per week for 21 d. The cells (differentiated and control) of one 6 well plate were used for staining of calcium deposits, the other wells for calcium quantification.

Staining:

The cells were washed twice with D-PBS and fixed with Fixation Solution (10% (v/v) formaldehyde in methanol) as described for adipogenic differentiation. 1 ml Staining Solution (5% (w/v) silver nitrate in $ddH_2O$) was added per well and incubated in the dark for 30 min at RT. After washing the cells with $dH_2O$ 3 times, 1 ml $dH_2O$ was pipetted onto the cells and the plate exposed to UV light on a transilluminator at 254 nm for 30 min. The $dH_2O$ was removed

and replaced with 1 ml Sodium thiosulfate Solution (5% (w/v) sodium thiosulfate in ddH$_2$O). After 5 min incubation at RT the reaction was stopped, the cells washed 3 times with dH$_2$O and the results documented. Calcium deposits stained brown/black.

Quantification:

Cells were washed twice with D-PBS, 500 µl 0.5 M HCl solution was added and incubated for 1 min at RT. Cells were scraped off and transferred to a 1.5 ml reaction tube. The wells were rinsed with 500 µl 0.5 M HCl and the solution added to the reaction tubes. Calcium was released from the cells by gentle shaking O/N at 4°C. Quantification was performed according to the manual of the Calcium CPC Liquicolor Kit and the calcium content calculated.

Chondrogenic differentiation:

5 x 10$^5$ cells were centrifuged for 5 min at 324 x g, the pellet washed with D-PBS and the cells pelleted again for 3 min at 3400 x g in 15 ml reaction tubes. Chondrogenesis medium (Advanced DMEM, 1 x non essential amino acids, 2 mM GlutaMAX, 10% FCS, 10 ng/ml TGF-ß) was carefully added to not disturb the pellet. The cells were incubated for 21 d with medium change twice per week. Thereafter, the medium was removed, the pellet washed twice with D-PBS and air dried. Further processing including papain digestion, quantification of glycosaminoglycans (GAG) and DNA quantification with the Pico Green Assay (Molecular probes) was performed by M.Sc. M. Durkovic (Lehrstuhl für Biotechnologie der Nutztiere, TUM).

### 2.2.3.11 Isolation of porcine fetal fibroblasts (poFF)

Fetuses were removed from the sow at day 32 of the pregnancy. The outside of the uterus was disinfected by spraying with Barricidal and 80% ethanol. It was then opened with sterile scissors and fetuses recovered within their amniotic sacs. The following steps were performed in a class II sterile hood. The amniotic sacs were opened and the fetuses extracted. The head, the liver and the heart were removed with sterile forceps and scalpel. The body was finely minced with a sterile scalpel and incubated for 30 min in Trypsin/EDTA solution containing 2% (v/v) chicken serum. The tissue clumps were disaggregated by pipetting with a 5 ml pipette every 5 min. The cell suspension was transferred to a 50 ml reaction tube and centrifuged for 5 min at 324 x g to pellet the cells. The pellet was resuspended in poFF medium containing 1 x Penicillin/Streptomycin and 2.5 µg/ml Amphotericin B and plated in one T150 flask per fetus. Medium was changed daily for 2 days, from day 4 onwards antibiotic/antimycotics were removed and the cells frozen on day 6. The cells were subcultured at 90% confluence.

### 2.2.3.12    Preparation of cells for somatic cell nuclear transfer

Positive cell clones after screening were mixed in equal amounts and 1 x 10$^5$ cells plated in 4 wells of a 6 well plate. The next day the medium was replaced in two wells with MSC Starvation medium to induce cell cycle synchronization. In the other two wells, the medium was replaced with MSC Starvation medium on the following day. The cells were then transported to the Institute of Molecular Breeding and Biotechnology (Oberschleißheim, Germany). The somatic cell nuclear transfer and embryo culture was performed by Dr. V. Zakhartchenko. The embryo transfer was conducted by Dr. B. Keßler.

## 2.2.4  Methods in microscopy

Cells were routinely observed with an AxioVert 25 inverted microscope to examine cell morphology and to exclude contamination. Expression of enhanced green fluorescent protein (EGFP) was visualized after excitation at 488 nm and emission at 507 nm. Cells were photographed with an AxioCam MRc camera.

## 2.2.5  Methods in protein chemistry

### 2.2.5.1  Western blot analysis

<u>Sample preparation</u>

Cell culture samples:

To prepare protein samples from cell culture cells, they were resuspended in 50 to 200 µl CytoBuster protein lysis buffer containing 1 x Complete Mini Protease Inhibitor Cocktail depending on the cell number. Cells were incubated at RT for 5 min and frozen at -80 °C. Before use, the suspension was centrifuged at 16000 x g for 5 min to remove insoluble material and the supernatant transferred to a new 1.5 ml reaction tube and either used directly for concentration measurement or frozen at -80 °C.

Porcine milk samples:

As a positive control for western blotting, skimmed milk was prepared from porcine milk. Approximately 30 ml of milk were obtained manually from lactating sows at the animal research station in Thalhausen, Germany. The milk was then aliquoted in 1.5 ml reaction tubes and centrifuged for 15 min at 16000 x g. The milk fat formed a dense layer on top of the skimmed milk and was removed. The skimmed milk was diluted 1:100 with ddH$_2$O containing 1 x Complete Mini Protease Inhibitor Cocktail, aliquoted and frozen at -80 °C.

## Determination of protein concentration

To determine the protein concentration the Bradford assay based Advanced Protein Assay Reagent was used according to manufacturers instruction either in single tube or 96 well format.

## Sodiumdodecylsulfate Polyacrylamide Gelelectrophoresis (SDS-PAGE)

To separate proteins according to size in an electric field, SDS-PAGE was used. To separate proteins, 12% or 20% separating gels with a 5% stacking gel were used. The gels were prepared according to Table 5.

**Table 5: Formulation of 12%, 20% and 5% gel for SDS-PAGE**

| Reagent | 12% separating gel | 20% separating gel | 5% stacking gel |
|---|---|---|---|
| Polyacrylamide 40% (29:1) | 900 µl | 1500 µl | 125 µl |
| 1 M Trizma base pH 8.8 | 1125 µl | 1125 µl | --- |
| 0.5 M Trizma base pH 6.8 | --- | --- | 250 µl |
| 10% SDS | 30 µl | 30 µl | 10 µl |
| 10% APS | 30 µl | 30 µl | 10 µl |
| TEMED | 1.2 µl | 1.2 µl | 1 µl |
| ddH$_2$O | 913.8 µl | 313.8 µl | 604 µl |
| Total volume per gel | 3 ml | 3 ml | 1 ml |

Up to 50 µg of protein solution was mixed with 5 µl of 4 x Lämmli Buffer (250 mM Trizma-HCl, pH 6.8, 1% (w/v) SDS, 1.1 M sucrose, 26 mM dithiothreitol, including a trace of bromophenol blue) and heated to 95°C for 5 min in a PCR cycler. The Mini-PROTEAN 3 cell was assembled and filled with 1 x SDS-PAGE Running Buffer (25 mM Trizma base, 200 mM glycine, 0.1% (w/v) SDS) according to manufacturer's instructions. Then the samples and 10 µl of SeeBlue Plus2 pre-stained Standard for size estimation were loaded onto the gel. Empty wells were filled with 10 µl 4 x Lämmli Buffer to ensure even running of samples. The gel was run for 45 min at a constant voltage of 200 V.

## Transfer to PVDF membrane

The gel was removed from the glass plates and incubated in Semidry Blotting Buffer (25 mM Trizma Base, 200 mM glycine, 20% (v/v) methanol) for 5 to 10 min. The PVDF membrane (Immobilon-P) was activated for 1 min in 100% methanol and equilibrated in Semidry Blotting Buffer for 5 min. Filter papers were soaked in Semidry Blotting Buffer. The Trans-Blot SD Semi-dry Electrophoretic Transfer Cell was then assembled according to manufacturer's instructions and blotted at a constant voltage of 10 V per minigel (7 x 8 cm) for 30 min.

Blocking and antibody binding

After the Semidry Blotting procedure, the PVDF membrane with the transferred proteins was removed from the Blotting cell and incubated for 60 min at RT in 10 ml Blocking Buffer (5% (w/v) BSA, 20 mM Trizma base, 140 mM NaCl, 0.1% (v/v) TWEEN 20) on an orbital shaker. Afterwards the membrane was washed 3 x 5 min in 20 ml Washing Buffer (20 mM Trizma base, 140 mM NaCl, 0.1% (v/v) TWEEN 20) under agitation followed by incubation ON at 4°C with primary antibody diluted in 10 ml Blocking Buffer according to Table 6. The membrane was rinsed 3 x followed by washing 3 x 15 min in 20 ml Washing Buffer. Incubation with secondary antibody, diluted in 10 ml Blocking Buffer according to Table 6, was carried out at RT for 60 min on an orbital shaker. The membrane was washed again as described above.

**Table 6: Antibodies and dilutions**

| Antibody | Antigen | Raised in | Company | Dilution |
| --- | --- | --- | --- | --- |
| **Primary antibodies** | | | | |
| Pab240 | murine p53 | mouse | Santa Cruz | 1:2500 |
| DO-1 | human p53 | mouse | Sigma Aldrich | 1:5000 |
| Anti-bovine BLG | Bovine Beta-lactoglobulin | rabbit | Bethyl Laboratories | 1:5000 |
| Anti-Actin | Actin | Rabbit | Rockland Inc. | 1:5000 |
| Anti-Actin | Actin | Mouse | Sigma Aldrich | 1:100000 |
| Anti-GAPDH | Glyeraldehyd-3-phosphate dehydrogenase | Mouse | Sigma Aldrich | 1:5000 |
| **Secondary antibodies** | | | | |
| Anti-mouse | murine IgG | Rabbit | Abcam | 1:6000 |
| Anti-rabbit | rabbit IgG | Donkey | Rockland Inc. | 1:30000 |

Chemiluminescent detection

After washing the membrane again, excess liquid was removed, 500 µl of Detection Reagent 1 and 500 µl of Detection Reagent 2 from Pierce ECL western blotting Substrate Kit were mixed and applied to the membrane. After 1 min incubation excess liquid was removed and the membrane placed in a plastic wrap. In a dark room, the membrane was placed in a cassette with X-Ray film and incubated for 10 sec to 30 min depending on the signal intensity. The results were visualized by incubation AGFA developer solution and fixation solution for 3 min.

### 2.2.5.2  Dual-Luciferase Assays

Cells were transfected with 3 µg DNA with Nanofectin and at the indicated molar ratios.

| Plasmids | molar ratio |
|---|---|
| psiCHECK2 : shRNA construct | 1:5 |
| psiCHECK2 : miRNA construct | 1:5 |
| pRGC-Luc : pcDNA-p53 : p*Renilla* | 1 : 1 : 0,02 |
| pRGC-Luc : pcDNA-BLG : p*Renilla* | 1 : 1 : 0,02 |
| pRGC-Luc : p*Renilla* | 1 : 0,02 |

After 48 h cells were washed with D-PBS and lysed with 500 µl 1 x Passive Lysis Buffer for 15 min at RT. The suspension was transferred into 1.5 ml reaction tubes and centrifuged for 5 min at 16000 x g to remove cell debris. 75 µl of the supernatant were mixed with 75 µl Dual-Glo Reagent and incubated at RT for 15 min. Luminescence generated by firefly luciferase was measured with a GloMAX 20/20 luminometer with an integration time of 2 s. 75 µl Dual-Glo Stop & Glo solution was added and incubated for 15 min at RT. Luminescence generated by *Renilla* luciferase was determined as before for firefly luciferase. Dual-Luciferase Assays were performed in triplicate and performed at least twice. Relative light units (RLU) were calculated by normalization of the regulated luciferase with the respective control luciferase. RLU are stated as mean values of the triplicates and error bars represent standard deviations. The significance of the RLU compared to control values was determined by ANOVA. Representative diagrams of the independent experiments are shown.

# 3 Results

## 3.1 Sequence-specific gene silencing of porcine beta-Lactoglobulin

Beta-Lactoglobulin (BLG) is one of the major proteins in the whey fraction of milk in mammalian species with the exception of humans, rodents and lagomorphs. The function of the protein is unknown. Some biological functions have been proposed for bovine BLG such as retinol transport or absorption, involvement in intestinal fatty acid absorption and the digestion of milk fat but no definitive proof is available (Papiz *et al.,* 1986; Kushibiki *et al.,* 2001; Pérez *et al.,* 1992). For porcine BLG no functions other than as a source of amino acids for the offspring have been suggested. Moreover, bovine BLG is known to be a major factor in cow's milk intolerance/allergy (CMI/CMA) (Wal, 1998a). Elimination of BLG from bovine and porcine milk could give further insight into the function of the protein. Removal of BLG from cow's milk would have a beneficial effect for children suffering from CMI/CMA. Knockout of BLG by gene targeting has been attempted in the past but without success. The main hurdle for knockout by gene targeting is the fact that milk proteins are generally not expressed in primary cells in culture. This impedes the use of promoter-trap vectors for the enrichment of gene targeted cells (Thomson *et al.,* 2003). Therefore the aim of this project was to determine whether RNA mediated gene silencing (RNAi), offers a useful alternative approach. In this part of the project pigs with RNAi mediated knockdown of BLG should be generated. The pig was chosen as a convenient species because the generation time of pigs is much shorter than in cattle and pigs have bigger litter sizes. Success in this pilot work could lead to the identification of gene function of porcine BLG and a suitable method for the generation of cows with BLG knockdown.

### 3.1.1 Construction of short hairpin RNA vectors for BLG knockdown

To achieve stable downregulation of porcine BLG, different RNAi expression constructs were employed. These were based either on short hairpin RNAs (shRNAs) or artificial microRNAs (miRNAs). The first step was to design shRNAs for knockdown of BLG with the siRNA Target Finder (Ambion) or Block-iT RNAi Designer (Invitrogen) programs using the NCBI BLG Reference Sequence X54976. Sequences against BLG were retrieved and a BLAST search in the known porcine database performed to exclude homologies with other porcine mRNAs. Sequences showing more that 15 nt stretches of homology with other porcine genes were avoided to minimize off-target effects because siRNAs can bind to sequences with partial complementarity (Hajeri and Singh, 2009). Six sequences were chosen for shRNA mediated BLG downregulation and were named according to their position relative to the 5' end of the NCBI BLG mRNA Reference Sequence X54976. The shRNA target sequences started at nt 219, 279, 334, 369, 380 and 489 and are illustrated in Figure 13.
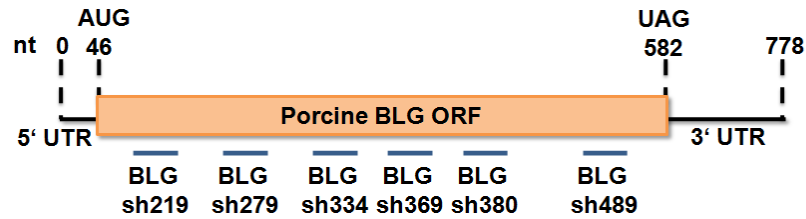
**Figure 13: Porcine BLG mRNA from Reference Sequence X54976 with shRNA target sites.** Target sites are located in the 537 nt open reading frame (ORF) of porcine BLG and are named according to their position from the 5' end of the NCBI BLG Reference Sequence X54976.

The RNAi expression sequences were obtained as single stranded DNA oligonucleotides. Before insertion into the shRNA expression plasmid pSuppressorNeo (named IMG-800 in the following sections), they were annealed to generate double stranded DNA oligonucleotides. The schematic structure of annealed shRNA oligonucleotides is illustrated in Figure 14.
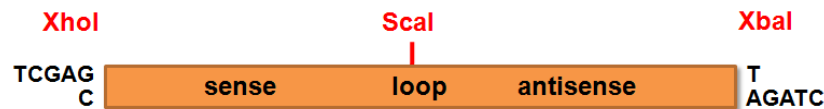


**Figure 14: General configuration of double stranded shRNA DNA oligonucleotides.** DNA oligonucleotides were designed with XhoI and XbaI restriction site overhangs. The 6 nt spacer (loop) sequence contains a ScaI restriction site. The antisense sequence includes a transcription terminator (TTTTT).

The double stranded shRNA oligonucleotides were inserted into the SalI and XbaI restriction sites of the shRNA plasmid IMG-800. The general configuration of the shRNA expression cassette in this plasmid is presented in Figure 15. The transcription of the shRNA is directed by the U6 Pol III promoter and terminated by a stretch of five thymidine residues at the 3' end of the antisense sequence. Moreover, a second terminator is included downstream of the cassette to ensure the production of small RNAs. This cassette results in the expression of shRNAs within mammalian cells.
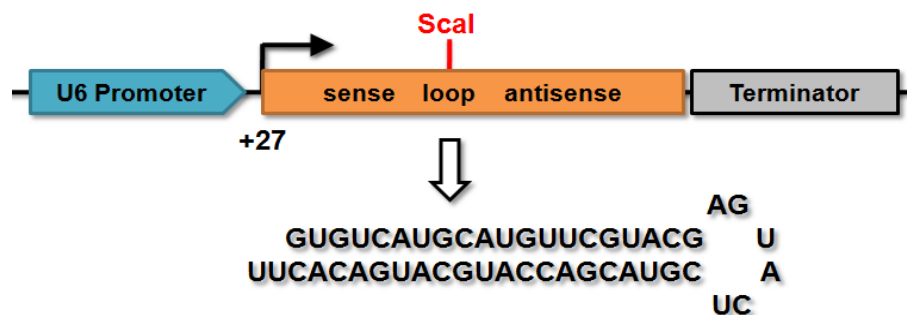


**Figure 15: Configuration of shRNA expression cassette in IMG-800.** The U6 promoter (blue) is a polymerase III promoter directing the expression of short RNAs. The shRNA coding sequence is connected to a complementary coding sequence by a 6 nt spacer (loop) containing a ScaI restriction site (orange). In addition to five thymidine residues at the 3' end of the antisense sequence, a second transcription terminator (grey) is included in the construct. After transcription, the RNA folds into a hairpin structure with 3' uracil overhangs.

Insertion of the shRNA oligonucleotides into IMG-800 was tested by restriction enzyme digestion with SalI and ScaI. Due to the fact that the shRNA oligonucleotides contain an XhoI overhang compatible with the SalI site of IMG-800, this restriction site was eliminated during

insertion of the oligonucleotide. The loop sequence of the shRNA includes a ScaI restriction site which facilitates the identification of plasmids with shRNA insertion. The resulting construct, IMG-BLGshRNA, is depicted in Figure 16.
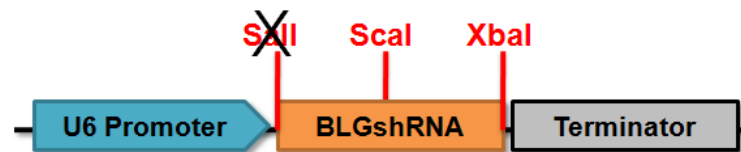


**Figure 16: Schematic diagram of the shRNA plasmid IMG-BLGshRNA.** Vector IMG-800 with shRNAs against porcine BLG. The shRNAs are inserted into the SalI and XhoI sites of IMG-800. Blue: RNA polymerase III promoter U6; Orange: shRNA against porcine BLG; Grey: second terminator for shRNA expression cassette.

After confirmation by restriction enzyme digestion, constructs containing the shRNA cassette were sequenced. A negative control shRNA plasmid, named IMG-shnc, containing a hairpin sequence with no homology to the rat, mouse and human genome was included in the pSuppressorNeo kit. Furthermore, a positive control shRNA sequence starting at position 61 relative to the *Renilla* luciferase (hRluc, Promega) start codon was designed with the Block-iT RNAi Designer program to silence *Renilla* luciferase. This sequence was also inserted into IMG-800 and the control plasmid IMG-shLuc1 generated.

### 3.1.2   Cloning of the RNA interference test vector psiCHECK-BLG

The knockdown ability of the selected shRNA sequences was first tested in a Dual-Luciferase RNAi reporter assay. This evaluation of knockdown efficiencies is based on the RNA interference test plasmid psiCHECK2 and a schematic diagram illustrating the test system is presented in Figure 17.

The plasmid psiCHECK2 contains two luciferase genes which can be expressed in mammalian cells. The cDNA of the gene of interest can be placed behind the stop codon of *Renilla* luciferase. This leads to a fusion mRNA of *Renilla* luciferase and the gene of interest. When *Renilla* luciferase is translated it catalyzes the oxidation of coelenterazine to coelenteramide, $CO_2$ and light (Promega). If a functional shRNA or artificial miRNA directed against the gene of interest or the *Renilla* luciferase sequence is added, the fusion mRNA is degraded, leading to a reduction of *Renilla* luciferase and thereby to a decrease in light emission. The second luciferase from firefly catalyzes the oxidation of luciferin to oxyluciferin under light emission (Promega). This luciferase serves as an internal control allowing results to be normalized for transfection efficiency.
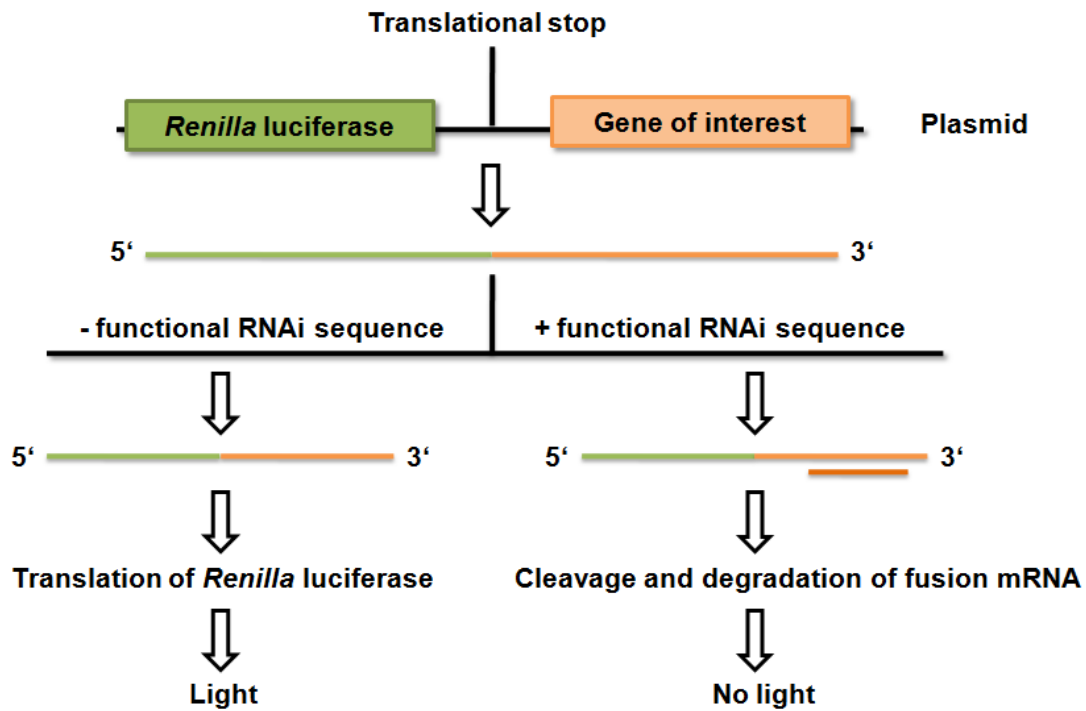
**Figure 17: psiCHECK2 RNA interference test system (adapted, from Promega).** The cDNA of the gene of interest is cloned into the 3' UTR of *Renilla* luciferase. The *Renilla* luciferase is translated and light emission can be measured. If functional RNAi constructs directed against the gene of interest or the *Renilla* luciferase are introduced into the cell, the fusion mRNA is cleaved and degraded by the RNA interference pathway. This leads to reduced substrate conversion and a decrease of light emission.

To employ the RNAi test vector for a first evaluation of the knockdown potential of the shRNA sequences against porcine BLG, it was necessary to insert the porcine BLG cDNA into the vector psiCHECK2. For this purpose, the cDNA for porcine BLG was excised from the plasmid pGEM-T-porcine BLG (see section 2.1.14) with NotI and inserted into the NotI site of the RNAi test vector psiCHECK2 (see Figure 18).
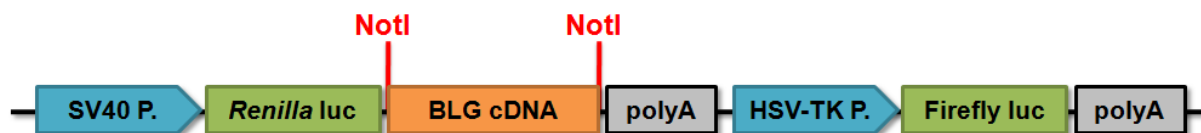


**Figure 18: Schematic diagram of the RNAi test vector psiCHECK-BLG.** Porcine BLG cDNA from pGEM-T-porcine BLG was inserted into the NotI restriction site of psiCHECK2. The fusion mRNA of *Renilla* luciferase and porcine BLG is expressed by a SV40 promoter and the firefly luciferase mRNA by a herpes simplex virus thymidine kinase promoter (blue). Polyadenylation signals are depicted in grey.

The plasmid was sequenced with the primer psiCHECK2 Seq f to exclude mutations in the BLG cDNA sequence.

### 3.1.3 Dual-Luciferase assay to determine knockdown potential of BLG shRNAs

ShRNA sequences directed against porcine BLG were examined for their knockdown efficiency in a Dual-Luciferase assay. For this purpose, the vector psiCHECK-BLG was either cotransfected with the shRNA plasmids against BLG (IMG-BLGsh219, IMG-BLGsh279, IMG-BLGsh334, IMG-BLGsh369, IMG-BLGsh380, IMG-BLGsh489), the negative control plasmid

IMG-shnc or the positive control vector IMG-shLuc1 against *Renilla* luciferase into HEK293 cells. Two days later *Renilla* and firefly luciferase catalyzed light emission was determined. Silencing efficiencies were calculated as percentage of the negative control IMG-shnc. BLGsh219 showed approximately 22%, BLGsh334 74%, BLGsh380 30% and BLGsh489 50% knockdown compared to the negative control. The positive control shLuc1 resulted in a 64% downregulation of relative *Renilla* luciferase expression (see Figure 19).
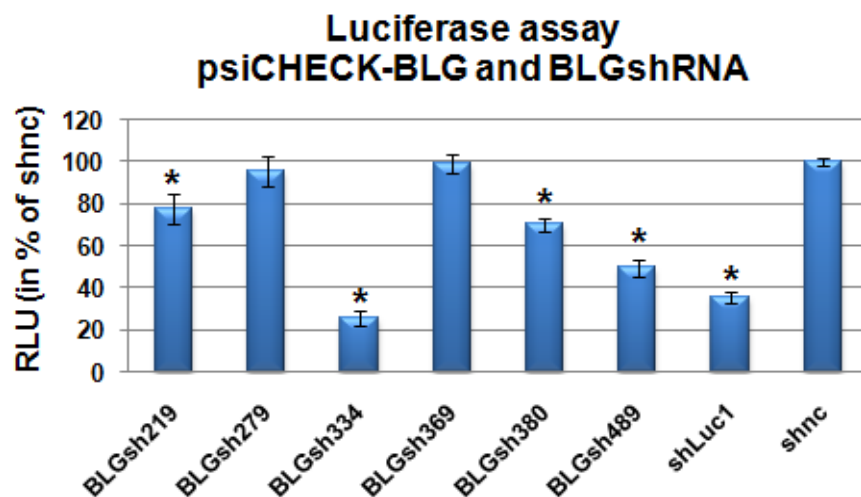


**Figure 19: Dual-Luciferase assay with psiCHECK-BLG and shRNA plasmids against BLG.** Relative light units (RLU) are presented. The negative control IMG-shnc (shnc) is set to 100%. Knockdown of *Renilla* is shown in comparison with the negative control. Measurements were performed in triplicates. All constructs with the exception of BLGsh279 and BLGsh369 showed significant downregulation of relative *Renilla* luciferase expression. Details see text (*: $p<0.05$; n=3).

Two of the designed shRNA sequences, BLGsh279 and BLGsh369, did not result in a significant downregulation of the relative *Renilla* luciferase expression. The frequency of two non functional shRNAs out of six designed sequences is similar to other published findings (Kumar *et al.,* 2003). The other four shRNAs showed downregulation of the relative *Renilla* luciferase expression ranging between 22 to 75% which was comparable to the positive control shLuc1 against *Renilla* luciferase (about 65%). Because of the best downregulation of relative *Renilla* luciferase expression, the shRNA sequences BLGsh334 and BLGsh489 were chosen for further experiments.

### 3.1.4  Construction of the lentiviral shRNA vector pLenti-shRNA

Since the long-term goal of this project was to generate pigs with stable knockdown of BLG with efficient RNAi sequences, a lentiviral vector was constructed for shRNA expression. These lentiviral vectors can be used to produce infectious lentiviral particles for the infection of cells or the generation of shRNA transgenic pig by lentiviral transgenesis (Hofmann *et al.,* 2003). For this purpose a lentiviral shRNA vector containing a shRNA expression cassette, a visual marker (EGFP) and also a selectable marker (Blasticidin resistance) was generated.

The visual and selectable markers were included to monitor the transfection efficiency and to facilitate the generation of stable cell pools and clones.

The construction of the vector will be briefly described, more detailed information about the cloning steps involved is shown in section 8.1. The first step was to insert a stuffer sequence downstream of the U6 promoter into the shRNA plasmid IMG-800. This was important to facilitate the insertion of shRNA oligonucleotides into the final lentiviral plasmid, because the distance between the SalI and XbaI restriction site in the original shRNA vector IMG-800 was only 9 bp. The insertion of the 118 bp stuffer sequence from pSL1180 Amersham allowed a better visualization of the restriction digestion fragments. The resulting plasmid was named IMG-Stuffer (see Figure 20).
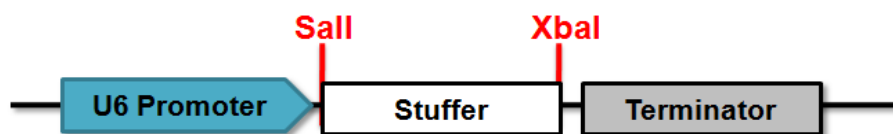


**Figure 20: Schematic diagram of the plasmid IMG-Stuffer.** A 118 bp stuffer sequence was excised with SalI and XbaI from pSL1180 Amersham and inserted into the SalI and XbaI restrictions sites of the plasmid IMG-800.

The U6 promoter cassette with the stuffer region and the secondary terminator sequence was then inserted into the lentiviral plasmid pLenti4s-XbaI (see section 2.1.14 and 8.2) downstream of the lentiviral packaging signal and a schematic diagram of the resulting plasmid pLenti4s-U6-Stuffer is presented in Figure 21.
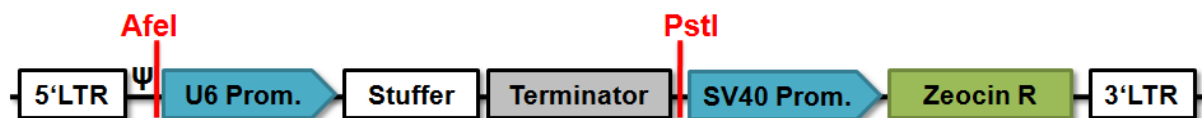


**Figure 21: Schematic diagram of the plasmid pLenti4s-U6-Stuffer.** The U6 promoter (blue) cassette containing the pSL1180 stuffer sequence (white) and the second terminator (grey) was inserted into the AfeI and PstI sites of the lentiviral plasmid pLenti4s-XbaI.

The plasmid pLenti4s-U6-Stuffer contained a Zeocin resistance cassette. Previous experience selecting various mammalian cell lines using Zeocin showed that it was less efficient than Blasticidin (Dr. H. Wieland, personal communication). Therefore the Zeocin resistance cassette was replaced with a PGK promoter directing the expression of EGFP linked to a Blasticidin resistance by an internal ribosome entry site (IRES) to allow selection and visual identification of transfected cells. For this purpose, a PGK promoter-EGFP fragment was inserted into the vector pIRES2-EGFP to obtain the vector pPGK-EGFP-IRES-EGFP1. The PGK promoter-EGFP-IRES cassette was obtained from the plasmid by PCR amplification with the primers IRESPGKEGFP f/r and digested with XhoI and EcoRV (see Figure 22).
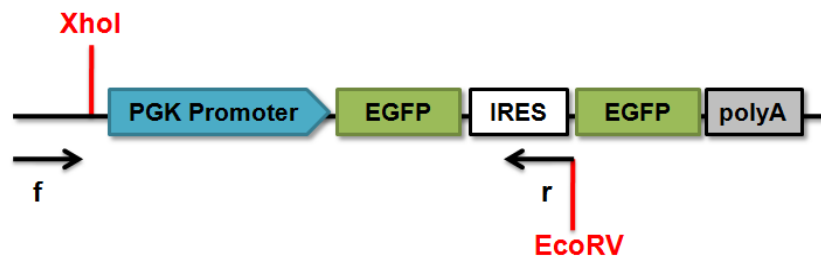
**Figure 22: Schematic diagram of the plasmid pPGK-EGFP-IRES-EGFP1.** The PGK promoter (blue) and the EGFP coding sequence (green) were inserted into the vector pIRES2-EGFP. PCR amplification with the primers IRESPGKEGFP f/r yielded a 2300 bp fragment which was digested with XhoI and EcoRV to generate a 1900 bp fragment containing the PGK promoter (blue), the EGFP coding region (green) and the internal ribosome entry site (IRES, white).

As a next step, the Blasticidin resistance gene together with the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), the 3' delta U3 LTR sequence and SV40 polyA signal were isolated from the vector pLenti6/TR-woodchuck (see section 2.1.14 and 8.2). The WPRE is a *cis*-acting element known to enhance transgene expression and viral titers, although the exact mechanism is unclear (Zufferey *et al.,* 1999). The PGK promoter-EGFP-IRES cassette and the Blasticidin-WPRE-SV40 polyA fragment were ligated with pLenti4s-U6-Stuffer in a three fragment ligation to generate the lentiviral shRNA vector pLenti-shRNA (see Figure 23).



**Figure 23: Schematic diagram of the lentiviral vector pLenti-shRNA.** The PCR fragment containing PGK-EGFP-IRES (blue, green), the cassette from pLenti6/TR-woodchuck with the Blasticidin resistance gene (BS, green), the woodchuck hepatitis virus post-transcriptional regulatory element (W, white) and delta U3 3' LTR (white) were ligated with the pLenti4s-U6-Stuffer backbone to create pLenti-shRNA. ψ: HIV packaging signal. Double stranded shRNA oligonucleotides can be inserted into the SalI and XbaI restriction sites of pLenti-shRNA.

The functionality of the PGK promoter expressing EGFP linked to the Blasticidin cassette by the IRES was tested in tissue culture. HEK293 cells were transfected with pLenti-shRNA and 48 h later EGFP expression was observed (see No. 2 in Figure 24). Resistant cells were obtained by selection with Blasticidin whereas non-transfected cells started to round up and detach from the tissue culture plate on day 5 (see No. 5 and 6 in Figure 24).
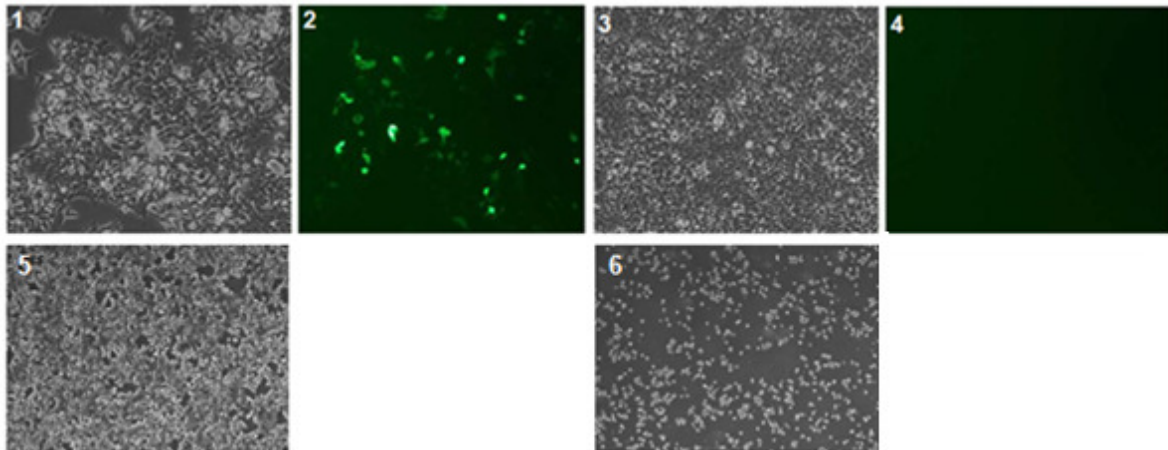
**Figure 24: Functional test of pLenti-shRNA in HEK293 cells.** 1 and 2: Phase contrast (1) and fluorescence image (2) of HEK293 cells 48 h after transfection with pLenti-shRNA. 3 and 4: Phase contrast (3) and fluorescence image (4) of untransfected HEK293 cells. 5 and 6: HEK 293 cells transfected with pLenti-shRNA (5) or untransfected HEK293 cells (6) after 5 d of Blasticidin selection.

These cell culture tests showed that of the visual and the selectable marker on the lentiviral shRNA plasmid pLenti-shRNA were functional.

### 3.1.5  Dual-Luciferase assay to determine knockdown with lentiviral shRNA constructs

One goal of this project was to generate shRNA transgenic pigs with downregulation of porcine BLG by lentiviral transgenesis (Hofmann *et al.,* 2003). Therefore the two most effective sequences, BLGsh334 and BLGsh489, the positive control against *Renilla* luciferase (shLuc1) and the negative control shRNA sequence (shnc) were cloned into the SalI and XbaI restriction sites of the lentiviral shRNA vector pLenti-shRNA. The ability of these plasmids to downregulate BLG was tested in the Dual-Luciferase Assay with psiCHECK-BLG and the results of the assay are shown in Figure 25.
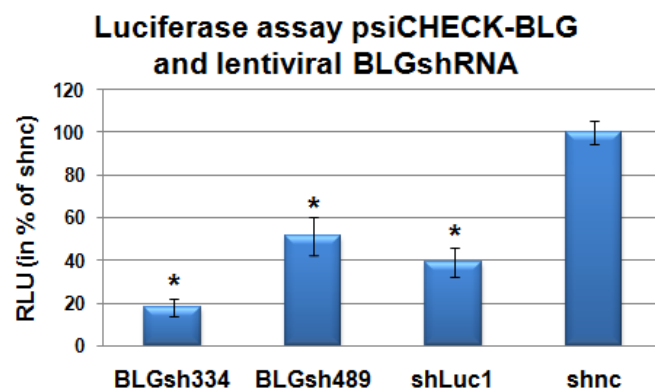


**Figure 25: Dual-Luciferase assay with lentiviral shRNA constructs against porcine BLG.** Relative light units (RLU) are presented. The negative control pLenti-shnc (shnc) is set to 100%. Knockdown of *Renilla* is shown in comparison with the negative control. All constructs showed significant downregulation of relative *Renilla* luciferase expression compared to the negative control shnc (*: $p < 0.05$; n=3).

The plasmid pLenti-BLGsh334 resulted in 82% downregulation of the relative *Renilla* luciferase expression, pLenti-BLGsh489 of 49% and pLenti-shLuc1 of 61% compared to the negative control lentiviral vector pLenti-shnc.

Both sequences directed against porcine BLG showed comparable knockdown to the non-viral shRNA plasmids IMG-BLGshRNA. IMG-BLGsh334 downregulated the relative *Renilla* luciferase expression by 74%, pLenti-BLGsh334 by 82%, IMG-BLGsh489 by 55% and the lentiviral construct by 49%. The same was true for the positive control sequence against *Renilla* luciferase (55% and 61%). Therefore the lentiviral sequences on the plasmid did not interfere with the efficiency of the U6 promoter driven shRNA cassette.

### 3.1.6  Virus production with lentiviral BLGshRNA constructs

The production of infectious lentiviral particles with pLenti-BLGsh334, pLenti-BLGsh489 and pLenti-shLuc1 was performed in HEK293FT cells as described in section 2.2.3.6. Titration of the supernatant was performed by infection of HT1080 cells and counting of Blasticidin resistant colonies. The maximum titer determined by colony forming assay was $6 \times 10^5$ transduction units per ml. These titers were however not judged sufficient to generate animals via lentiviral transgenesis, because titers of $10^9$ to $10^{10}$ TU/ml are necessary for this method (personal communication, Prof. Dr. E. Wolf; Lois *et al.,* 2002). Optimization of the transfection procedure by varying the DNA amount did not yield higher lentiviral titers (data not shown). The generation of lentiviruses with reduced viral titers has been previously described for shRNA containing constructs. This is most likely due to the presence of the shRNA hairpin in the viral mRNA, leading to Dicer mediated cleavage of the viral mRNA (Poluri and Sutton, 2008). Therefore, all following experiments were performed by transfection of the lentiviral plasmids instead of infection with lentiviruses. The plan to generate transgenic pigs by lentiviral transgenesis was replaced by the production via somatic cell nuclear transfer of transfected cell clones (section 3.1.15 and 3.1.16).

### 3.1.7  Cloning of artificial miRNA vectors for downregulation of BLG

As tissue-specific downregulation of BLG *in vivo* restricted to the lactating mammary gland should be achieved in the long term, artificial miRNA based RNAi constructs were also evaluated for their knockdown potential. As these constructs are directed by RNA polymerase II promoters, they can be expressed in a tissue-specific manner (Zeng *et al.,* 2002; Rao *et al.,* 2006) for example by using mammary gland specific promoters such as the BLG or beta-casein promoter. In addition, even ubiquitous downregulation of target mRNAs in an artificial miRNA context has been reported as less toxic than shRNAs *in vivo* (McBride *et al.,* 2008).

To this end the commercial vector pcDNA6.2-GW/miR (named pCMV-miRNA in the following sections) from Invitrogen was chosen (see section 2.1.14 and 8.2). In this plasmid the miRNA cassette is expressed from a RNA polymerase II promoter derived from human cytomegalovirus promoter (CMV). The transcript mimics natural pri-miRNAs after expression and consists of 5' and 3' flanking regions from murine miR-155, a hairpin structure containing the mature artificial miRNA with internal bulges and a polyadenylation signal from the herpes simplex virus thymidine kinase (HSV-TK polyA). The 5' and 3' flanking regions are removed in the cell by the endoribonuclease Drosha generating an artificial pre-miRNA. The schematic structure of the plasmid and an example of a resulting pre-miRNA are shown in Figure 26. Furthermore the plasmid contains a Blasticidin resistance cassette for the generation of stable cell clones and pools.
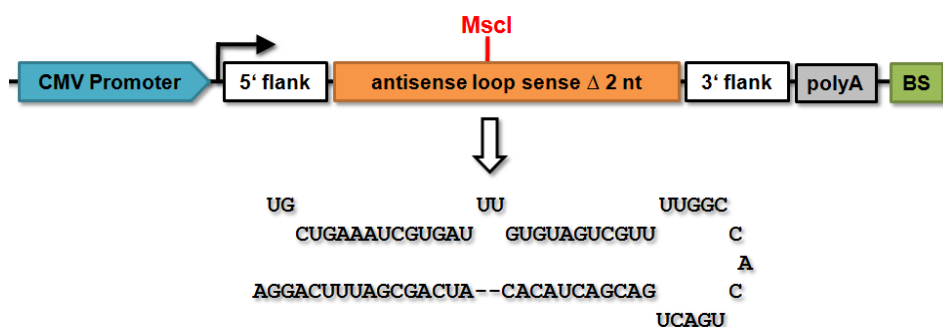


**Figure 26: Schematic diagram of the artificial miRNA expression vector pCMV-miRNA containing an artificial miRNA sequence.** The polymerase II promoter CMV (blue) directs the expression of a pri-miRNA cassette. The cassette includes 5' and 3' flanking regions derived from murine miR-155 (white), a stem loop structure with internal bulges (orange) and a HSV-TK polyadenylation signal (grey). A SV40 promoter directed Blasticidin resistance cassette (BS, green) is also present on the plasmid. An example of an artificial pre-miRNA after removal of the 5' and 3' flanking regions is also shown.

The plasmid pCMV-miRNA was obtained from the supplier in linearized form and thus could not be propagated in bacteria. This was circumvented by insertion of a stuffer sequence containing two BsaI restriction sites into pCMV-miRNA generating the plasmid pCMV-circ. Digestion with BsaI resulted in the overhangs necessary for insertion of the miRNA oligonucleotides. A schematic overview of the plasmid pCMV-circ is depicted in Figure 27.
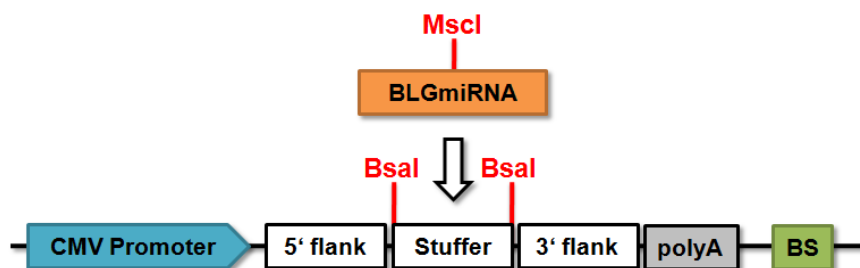


**Figure 27: Schematic diagram of the artificial miRNA vector pCMV-circ.** The artificial miRNA expression vector pCMV-circ contains a CMV promoter (blue), the 5' and 3' flanking regions (white), the stuffer sequence enabling propagation of the plasmid in bacteria (white), the HSV-TK polyadenylation sequence (grey) and a Blasticidin resistance cassette (green). Artificial miRNA against BLG (orange) can be inserted into the BsaI restriction sites. The artificial miRNAs contain an MscI restriction enzyme recognition site to facilitate the identification of plasmids containing miRNA sequences.

Sequences for artificial miRNA mediated knockdown of porcine BLG were selected with the Block-iT RNAi Designer program. Artificial miRNA sequences located in similar sequence positions to the previously determined effective shRNA sequences BLGsh334 and BLGsh489 on the porcine BLG mRNA Reference Sequence X54976 were designed. This allowed a comparison of shRNA and artificial miRNA vectors against similar target regions. The Block-iT RNAi Designer program suggested an artificial miRNA sequence with predicted high knockdown ability close to the target sequence BLGsh489. This sequence, BLGmi490, started at nucleotide position 490 relative to the 5' end of X54976. In contrast to this, no artificial miRNA sequence was found in the target region of BLGsh334. Therefore the BLGsh334 sequence was redesigned so that it could be inserted into the BsaI digested vector pCMV-circ and was named BLGmi334. The artificial miRNA sequences were obtained as single stranded DNA oligonucleotides. Before the insertion into pCMV-circ they were annealed to double stranded oligonucleotides. The general configuration of artificial miRNA oligonucleotides is presented in Figure 28.
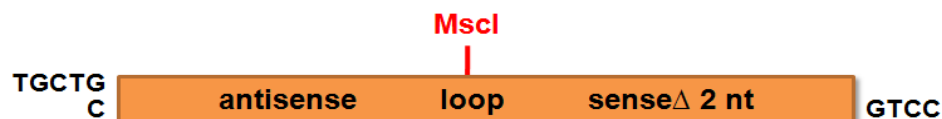


**Figure 28: General configuration of double stranded artificial miRNA DNA oligonucleotides.** DNA oligonucleotides were designed with overhangs as shown above. These were compatible with BsaI restriction sites of pCMV-circ. The 19 nt loop sequence contains a MscI restriction site and the sense sequence is 2 nt shorter than the 21 bp antisense sequence (mature miRNA) creating an internal loop.

The loop sequence of all BLG miRNA oligonucleotides contained a recognition site for the restriction endonuclease MscI. Insertion of the oligonucleotides BLGmi334 and BLGmi490 was therefore confirmed by MscI restriction digestion. Plasmids with correct restriction patterns were sequenced to ensure proper miRNA sequences.

The RNAi sequences targeting positions 334 and 490 of the porcine BLG mRNA were successfully inserted into the artificial miRNA vector pCMV-circ to generate pCMV-BLGmi334 and pCMV-BLGmi490. Moreover, a positive control plasmid against *Renilla* luciferase, pCMV-miLuc1, was also designed with a target sequence starting at nucleotide 61 relative to the start codon of hRluc (Promega), identical to the beginning of the target position of shLuc1. A negative control plasmid showing no homology to known vertebrate genes, pcDNA6.2-GW/miR-neg (named pCMV-minc in the following sections), was included in the pcDNA6.2-GW/miR kit.

### 3.1.8 Dual-Luciferase assay with pCMV-BLGmiRNA constructs

A Dual-Luciferase assay was performed to evaluate the knockdown potential of the vectors pCMV-BLGmi334 and pCMV-BLGmi490. These two artificial miRNA constructs against porcine BLG, the positive control plasmid pCMV-miLuc1 against *Renilla* luciferase and the negative control plasmid pCMV-minc were cotransfected into HEK293 cells with psiCHECK-

BLG. The plasmid pCMV-BLGmi334 showed 30%, pCMV-BLGmi490 60% and pCMV-miLuc1 35% knockdown of relative *Renilla* luciferase expression compared to the negative control pCMV-minc (see Figure 29).
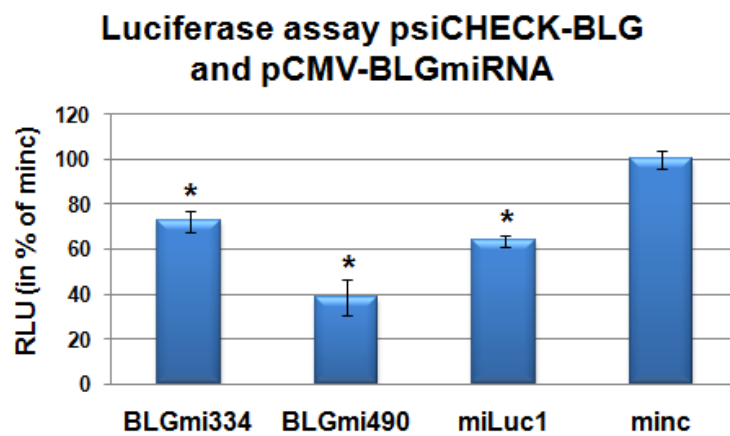


**Figure 29: Dual-Luciferase assay with psiCHECK-BLG and pCMV-BLGmiRNA plasmids against BLG.** Relative light units (RLU) are presented. The negative control pCMV-minc (minc) is set to 100%. Knockdown of *Renilla* is shown in comparison with the negative control. All constructs showed significant downregulation of relative *Renilla* luciferase expression compared to the negative control minc (*: $p < 0.05$; n=3).

The Dual-Luciferase assay showed that both artificial miRNA sequences led to a significant knockdown of the relative *Renilla* luciferase expression. The extent of downregulation obtained with the BLGsh489 and BLGmi490 constructs was similar (55% and 60%). In contrast to this, the other two constructs, BLGmi334 and miLuc1, were not as effective as the respective shRNA constructs (BLGsh334: 74%, BLGmi334: 30%; shLuc1: 64%; miLuc1: 35%).

### 3.1.9 Primary porcine mammary epithelial cell culture for evaluation of RNAi constructs

The RNAi constructs against porcine BLG had been tested in HEK293 cells for their efficiency in downregulating a fusion mRNA between *Renilla* luciferase and BLG. Next the inhibition of endogenous porcine BLG should be examined. For this purpose primary porcine mammary epithelial cells (PMEC) were isolated and functional differentiation of these cells was attempted. After a successful knockdown of endogenous porcine BLG, these cells could be directly used as donor cells for somatic cell nuclear transfer (SCNT) to generate RNAi transgenic pigs.

Only two research groups had reported successful culture of primary PMEC (Kumura *et al.,* 2001; Sun *et al.,* 2005). This indicates that the culture and functional differentiation of PMEC is not trivial. Kumura *et al.* (2001) isolated primary cells from mammary glands of non-lactating pigs and cultured them on floating collagen gels. After two days in culture, the cells expressed the porcine milk proteins beta-casein, BLG and lactoferrin. The expression of BLG and lactoferrin did not require hormonal induction with prolactin, supplementation of the

medium with hydrocortisone was sufficient to induce expression. In contrast, the expression of casein required prolactin induction. Unlike Kumura *et al.* (2001), Sun *et al.* (2005) isolated primary PMEC from lactating sows. In addition, there were other differences between the two culture protocols for PMEC. Sun *et al.* used a different cell culture medium to Kumura *et al.*, the induction of milk protein expression was carried out on extracellular basement membrane (Matrigel) instead of floating collagen gels and the expression of BLG as well as beta-casein and alpha-lactalbumin required the presence of the lactogenic hormone prolactin (Sun *et al.*, 2005).

Because of these two reports describing PMEC culture and the induction of milk protein expression, cells were isolated from mammary gland tissue of eleven non-lactating and also three lactating sows. The isolation procedure was performed according to Sun *et al.* (2005) and is described in section 2.2.3.7. Of these, four isolations from non-lactating (PMEC 4NL, 5NL, 8NL and 11NL) and one isolation from lactating (PMEC 14L) pigs, which showed a predominantly epithelial phenotype and good growth characteristics, were used for various experiments.

The first step was to establish appropriate culture conditions for PMEC. PMEC 4NL was cultured in MEC medium for up to 56 days (passage 8). This medium was essentially identical to the medium used by Sun *et al.* (2005) but without the antibiotics (Penicillin, Streptomycin, Neomycin). During the culture period, the morphology changed from epithelial to fibroblast-like and then senescent cells. Figure 30 shows the cell morphology in passage 0 (P0) on day 2 and 8, P4 on day 30 and P8 on day 56.
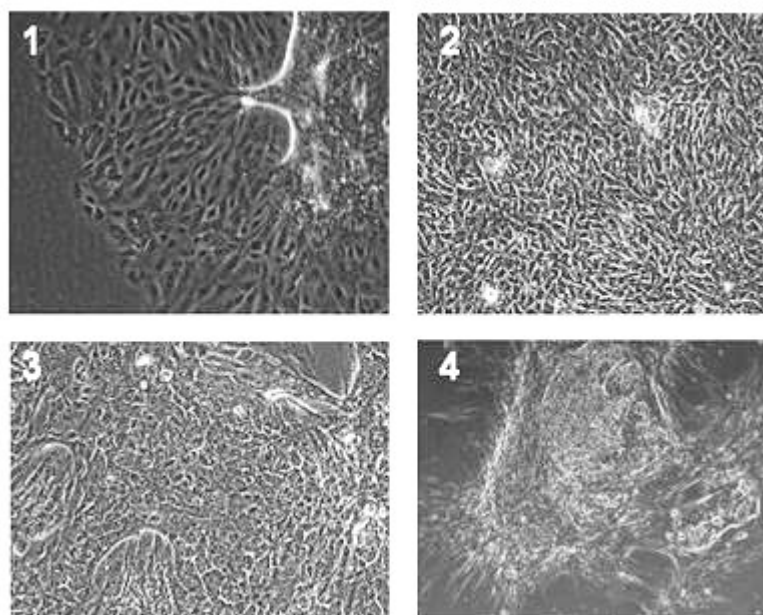


**Figure 30: Morphology of PMEC 4NL in MEC medium.** 1: Islets with epithelial morphology arose after attachment of PMEC (P0, d2). 2: On day 8 cells formed confluent monolayers with epithelial morphology (P0, d8). 3: Cell morphology changed with prolonged culture to a less uniform monolayer (P4, d30). 4: Cells formed clusters surrounded by fibroblast-like and senescent cells (P8, d56).

On day 2, many islets with epithelial cuboidal cells were present. The predominant epithelial phenotype changed during the culture period to a mixture of epithelial and fibroblast-like cells. On day 56 (P8), most of the cells ceased to grow and appeared senescent. The number of passages before the cells reached senescence was lower than described by Sun *et al.* (2005) (P15 to 20). Furthermore, Sun *et al.* reported almost complete loss of contaminating fibroblast cells by passage 5. This was not observed in this project although selective Accutase treatment (see section 2.2.3.7) was used to remove the contaminating fibroblasts.

Because of these results it was investigated whether other growth media would support longer growth of more uniform epithelial cells. Five different growth media were tested with freshly isolated cells (PMEC 8NL), the compositions are described in section 2.2.3.7. These included the medium used by Sun *et al.* and the same medium substituting the fetal calf serum for porcine serum (MEC PS medium). This modification was performed because a positive effect of autologous serum on the growth characteristics of human MEC has been reported (Emerman *et al.,* 1987).  A simplified medium similar to Kumura *et al.* (2001) for the culture of PMEC was also used. The fourth medium was a serum-free medium supplemented with bovine pituitary extract and EGF used to culture human MEC (Hammond *et al.,* 1984; MCDB170EGF) and the last medium was a defined commercial medium for the culture of epithelial cells (Q286).

PMEC 8NL was plated into the five different media directly after cell isolation from mammary gland tissue. Figure 31 A shows the cells on day 3 after plating. All five media supported attachment of PMEC to plastic surfaces. Islets with epithelial cell morphology were detected in all five media, but in modified Kumura and MCDB170EGF medium, the number of colonies was lower than in the other media. PMEC 8NL was cultured for another week in the each medium. Cells in MEC medium had to be excluded from the test due to fungal contamination. PMEC proliferation in modified Kumura and MCDB170EGF medium was limited and only a few colonies were visible on day 10. Q286 medium led to fast proliferating cells but the morphology changed from epithelial to fibroblast-like. MEC PS medium promoted the growth of epithelial cells. Figure 31 B shows the cells in various media at day 10.

The initial culture experiment with PMEC 4NL and the comparison of growth media with PMEC 8NL showed that the original culture medium for PMEC, MEC medium, and the modified MEC PS medium with porcine instead of fetal calf serum resulted in cells with epithelial morphology which proliferated well. The other tested media did not improve the culture of PMEC. Due to these results, further isolations were cultured in either MEC medium or MEC PS medium.
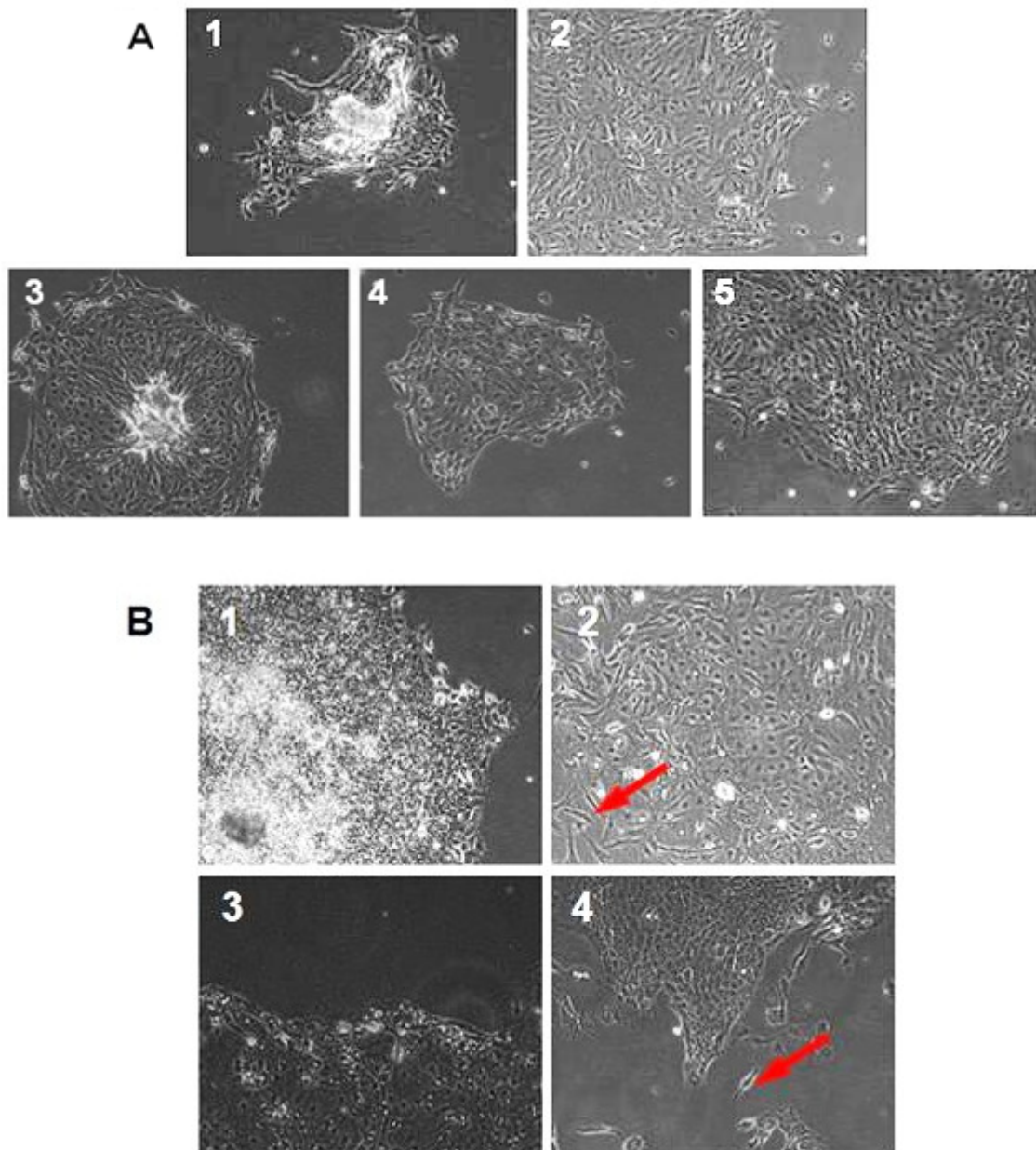
**Figure 31: PMEC 8NL 3 days (A) and 10 days (B) after isolation in different media.** 1: Cells in modified Kumura medium. 2: Cells in Q286 medium. 3: Cells in MCDB170EGF medium. 4: Cells in MEC PS medium. 5: Cells in MEC medium. Primary cultures are shown at P0. All media supported growth of epithelial cell islets after cell attachment. Only few colonies formed in modified Kumura medium and MCDB170EGF medium and proliferated slowly. MEC and MEC PS medium yielded many colonies with epithelial morphology. In Q286 medium cells proliferated well but the morphology changed to fibroblast-like cells. Red arrows: examples of fibroblast-like cells.

The goal of primary PMEC culture was to establish cell culture conditions which lead to the expression of milk proteins, allowing an evaluation of RNAi mediated downregulation of endogenous porcine BLG. To induce milk protein expression in primary MEC it is important to mimic the *in vivo* 3D organization of mammary cells (Emerman and Pitelka, 1977; Li *et al.,* 1987; Barcellos-Hoff *et al.,* 1989). Mammary epithelial cells in the intact mammary gland are organized in spheroids, so called acini or alveoli. They are characterized by polarized epithelial cells, attached to a basement membrane surrounding a hollow lumen. This organization is important in controlling cell survival, proliferation, differentiation and milk

protein expression (McGee *et al.,* 2006). 3D culture of mammary epithelial cells on floating collagen gels or on/embedded in extracellular basement membrane matrix (e.g. Matrigel) has been used to mimic the alveolar structure *in vitro.* Matrigel is a commercial preparation of a soluble basement membrane extract from the murine Engelbrecht Holm-Swarm (EHS) sarcoma model that supports 3D growth of polarized cells (reviewed in LeBleu *et al.,* 2007; Kleinman and Martin, 2005). This EHS basement membrane matrix has been used by many groups for the 3D culture and functional differentiation of primary MEC and MEC cells lines of different origin (for example: Medina *et al.,* 1987; Li *et al.,* 1987; Barcellos-Hoff *et al.,* 1989; Ip and Darcy, 1996; Talhouk *et al.,* 1998; Debnath *et al.,* 2003). Functional differentiation of human MEC cultured on Matrigel is shown in Figure 32.
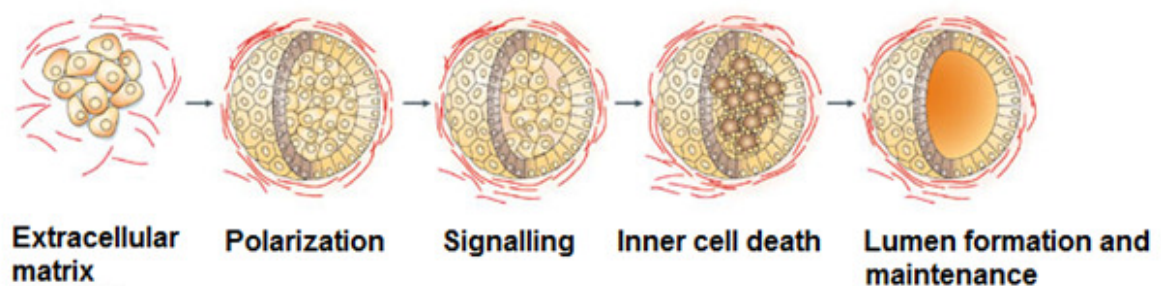


**Figure 32: Functional differentiation of MEC on or embedded in Matrigel (adapted, from Debnath and Brugge, 2005).** MEC surrounded by extracellular matrix show polarization on the cells followed by extensive signalling. This signalling induces apoptosis of the cells on the inside of the 3D structure resulting in the formation of alveolar-like hollow structures.

Two different approaches for the 3D growth of mammary epithelial cells with Matrigel have been described. The first method, called overlay, is based on culturing the cells on top of a layer of solidified Matrigel. For the second method, the so-called embedment, a thin layer of Matrigel is used to cover the culture dish. The cells are mixed with liquid Matrigel and are then added to the pre-coated culture dish (Debnath and Brugge, 2005). Both types of culture lead to the formation of 3D structures by MECs resembling alveoli. A schematic overview of both systems is presented in Figure 33.
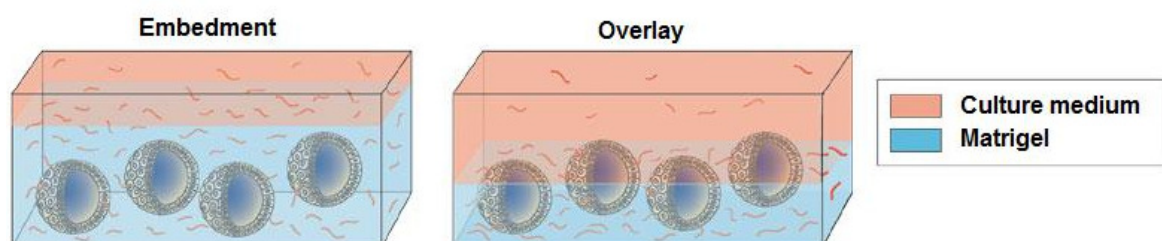


**Figure 33: The two different types of Matrigel assay used to culture MECs (adapted, from Debnath and Brugge, 2005).** Functional differentiation of MEC can be induced by culturing these cells inside (embedment) or on top of Matrigel (overlay).

3D culture on Matrigel was used to induce milk protein expression in PMEC according to Sun *et al.* (2005). To establish the conditions for 3D culture of PMEC, cells from isolation PMEC 5NL were cultured in 6 well plates. Each well was coated with 300 µl Matrigel and $8 \times 10^5$ cells plated in MEC medium with 5 µg/ml ovine prolactin. The cells formed alveolar-like structures after 2 days which appeared hollow at day 4 (see Figure 34).
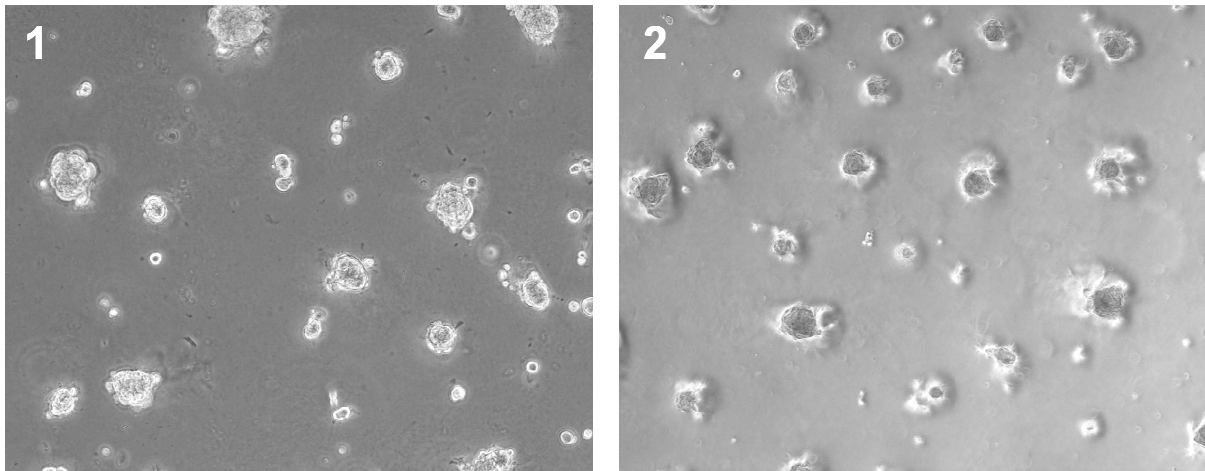


**Figure 34: PMEC isolation 5NL at P5 cultured on Matrigel.** 1: Alveolar-like structures on day 2 on Matrigel in MEC medium containing 5 µg/ml ovine prolactin. 2: Hollow alveolar-like structures on day 4 on Matrigel in MEC medium containing 5 µg/ml ovine prolactin.

On day 5, RNA was extracted to examine milk gene expression. One step RT-PCR was used to detect mammary gland specific mRNAs (Whey acidic protein, beta-casein, BLG and α-lactalbumin) using primers which had been shown to be functional on mRNA isolated from cells in porcine milk. None of these mRNAs could be detected in PMEC 5NL (data not shown).

Because of the lack of expression of milk protein genes, cells cultured in MEC PS instead of MEC medium were analyzed to determine whether the fetal calf serum in MEC medium inhibited milk gene expression. For these experiments, cells from isolation 8NL cultured in MEC PS medium were used. In addition serum free conditions were examined to determine if the porcine serum had inhibitory effects on milk protein gene expression. Cells cultured in MEC PS medium with 5 and 10 µg/ml ovine prolactin were also compared. PMEC 8NL was cultured under these different conditions to induce milk protein expression. $5.5 \times 10^5$ cells were plated and cultured according to Table 7. One day after culture initiation, 3D structures formed under all culture conditions except in the well without Matrigel. The presence or absence of ovine prolactin or porcine serum did not lead to major morphological differences. The alveolar-like structures observed at day 1 are shown in Figure 35.

**Table 7: Culture conditions for PMEC 8NL Matrigel assay.**

| 1 | MEC PS medium<br>5 µg/ml ovine prolactin | 2 | Serum free MEC PS<br>5 µg/ml ovine prolactin | 3 | MEC PS medium<br>10 µg/ml ovine prolactin |
|---|---|---|---|---|---|
| 4 | MEC PS medium<br>without ovine prolactin | 5 | Serum free MEC PS<br>without ovine prolactin | 6 | MEC PS medium<br>5 µg/ml ovine prolactin<br>without Matrigel |
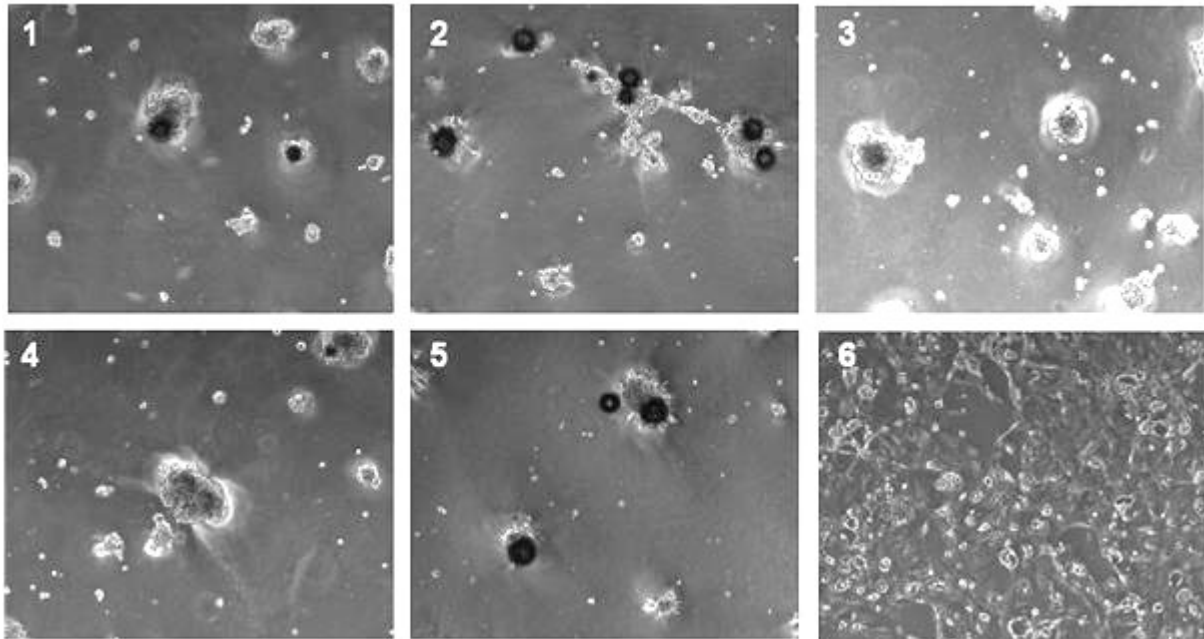


**Figure 35: Matrigel assay on day 1 with PMEC 8NL at P4 in MEC PS medium.** 1: Cells in MEC PS medium with 5 µg/ml ovine prolactin. 2: Cells in serum free MEC PS medium with 5 µg/ml ovine prolactin. 3: Cells in MEC PS medium with 10 µg/ml ovine prolactin. 4: Cells in MEC PS medium. 5: Cells in serum free MEC PS medium. 6: Cells in MEC PS medium with 5 µg/ml ovine prolactin without Matrigel. No differences in morphology were visible in culture conditions 1 to 5. Cells formed alveolar-like structures independent of prolactin supplementation. Cells cultured without Matrigel did not form 3D structures.

After the cells were cultured for 7 days, differences in morphology became apparent. Cells cultured with porcine serum had proliferated and overgrown the Matrigel surface. In medium without porcine serum, the cells did not proliferate and formed small alveolar-like structures. Culture conditions with ovine prolactin improved the preservation of 3D structures resembling alveoli. Cells cultured without Matrigel showed a senescent phenotype. The 3D assay on day 7 is presented in Figure 36. RNA was isolated on day 7 and RT-PCR analysis performed for casein and β-actin mRNA. Expression of β-actin but not of beta-casein could be detected (data not shown).
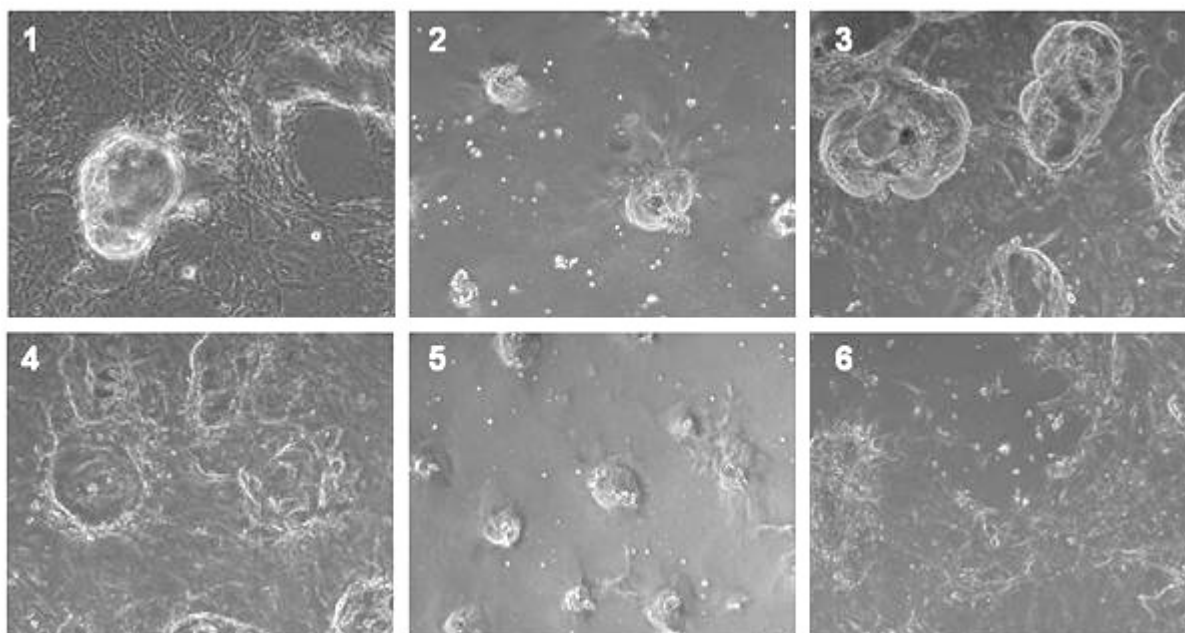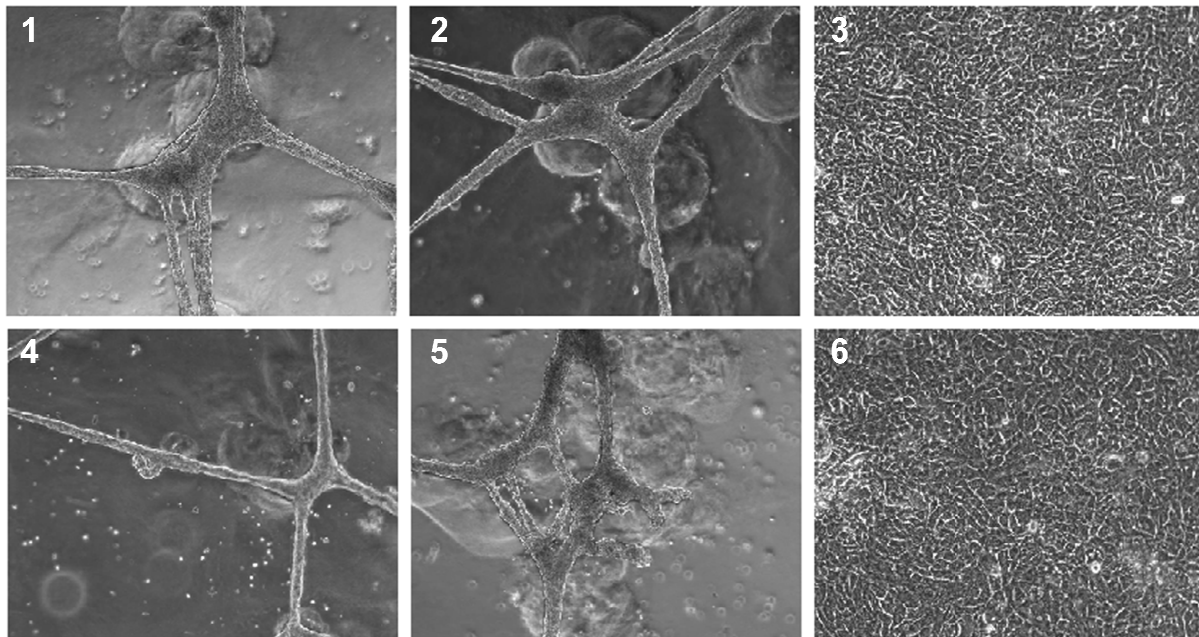
**Figure 36: Matrigel assay on day 7 with PMEC 8NL at P4 in MEC PS medium.** 1: Cells in MEC PS medium with 5 µg/ml ovine prolactin. 2: Cells in serum free MEC PS medium with 5 µg/ml ovine prolactin. 3: Cells in MEC PS medium with 10 µg/ml ovine prolactin. 4: Cells in MEC PS medium. 5: Cells in serum free MEC PS medium. 6: Cells in MEC PS medium with 5 µg/ml ovine prolactin without Matrigel. Differences in morphology were visible in culture conditions 1 to 5. Cultures receiving porcine serum showed cell proliferation in contrast to cells lacking porcine serum. Alveolar-like structures were more preserved with prolactin supplementation. Cells cultured without Matrigel did not form 3D structures.

PMEC isolation 11NL was cultured in MEC medium because the replacement of FCS with porcine serum showed no improvement of milk protein expression. To exclude the possibility that the lack of milk protein expression was due to low cell numbers, $1.5 \times 10^{6}$ cells were plated per well on 500 µl of Matrigel. Culture media were according to Table 8. Four days after plating, extensive 3D growth resembling mammary duct-like structures was observed in all wells with Matrigel. The presence or absence of ovine prolactin or FCS had no effect on the duct-like morphology. Cells grown in wells without Matrigel did not form 3D structures (see Figure 37).

**Table 8: Culture conditions for PMEC 11NL Matrigel assay.**

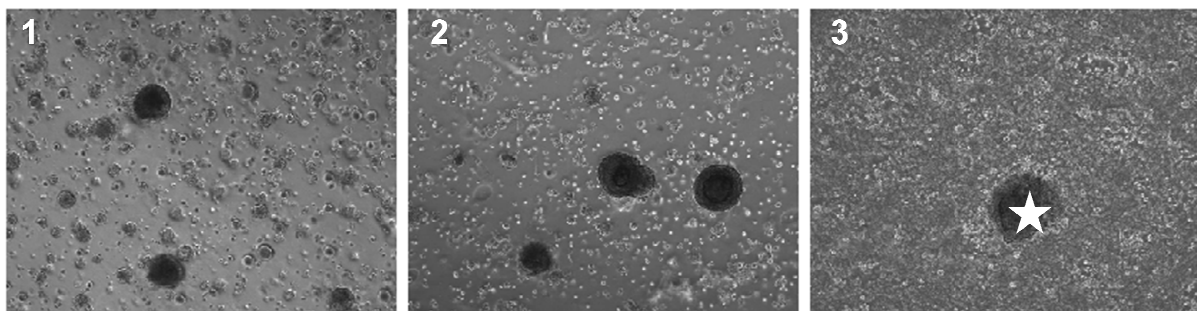| 1  MEC medium 5 µg/ml ovine prolactin | 2  MEC medium without ovine prolactin | 3  MEC medium 5 µg/ml ovine prolactin without Matrigel |
|---|---|---|
| 4  serum free MEC medium 5 µg/ml ovine prolactin | 5  serum free MEC medium without ovine prolactin | 6  serum free MEC medium 5 µg/ml ovine prolactin without Matrigel |

**Figure 37: Matrigel assay on day 4 with PMEC 11NL at P6 in MEC medium.** 1: Cells in MEC medium with 5 µg/ml ovine prolactin. 2: Cells in MEC medium without ovine prolactin. 3: Cells in MEC medium with 5 µg/ml ovine prolactin without Matrigel. 4: Cells in serum free MEC medium with 5 µg/ml ovine prolactin. 5: Cells in serum free MEC medium without prolactin. 6: Cells in serum free MEC medium with 5 µg/ml ovine prolactin without Matrigel. No differences in morphology were visible in culture conditions 1, 2, 4 and 5. Mammary duct-like structures were visible independent of prolactin supplementation. Cells cultured without Matrigel did not form 3D structures.

On day five after culture initiation, RNA was isolated to analyze expression of milk genes. RT-PCR analysis did not show expression of casein or BLG but the expression of β-actin was detected (data not shown). The experiments were repeated with different cell isolations and varying culture durations (up to 14 d) were evaluated, but none of them led to the expression of BLG.

All of these experiments were conducted using primary MECs from non-lactating sows according to Kumura *et al.* (2001). The lack of success with cells from this source led to the isolation of primary PMECs from lactating sows according to Sun *et al.* (2005).

One cell isolation with good growth characteristics, PMEC 14L, was studied in more detail. PMEC 14L was cultured in MEC medium and showed epithelial morphology. At high density (post-confluence) these cells formed spontaneous 3D dome-like hollow structures (see Figure 38). This feature of mammary epithelial cell has been described before and has been associated with contact-induced differentiation (McGrath, 1975; Pantschenko *et al.,* 2000).
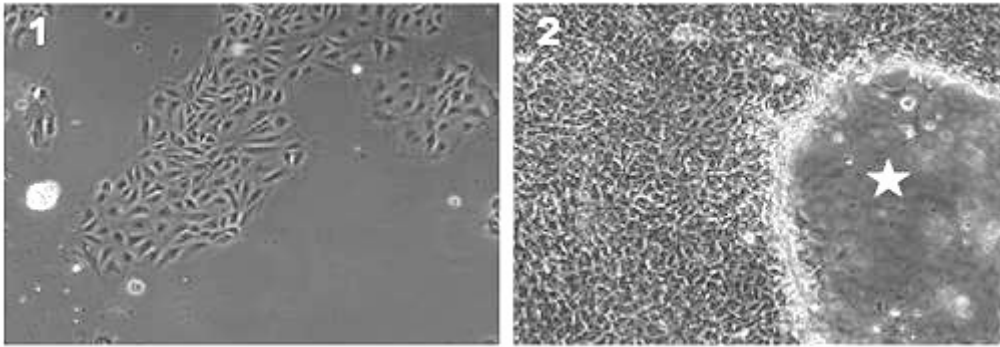
**Figure 38: Morphology of PMEC isolation 14L at low and high density.** 1: PMEC 14L (P5) at low density with typical epithelial morphology. 2: PMEC 14L (P6) at confluence forming dome-like structures (indicated by white star).

These cells were cultured on top of and embedded in Matrigel in MEC medium under various conditions. For the embedding, 2 x $10^6$ cells were cultured in 800 µl Matrigel for 7 days under conditions stated in Table 9. The 3D structures on day 7 are depicted in Figure 39. They did not form duct-like but alveolar-like structures. Cells cultured without Matrigel spontaneously formed some dome-like structures. RNA was isolated after 7 days in culture and RT-PCR for the expression of porcine BLG performed. Only porcine GAPDH but no expression of BLG could be detected. The same result was obtained from cells cultured on top of Matrigel (not shown).

**Table 9: Culture conditions for PMEC 14L Matrigel assay**

| 1 MEC medium | 2 serum free MEC med. | 3 MEC medium |
|---|---|---|
| 5 µg/ml ovine prolactin | 5 µg/ml ovine prolactin | without prolactin/Matrigel |



**Figure 39: Matrigel assay on day 7 with PMEC 14L at P12 in MEC medium**. 1: Cells in MEC medium with 5 µg/ml ovine prolactin. 2: Cells in serum free MEC medium with 5 µg/ml ovine prolactin. 3: Cells in MEC medium without ovine prolactin and Matrigel. No differences in morphology were visible in culture conditions 1 and 2. Alveolar-like structures were visible. Cells cultured without Matrigel showed some spontaneously dome-like structures (indicated by white star).

Many different culture conditions, induction times, cell isolations and media have been used to induce milk protein expression in primary porcine MEC from lactating and non-lactating sows. In contrast to published work concerning porcine mammary gland epithelial cells

(Kumura *et al.,* 2001; Sun *et al.,* 2005) none of these conditions led to the expression of milk protein genes such as beta-casein, α-lactalbumin, whey acidic protein or BLG.

## 3.1.10 Generation of cell lines with recombinant porcine BLG expression

Because isolated porcine primary mammary epithelial cells could not be induced to express milk genes, it was necessary to try a different approach to further investigate the knockdown effect of shRNA and artificial miRNA constructs identified in the Dual-Luciferase assays (section 3.1.3 and 3.1.8). Cultured cell lines stably expressing exogenous porcine BLG were generated. For this, CHO-K1 and HEK293 cells were transfected with the porcine BLG expression plasmid pcDNA-BLG (Dr. H. Wieland). A schematic diagram of the plasmid is presented in Figure 40.



**Figure 40: Expression construct for porcine BLG.** The porcine BLG cDNA (orange) is under the control of a CMV promoter (blue). A bovine growth hormone polyadenylation signal (polyA, grey) is also included in the construct. The Neomycin resistance gene under the control of an SV40 promoter (green) was used for selection of transfected clones with G418.

After selection with G418 for about 21 days, twelve clones from CHO-K1 were expanded and three of them analyzed by RT-PCR for the expression of BLG. All three clones expressed BLG (data not shown) and one clone, named CB1, was used for further experiments and analyzed by western blot. The RT-PCR and western blot analysis of CB1 are shown in Figure 41 A and B.
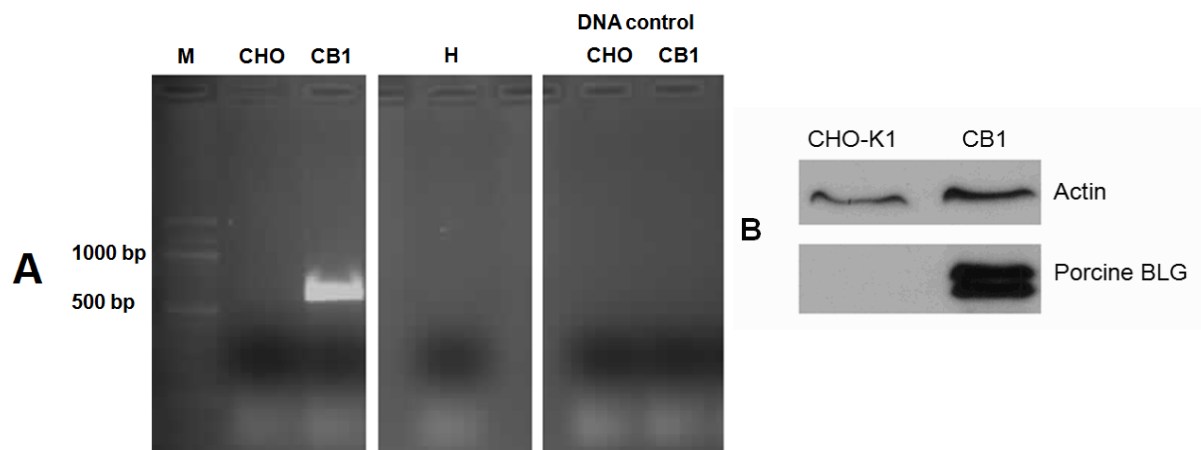


**Figure 41: RT-PCR and western blot analysis of CHO-K1 and CB1.** (A): RT-PCR analysis on 1% agarose gel. M: 100 bp DNA ladder. CHO: RT-PCR of wildtype CHO-K1 cells; CB1: RT-PCR product of clone CB1; H: water control; DNA control of CHO-K1 and CB1. The clone CB1 expressed the porcine BLG cDNA (product size: 624 bp). (B): Western blot with 20 µg protein per lane for β-actin (42 kDa) and porcine BLG (18.5 kDa) on a 20% reducing Tris/glycine gel. CHO-K1: CHO-K1 cells. CB1: BLG expression clone CB1. CHO-K1 did not express porcine BLG whereas CB1 showed persistent BLG expression.

After the transfection of HEK293 with pcDNA-BLG, twelve clones were expanded and six of them analyzed by RT-PCR. Four out of these six clones expressed porcine BLG. The RT-PCR analysis of these four clones is presented in Figure 42 A. One clone, HpB9, was

examined by western blot, showed persistent expression of recombinant BLG (see Figure 42 B) and was therefore chosen for further experiments.
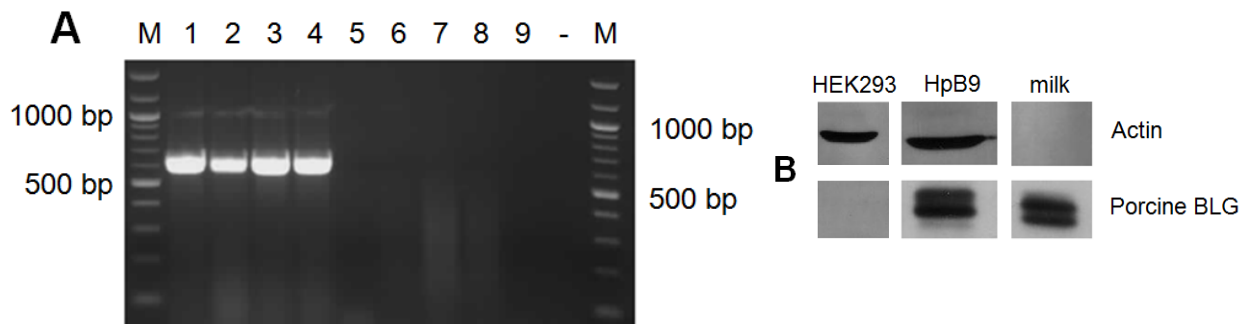


**Figure 42: RT-PCR analysis of HpB clones 6, 7, 9 and 11 and western blot analysis of HEK293 and HpB9.** (A): RT-PCR analysis on 1% agarose gel.  M: 100 bp DNA ladder. 1: RT-PCR product of clone HpB 6. 2: RT-PCR product of clone HpB7. 3: RT-PCR product of clone HpB9. 4: RT-PCR product of clone HpB11. 5: RT-PCR control without template. 6: DNA control of clone HpB6. 7: DNA control of clone HpB7. 8: DNA control of clone HpB9. 9: DNA control of clone HpB11. -: PCR control without template. Four clones expressed the porcine BLG cDNA (product size: 624 bp). (B): Western blot with 20 µg protein per lane for β-actin (42 kDa) and porcine BLG (18.5 kDa) on a 20% reducing Tris/glycine gel. HEK293: untransfected control cell line HEK293; HpB9: BLG expression clone HpB9. Milk: 5 µl 1:100 diluted porcine skim milk. HpB9 showed persistent BLG expression.

Transfection of CHO-K1 and HEK293 cells with a porcine BLG expression construct resulted in cell clones expressing BLG. Two cell clones, CB1 and HpB9, showed stable expression over many passages. The expression was detected by RT-PCR and western blot and these two cell clones were used to further experiments.

## 3.1.11 Knockdown of porcine BLG in CB1 and HpB9 cell lines

The clonal cell lines CB1 and HpB9 were used to further evaluate the knockdown potential of the shRNA and miRNA constructs which had shown the highest silencing efficiencies in the Dual-Luciferase assays. The knockdown was evaluated in cell pools after stable transfection of the RNAi constructs into the two BLG expressing cell lines.

For this, the cell lines CB1 and HpB9 were transfected with the plasmids pLenti-BLGsh334, pLenti-BLGsh489, pLenti-shnc, pCMV-BLGmi334, pCMV-BLGmi490 and pCMV-minc. Stable pools were selected with Blasticidin, protein extracts of these pools prepared and used for detection of knockdown by western blot analysis. The western blots of CB1 and HpB9 with the knockdown constructs is displayed in Figure 43 A and B.
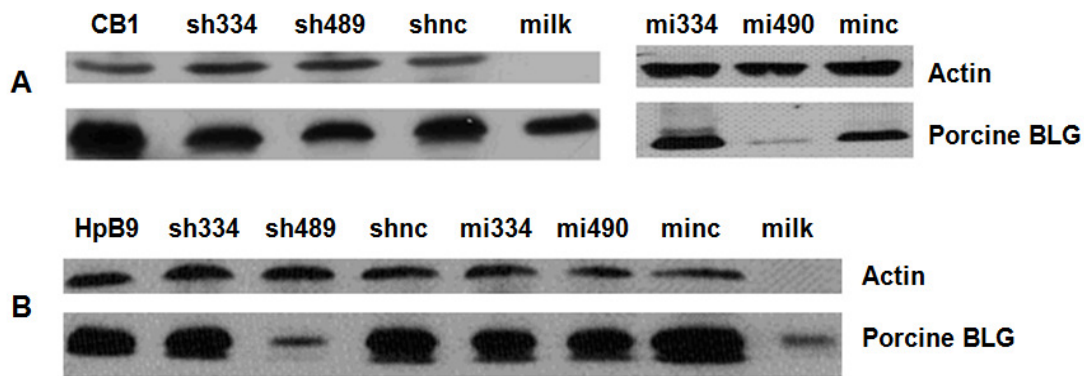
**Figure 43: Western blot analysis of CB1 and HpB9 with lentiviral shRNA and artificial miRNA knockdown constructs against porcine BLG.** Western blots with 20 µg protein per lane on a 12% reducing Tris/glycine gel A: Western blot analysis of CB1 with RNAi constructs. CB1: untransfected CB1 cell line. sh334: CB1 transfected with pLenti-BLGsh334. sh489: CB1 transfected with pLenti-BLGsh489. shnc: CB1 transfected with negative control plasmid pLenti-shnc. Milk: 5 µl 1:100 diluted porcine skim milk. mi334: CB1 transfected with pCMV-BLGmi334. mi490: CB1 transfected with pCMV-BLGmi490. minc: CB1 transfected with negative control plasmid pCMV-minc. Stable cell pools with knockdown constructs were used for western blot. Actin (42 kDa) was used as loading control. 74% knockdown of porcine BLG (18.5 kDa) expression was detected in the cell pools containing pCMV-BLGmi490. B: Western blot analysis of HpB9 with RNAi constructs. HpB9: untransfected HpB9 cell line. sh334: HpB9 transfected with pLenti-BLGsh334. sh489: HpB9 transfected with pLenti-BLGsh489. shnc: HpB9 transfected with negative control plasmid pLenti-shnc. Milk: 2 µl 1:100 diluted porcine skim milk. mi334: HpB9 transfected with pCMV-BLGmi334. mi490: HpB9 transfected with pCMV-BLGmi490. minc: HpB9 transfected with negative control plasmid pCMV-minc. Stable cell pools with knockdown constructs were used for western blot. Actin (42 kDa) was used as loading control. 67% knockdown of porcine BLG (18.5 kDa) expression was detected in the cell pools containing pLenti-BLGsh489.

The results of the western blot analysis showed different knockdown efficiencies compared to the Dual-Luciferase assays. Whereas the construct pLenti-BLGsh334 showed consistent downregulation of about 70% in the Dual-Luciferase assay, no knockdown of BLG expression was observed in the western blots with CB1 or HpB9 cells. The same was true for the artificial miRNA construct pCMV-BLGmi334 which resulted in 30% downregulation in the Dual-Luciferase assays but had no effect in the stable BLG expressing cell lines.

The shRNA construct pLenti-BLGsh489 resulted in 49% downregulation of the relative *Renilla* luciferase expression in the Dual-Luciferase assays. Image analysis with the Image J software showed that this shRNA construct resulted in 67% knockdown of recombinant porcine BLG in HpB9 compared to the negative control pLenti-shnc whereas it had no effect in the second stable BLG expressing cell line, CB1. The opposite result was obtained for the artificial miRNA construct pCMV-BLGmi490. The construct was effective in the Dual-Luciferase assay (60% knockdown) but showed no reduction of BLG expression in HpB9. In CB1, the construct reduced the BLG amount about 74% compared to the negative control pCMV-minc as detected by image analysis (Image J). Possible reasons for these differences are discussed later (section 4.2.4).

Due to these results, it was not evident which type of construct, shRNA or artificial miRNA, was the best choice for the downregulation of porcine BLG in a transgenic animal. Therefore both construct types were used later to generate RNAi transgenic fetuses.

### 3.1.12 Isolation and characterization of porcine bone marrow derived and adipose tissue derived mesenchymal stem cells

Insufficient lentiviral titers excluded the production of transgenic pigs by direct transduction of zygotes (see section 3.1.6). Another way to obtain transgenic pigs is the *in vitro* manipulation of primary cells which are then used for somatic cell nuclear transfer (SCNT). For this approach, cells are transfected with a DNA construct, selected for the presence of a selectable marker gene, cell pools or single colonies obtained and the presence of the construct confirmed. These cells can then be used as nuclear donors for SCNT (Schnieke *et al.,* 1997).

For this purpose, porcine mesenchymal stem cells from bone marrow (BMMSC) and adipose tissue (ADMSC) were chosen. BMMSCs were selected for the generation of RNAi transgenic pigs because our laboratory has extensive experience in the culture and genetic manipulation of this cell type. Moreover, BMMSCs have previously been evaluated as nuclear donors for SCNT in our laboratory and resulted in viable offspring (Landmann, unpublished results). For a comparison, mesenchymal stem cells derived from porcine adipose tissue (ADMSCs) were selected as donor cells for SCNT because they are easy to isolate in large amounts. No further experiments with this cell type had yet been carried out in our laboratory.

For this project, both cell types were isolated as described in section 2.2.3.8 and 2.2.3.9 from the same female pig to allow a direct comparison. Female cells were chosen so that the effect of the BLG knockdown could later be directly observed in the milk of the founder generation. Isolated BMMSCs and ADMSCs at passage 3 or 4 are shown in Figure 44.
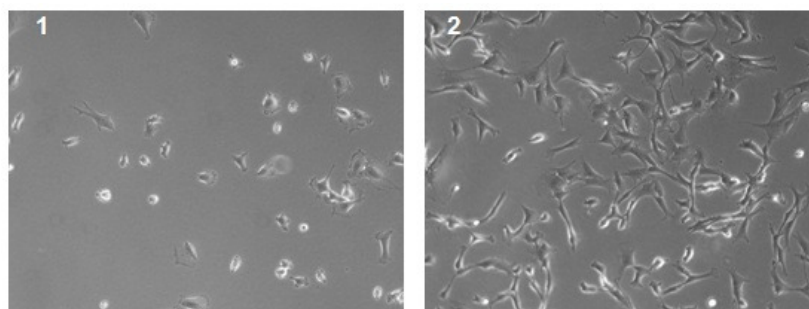


**Figure 44: Porcine mesenchymal stem cells from bone marrow and adipose tissue.** 1: Porcine bone marrow derived mesenchymal stem cells (BMMSC) at passage 3. 2: Porcine adipose tissue derived mesenchymal stem cells (ADMSC) at passage 4. Both cell types showed fibroblast like morphology. BMMSC were smaller and less elongated than ADMSC.

Mesenchymal stem cells (MSCs), also called multipotent mesenchymal stromal cells, are defined by three main criteria. These include adhesion to plastic surfaces, expression or absence of certain surface marker proteins and their potential to differentiate *in vitro* towards adipocytes, chondroblasts and osteoblasts (Dominici *et al.,* 2006). The isolated BMMSCs and ADMSCs adhered to plastic under standard culture conditions fulfilling the first criterion. Examination of cell surface proteins for the identification of MSCs was not performed since the availability of antibodies reactive with porcine surface marker proteins was limited. Moreover it is not known, whether porcine MSCs express the same surface proteins as human MSCs. The third criterion, the multipotent differentiation competence of the isolated BMMSCs and ADMSCs, was examined by *in vitro* differentiation assays. To this end, the cells were subjected to different media designed to induce adipogenesis, osteogenesis or chondrogenesis, and the differentiation of BMMSCs and ADMSCs was compared (media and culture conditions see section 2.2.3.10). Osteogenic and adipogenic differentiation of BMMSC is shown in Figure 45. Osteogenic differentiation was visualized by von Kossa staining of calcium deposits and adipogenic differentiation after staining of lipid droplets with Oil Red O. Bone marrow derived MSCs differentiated towards osteoblasts and adipocytes in induction medium whereas no differentiation was observed in control medium. In addition to von Kossa staining, the amount of produced calcium was measured to obtain a quantitative result (see section 2.2.3.10). 121.5 µg of calcium was produced per well of a six well plate in induced cells and 0.1 µg in control cells.
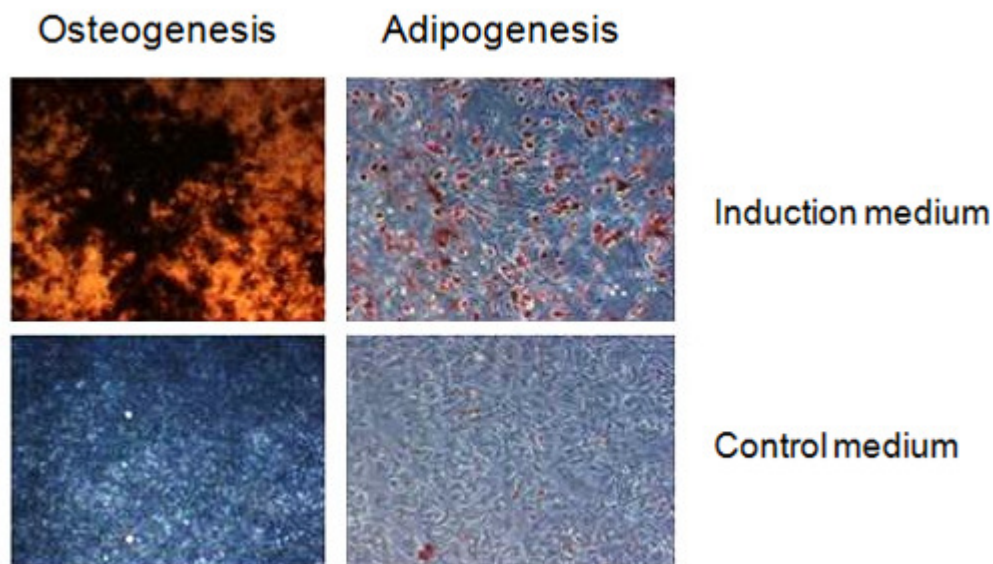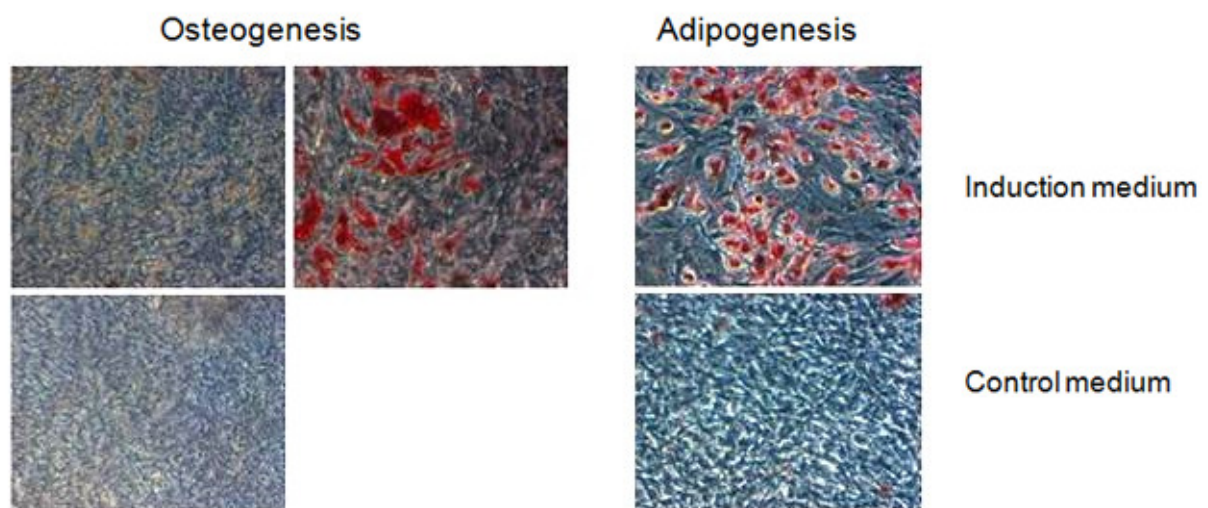


**Figure 45: Osteogenic and adipogenic differentiation of porcine bone marrow derived MSCs.** BMMSC were either cultured in induction medium or in control medium (MSC medium). Cells in osteogenesis medium showed extensive calcium deposits, stained black with von Kossa staining, whereas the control cells did not produce calcium deposits. BMMSC in adipogenesis medium formed lipid droplets which stained red with Oil Red O. Cells in control medium did not form lipid droplets.

Chondrogenic differentiation of BMMSC led to the formation of 3D pellets in the culture tubes. To quantify the chondrogenic differentiation of BMMSCs, the amount of produced glycosaminoglycans (GAG) was measured and normalized to the DNA content of the cell pellet (see section 2.2.3.10). About 53 ng GAG per µg DNA were produced during chondrogenic differentiation.

The differentiation of adipose tissue derived MSCs (ADMSCs) into the adipogenic, osteogenic and chondrogenic lineage was also performed to allow a comparison the two cell types. The osteogenic differentiation ability of ADMSCs was lower than that of BMMSCs but the cells differentiated to adipocytes more efficiently. In contrast to bone marrow derived MSCs, ADMSCs in osteogenic induction medium showed extensive differentiation towards adipocytes in addition to osteogenesis. The von Kossa staining detected only marginal calcium deposits and quantitative analysis showed that ADMSCs only produced 16.3 µg calcium per well. The cells in control medium did not differentiate (see Figure 46). Chondrogenic differentiation was also observed but ADMSCs formed smaller 3D pellets in culture tubes than BMMSCs. Nevertheless they produced a similar amount of GAG per µg DNA (40 ng GAG/µg DNA). This indicates that fewer cells participated in the chondrogenic differentiation but the cells which formed 3D pellets had a similar ability to produce GAG as BMMSCs.



**Figure 46: Osteogenic and adipogenic differentiation of porcine ADMSCs.** ADMSC were either cultured in induction medium or in control medium (MSC medium). Cells cultured in osteogenesis medium showed calcium deposits (stained black with von Kossa staining), whereas the control cells did not produce calcium deposits. ADMSC formed lipid droplets in osteogenesis and adipogenesis medium (stained with Oil Red O). Cells in control medium did not form lipid droplets.

Both cell types, BMMSC and ADMSC, showed multipotent differentiation ability. The bone marrow derived MSCs showed more extensive differentiation towards osteoblasts compared to the adipose tissue derived cells. Unexpectedly, the ADMSCs cultured in osteogenesis medium showed differentiation towards adipocytes. The low rate of osteoblast differentiation

together with extensive generation of adipocytes in osteogenesis medium was not in accordance to the published literature regarding porcine adipose tissue derived MSCs (Qu *et al.,* 2007; Wang *et al.,* 2008). The adipogenic differentiation was efficiently induced in both cell types with the ADMSCs showing more differentiation towards adipocytes than BMMSCs. The chondrogenesis was comparable between the two cell types indicated by a similar amount of GAG production. An overview of the differentiation results of BMMSCs and ADMSCs are presented in Table 10.

**Table 10: Comparison of the differentiation ability of porcine BMMSCs and ADMSCs.**

|  | **BMMSC** | **ADMSC** |
|---|---|---|
| Adipogenesis (Oil Red O) | ++ | +++ |
| Osteogenesis (von Kossa) | +++ | + |
| Osteogenesis (Calcium) | 121.5 µg/well | 16.3 µg/well |
| Chondrogenesis (GAG) | 53 ng GAG/µg DNA | 40 ng GAG/µg DNA |

### 3.1.13 Analysis of the Ryanodine receptor 1 (RYR1) in nuclear donor cells

In a previous project of our laboratory, gene-targeted mesenchymal stem cells were successfully used as donor cells for SCNT. One viable offspring was obtained but the piglet died after 126 days. Necropsy revealed that the death was most probably caused by stress induced malignant hyperthermia (MH), also called porcine stress syndrome. It is known that pigs with a certain mutation (Cys$^{615}$ to Arg$^{615}$) in the Ryanodine receptor 1 gene (RYR1) are susceptible to this syndrome (Fujii *et al.,* 1991; MacLennen and Phillips, 1992). This protein forms part of a calcium release channel and is involved in the control of calcium release from the skeletal muscle sarcoplasmatic reticulum. A mutation in RYR1 leads to abnormal calcium release which stimulates spontaneous muscle contractions (MacLennan and Phillips, 1992). Animals with a mutation in RYR1 are sensitive to stress induced MH, whereas animals homozygous for the normal allele are not. The mutation is detectable due to a restriction enzyme polymorphism. After PCR amplification of a fragment surrounding the mutation site, heterozygous and homozygous animal with the mutation can be identified by digestion of the PCR fragment with the restriction enzyme HhaI as the mutated RYR1 allele does not contain a HhaI restriction enzyme recognition site while the normal allele contains this recognition site. Cells intended for SCNT were therefore examined to reveal the status of RYR1 to exclude MH.

A PCR with the primers RYR1_forward and RYR1_reverse was performed with DNA isolated from the female MSCs which were intended for the generation of RNAi transgenic pigs with BLG knockdown. The PCR generated a 118 bp fragment. This fragment was digested with the restriction enzyme HhaI, resulting in two fragments of 85 bp and a 33 bp fragment (see Figure 47).
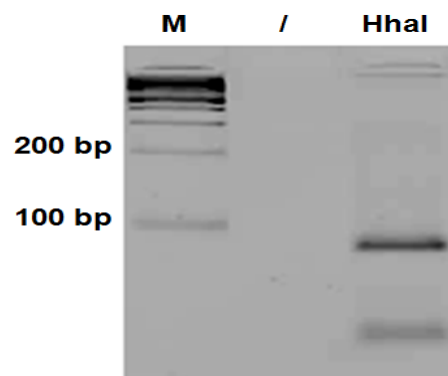
**Figure 47: Analysis of the Ryanodine receptor in nuclear donor MSCs**. M: 100 bp DNA ladder. HhaI: Digest of the 118 bp PCR product with HhaI. The fragments of 33 bp and 85 bp showed that the cells were derived from an animal with homozygous normal RYR1 allele.

This showed that the isolated porcine BMMSCs and ADMSCs were derived from a female pig which was homozygous for the normal allele. The results of the differentiation assays and the presence of the homozygous normal RYR1 allele supported the further use of this BMMSC and ADMSC preparation.

### 3.1.14 Modification of the artificial miRNA plasmids pCMV-BLGmi490 and pCMV-minc

The next step was to introduce the RNAi constructs for knockdown of porcine BLG into the primary porcine BMMSCs and ADMSCs characterized as suitable for the generation of transgenic pigs by SCNT. Before this step, the artificial miRNA construct pCMV-BLGmi490 was modified by exchanging the viral CMV promoter with a cellular PGK promoter. This was necessary as there is evidence that the CMV promoter leads to mosaic expression of transgenes in pigs (Deppenmeier *et al.,* 2006). In contrast, the PGK promoter has been shown to be active in all tissues of transgenic mice (McBurney *et al.,* 1994). Moreover, a construct was generated where the CMV promoter was replaced with the ovine BLG promoter. This promoter has previously been used for mammary gland specific expression of human Factor IX in transgenic sheep (Schnieke *et al.,* 1997). The intention was to achieve tissue-specific expression of the artificial miRNA only in the lactating mammary gland.

For this purpose, the CMV promoter was excised from the plasmid pCMV-BLGmi490. The PGK promoter from the plasmid pPGK-EGFP1 was obtained by restriction enzyme digestion and ligated into pCMV-BLGmi490. A schematic diagram of the artificial miRNA vector pPGK-BLGmi490 is presented in Figure 48. To insert the ovine BLG promoter in pCMV-BLGmi490, the BLG promoter was excised from the plasmid pMIX-FIX (section 2.1.14 and 8.2) and inserted into the digested vector pCMV-BLGmi490. The resulting vector pBLG-BLGmi490 is illustrated in Figure 48. The same approach was used to replace the CMV promoter of pCMV-minc with the PGK promoter.
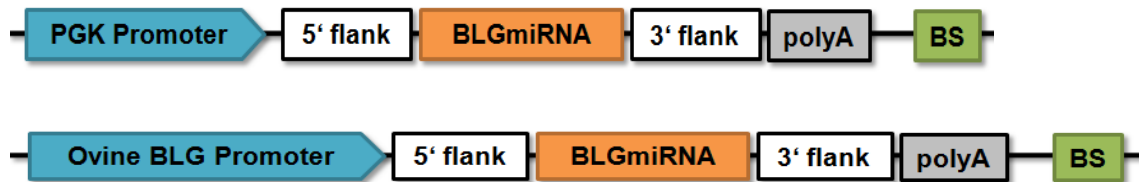
**Figure 48: Schematic diagram of the modified artificial miRNA plasmids with PGK or ovine BLG promoter.** Top: The CMV promoter of pCMV-BLGmi490 was excised with Bpu10I and DraI and the PGK promoter (blue) was obtained from pPGK-EGFP1 after digestion with SacI and SmaI. After the generation of blunt ends, the fragments were ligated to generate pPGK-BLGmi490 and pPGK-minc. Bottom: The CMV promoter of pCMV-BLGmi490 was replaced with the BLG promoter (blue) from pMIX-FIX. For this, the CMV promoter was removed after restriction digest with Bpu10I and DraI. The ovine BLG promoter was obtained from pMIX-FIX by restriction with SalI and XhoI. The blunted fragments were ligated to obtain pBLG-BLGmi490. A Blasticidin resistance cassette was included in the vector (BS, green).

The silencing efficiency of the modified miRNA vector with the PGK promoter was compared to the original CMV promoter directed miRNA vector pCMV-BLGmi490 in a Dual-Luciferase assay with psiCHECK-BLG. The RNAi construct pPGK-BLGmi490 showed a knockdown of 72% and pCMV-BLGmi490 of 65% compared to the respective negative control plasmids. This experiment showed that the PGK promoter can direct expression of the miRNA as efficiently as the CMV promoter based construct.
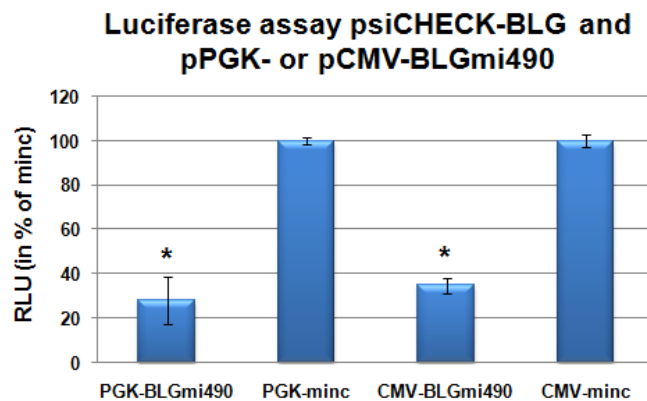


**Figure 49: Dual-Luciferase Assay with pPGK-BLGmi490 and pCMV-BLGmi490.** pPGK-BLGmi490 showed a knockdown of *Renilla* luciferase activity of 72% and pCMV-BLGmi490 of 65% compared to the respective negative controls pPGK-minc and pCMV-minc. Measurements were performed in triplicate The knockdown of the *Renilla* luciferase fusion mRNA was comparable between the constructs (*: $p < 0.05$; n=3).

The artificial miRNA plasmid pBLG-BLGmi490 could not be tested in the Dual-Luciferase assay since the ovine BLG promoter is a mammary gland specific promoter and therefore not active in HEK293 cells. The constructs with the PGK and BLG promoter were chosen for the generation of RNAi transgenic pigs for knockdown of porcine BLG.

### 3.1.15 Generation of stable BMMSC and ADMSC clones with RNAi constructs

To generate transgenic pigs with RNAi mediated knockdown of BLG, the previously characterized female ADMSCs and BMMSCs were used. As described before (see section 3.1.11), the western blot to determine the knockdown potential of the RNAi constructs did not result in a clear indication of which type of RNAi construct was more effective for

downregulation of porcine BLG. Therefore, shRNA and artificial miRNA constructs were used to generate stable BMMSC and ADMSC clones.

In a preliminary experiment, BMMSCs and ADMSCs were transfected with both artificial miRNA constructs (PGK and BLG promoter), selected with Blasticidin and single colonies expanded. Six ADMSC and two BMMSC colonies after transfection with the PGK promoter and eleven ADMSC and no BMMSC colonies with the BLG promoter directed construct were obtained. The low colony number obtained with BMMSCs was surprising because this cell type had previously been used successfully in our laboratory to generate cell clones with high efficiency after gene targeting. The colonies were screened by PCR with the primers miRNA cassette f/r. PCR analysis resulted in two out of eight clones with the PGK and eight out of eleven clones with the BLG promoter artificial miRNA cassette. The results of the PCR analysis are shown in Figure 50.
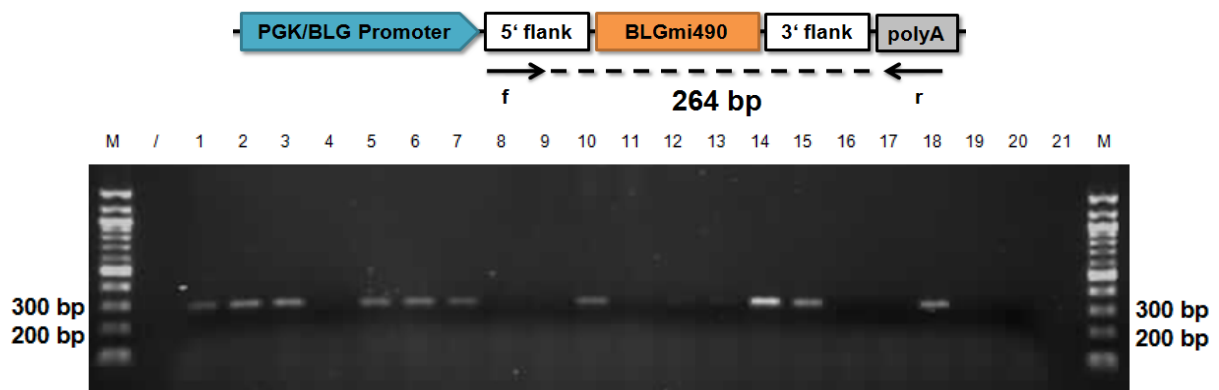


**Figure 50: PCR screening of BMMSC and ADMSC clones after transfection with pPGK-BLGmi490 and pBLG-BLGmi490.** M: 100 bp DNA Ladder. Fragment size with primers miRNA cassette f/r: 264 bp. Several positive clones containing the BLG-BLGmi490 cassette are shown (1, 2, 3, 5, 6, 7, 14, 15 : ADB2 I, ADB5 I, ADB9 I, ADB11 I, ADB12 I, ADB13 I, ADB7 I and ADB14 I). Positive clones with the PGK-BLGmi490 cassette were also generated (10: ADP3 I; 18: MP1). 20: wildtype DNA. 21: water control.

The transfection and selection procedure was performed again with ADMSCs but not with BMMSCs because of the few colonies obtained after the first transfection. The shRNA construct against porcine BLG and the two artificial miRNAs plasmids with either PGK or BLG promoter were used for transfection.

24 clones were selected from the transfection with pLenti-BLGsh489, eight showed normal ADMSC morphology and proliferated well. Six of them were analyzed by PCR with the primers shRNA cassette f/r for the presence of the BLGsh489 cassette and all clones showed the BLGsh489 cassette (see Figure 51).
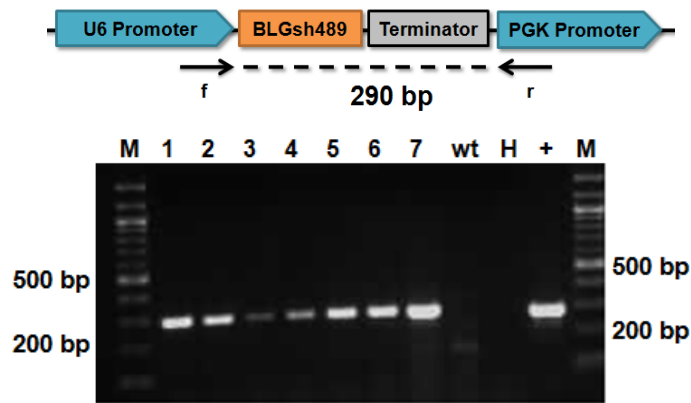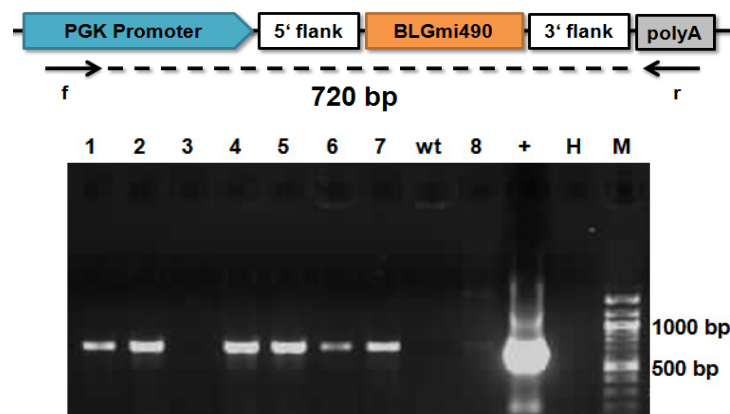
**Figure 51: PCR screening of ADMSC clones after transfection with pLenti-BLGsh489.** M: 100 bp DNA Ladder. H: water control. Fragment size with primers shRNA cassette f/r: 290 bp. All six tested cell clones contained the shRNA cassette (1, 2, 3, 4, 5 and 6: clone ADS5, ADS10, ADS15, ADS18, ADS19, ADS24). The two positive controls showed the expected bands (7: ADMSC pool with pLenti-BLGsh489; +: plasmid DNA pLenti-BLGsh489) and no amplification product could be seen in the negative control (wt: ADMSC).

After transfection with pPGK-BLGmi490, 21 colonies were chosen, six of which continued to proliferate as single clones. PCR screening with primers PGK-mi_F/R was performed to verify the presence of the miRNA cassette. The PGK-BLGmi490 cassette could be detected in five out of six colonies and the result of the PCR analysis is presented in Figure 52.



**Figure 52: PCR screening of ADMSC clones transfected with pPGK-BLGmi490.** M: 100 bp DNA Ladder. H: water control. Fragment size with primers PGK-mi_F/R: 720 bp. Several positive clones with insertion of the PGK-BLGmi490 cassette are shown (1, 2, 4, 5 and 6: clone ADP2 II, ADP3 II, ADP6 II, ADP9 II and ADP11 II). The two positive controls showed the expected bands (7: ADMSC pool with pPGK-BLGmi490; 8: diluted ADMSC pool; +: plasmid DNA pPGK-BLGmi490) and no amplification product could be seen in the negative control (wt: ADMSC).

Transfection of the pBLG-BLGmi490 construct produced 28 colonies, ten of which proliferated further as cell clones. They were screened by PCR with the primers BLG-mi_F/R. The PCR screening for the BLG-BLGmi490 cassette in cell lysates for the detection of the 2732 bp cassette was not possible. The reason might be that these crude lysates did not contain enough cells for successful amplification of this long fragment or that impurities in the lysates inhibited the PCR reaction. Genomic DNA was therefore obtained from four clones. Three of these clones showed the presence of the cassette after PCR from purified genomic DNA. One clone from the first transfection was also included in the analysis (Figure 53).
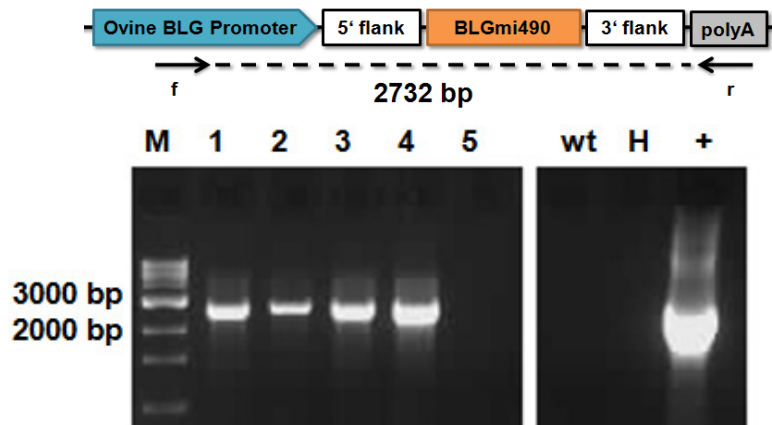
**Figure 53: PCR for the detection of the BLG-BLGmi490 cassette from genomic DNA of ADMSC cell clones.** M: 1 kb DNA Ladder. H: water control. Fragment size with primers BLG-mi_F/R: 2732 bp. 4 of the 5 clones contained the BLG-BLGmi490 cassette (1, 2, 3 and 4: clone ADB12 I, ADB6 II, ADB17 II, ADB22 II). The positive control showed the expected band (+: plasmid DNA pBLG-BLGmi490) and no amplification product could be seen in the negative control (wt: ADMSC).

In total, 24 colonies were selected after transfection of ADMSCs with pLenti-BLGsh489, 27 from pPGK-BLGmi490 and 39 from pBLG-BLGmi490. Not all of these single colonies proliferated well. It has also been shown by other groups that not all MSC clones proliferate as single cell clones, indicating heterogeneous proliferation potential of these cells (Boquest *et al.,* 2006). Of the analyzed colonies, six showed the presence of the U6 promoter directed BLGsh489 construct, six of the PGK promoter directed BLGmi490 and eleven of the BLGmi490 construct under the control of the BLG promoter. Positive cell clones with the highest proliferation rate and normal ADMSC morphology were chosen as donor cells for SCNT.

### 3.1.16 Cell culture of transfer cell pools and generation of transgenic fetuses

Cell clones containing RNAi constructs were used as donor cells for SCNT and cultured in parallel to evaluate the growth characteristics and differentiation potential. The cell clones with best morphological and proliferative properties were chosen for the generation of RNAi transgenic fetuses. Six clones with the shRNA cassette (ADS5, 10, 15, 18, 19 and 24), five clones with the PGK promoter directed artificial miRNA construct (ADP2 II, P3 II, P6 II, P9 II and P11 II) and four clones with the BLG promoter driven miRNA expression construct (ADB12 I, B6 II, B17 II and B22 II) were used for SCNT. The cell clones of each construct were combined. At passage 7 a total of $3.6 \times 10^5$ cells per construct was mixed and subjected to serum starvation prior to SCNT (see section 2.2.3.12). In parallel to the SCNT, $1 \times 10^5$ cells of the separate transfer pools (Transfer pool BLG-BLGmi490, PGK-BLGmi490 and BLGsh489) were cultured to monitor the growth characteristics and differentiation ability of these cell pools. An overview of the experiments is presented in Figure 54.
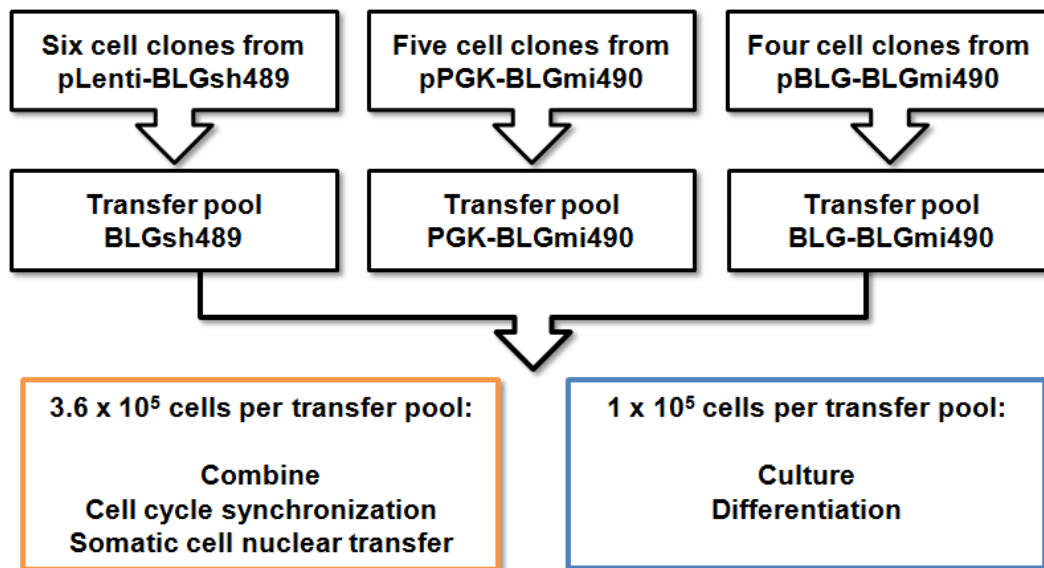
**Figure 54: Schematic diagram of the cell culture of transfer pools and somatic cell nuclear transfer.** Individual cell clones containing one type of construct were mixed to obtain transfer pool BLGsh489, PGK-BLGmi490 and BLG-BLGmi490. $1 \times 10^5$ cells per transfer pool were cultured to evaluate the growth characteristics and differentiation potential of single clone derived pools. $3.6 \times 10^5$ cells per transfer pool were combined and subjected to serum starvation prior to SCNT.

### 3.1.16.1    Culture of RNAi transgenic cell pools

The individual cell pools derived from 4 to 6 clones per construct (see 3.1.16) were cultured in parallel to the SCNT and embryo transfer. This analysis was performed to determine whether the cell pools derived from single colonies retained their differentiation ability and to monitor if the presence of RNAi constructs had any discernible effect on these cell pools.

During culture of the cell pools derived from ADMSC clones it became apparent, that the three separate pools had different proliferation rates. The cell pool containing the U6 promoter directed BLGsh489 construct grew more slowly than the pool containing the PGK promoter directed BLGmi490. The cell pool with the fastest proliferation rate was the pool with the BLG promoter directed BLGmi490 cassette. In 12 weeks, BLGsh489 reached passage 11, PGK-BLGmi490 passage 13 and BLG-BLGmi490 passage 17 under the same culture conditions (passage and cell number at the beginning: P7, $1 \times 10^5$ cells; cells passaged at 80% confluence; subculturing ratio: 1:10). This difference might be linked to the expression level of the shRNA or the artificial miRNA constructs. Stewart *et al.* (2008) have shown that a high level of shRNA expression in porcine fetal fibroblast had negative effects on these cells. The same could be seen here, the shRNA containing cell pool grew slower than the other two cell pools containing artificial miRNA expression cassettes.

The separate transfer pools were also used in a differentiation assay at passage 10 or 11 to determine whether any change in differentiation ability had occurred. The adipogenic differentiation potential of the cell pools containing the PGK and BLG promoter directed artificial miRNA construct showed reduced levels of adipocyte differentiation compared to the

initial ADMSC culture. In contrast, almost 100% of the cell pool containing BLGsh489 differentiated into adipocytes (see Figure 55).
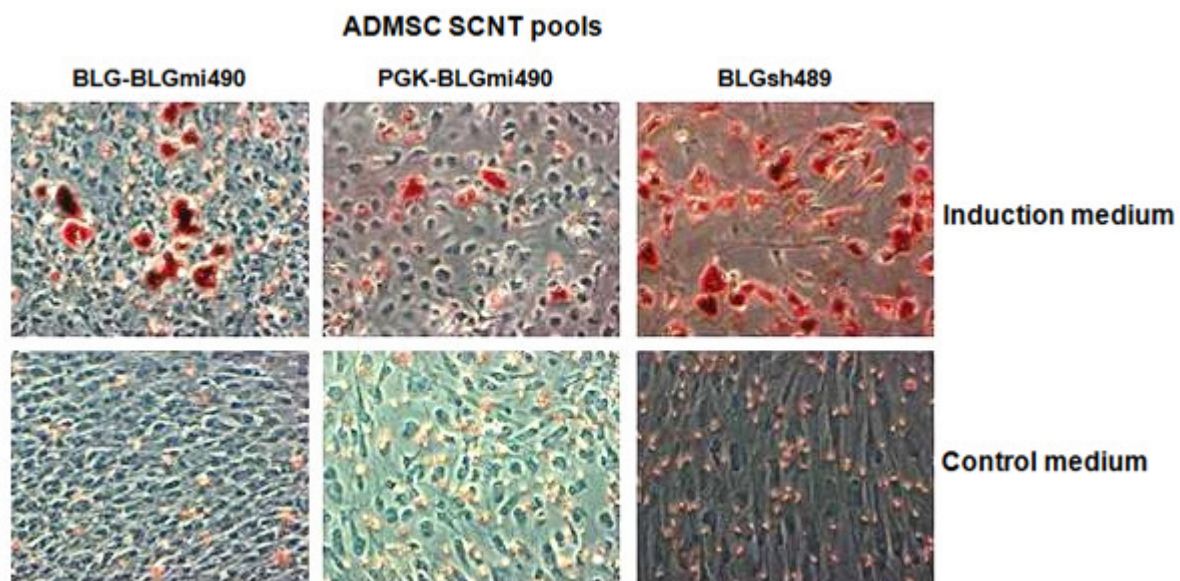
**ADMSC SCNT pools**



**Figure 55: Adipogenesis of transfer cell pools.** The cell pools containing the three different RNAi constructs against BLG, which were mixed for SCNT, were cultured individually in adipogenesis medium. The pool containing the BLG and PGK promoter directed artificial miRNAs showed lower adipogenetic differentiation potential compared to the early passage non clonal ADMSCs. In the cell pool containing the U6 promoter controlled shRNA against porcine BLG, adipogenesis was enhanced compared to the initial ADMSC culture.

No obvious differences in the osteogenic or chondrogenic differentiation potential between the three cell pools as visualized by von Kossa staining of calcium deposits or 3D pellet formation capacity in chondrogenesis medium were visible (data not shown). This showed that the cell pools derived from single colonies retained their differentiation potential towards adipocytes, osteoblast and chondroblasts with differences in the adipogenesis.

### 3.1.16.2     Generation of transgenic fetuses

SCNT with cell pools containing the three RNAi constructs against porcine BLG was performed at the Institute of Molecular Animal Breeding and Biotechnology (LMU). The transfer of 160 SCNT embryos into two synchronized recipient sows was performed at the TUM animal research station (Thalhausen) and resulted in one pregnancy which was terminated at day 32.

The termination of the pregnancy was performed because of different reasons. First, it was unknown whether porcine ADMSCs were a suitable donor cell type for SCNT and could support development to term. Second, it has been reported that a high level of shRNA expression from the U6 promoter can interfere with the endogenous miRNA pathway. This resulted in toxicity of the expressed shRNA in mice (Grimm *et al.,* 2006) and Cao *et al.* (2005) reported fetal and neonatal death of mice carrying shRNA constructs under the U6 and H1 promoter. Therefore the second reason to terminate the pregnancy at day 32 was to

investigate whether the shRNA construct led to the generation of fetuses. Third, nuclear transfer was performed with a cell pool. This should lead to multiple fetuses derived from different cells within the pool. The isolation of fetal fibroblasts allows the analysis of clones which support fetal development combined with the possibility of expression analysis of the shRNA and PGK promoter directed artificial miRNA construct. After this analysis the fetal fibroblast can then be used for another round of cloning, so-called recloning, after minimal time in culture. Recloning has been shown to be more efficient than using cells which have been cultured, transfected and selected prior to SCNT (Fujimura *et al.,* 2008).

A total of three fetuses were recovered on day 32. Two of the three obtained fetuses showed normal morphology, the other fetus was dead and showed signs of degradation. The sex of the fetuses was determined by PCR as described in section 2.2.2.2 and all three fetuses were confirmed as female (see Figure 56).
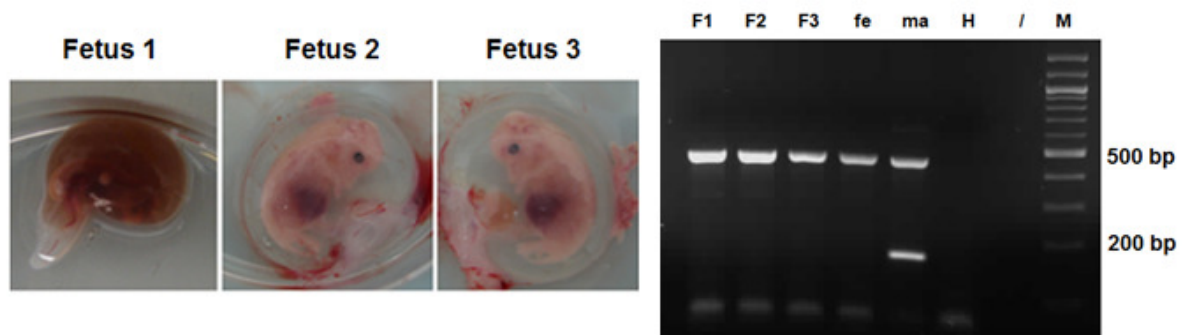


**Figure 56:  Fetuses recovered on day 32 after SCNT/embryo transfer and sex determination of the fetuses.** Left: Three fetuses were recovered from one pregnant recipient sow 32 days after embryo transfer. Fetus 1 was dead and showed signs of degradation. Fetus 2 and 3 showed normal morphology. Right: The sex of the recovered fetuses was determined by PCR. PCR fragments for male fetuses (163 + 445 bp) and female fetuses (445 bp) were detected. M: 100 bp marker. F1 to F3: DNA from the three fetuses.  A female (fe) and male (ma) positive control and the water control (H) are shown as well. All fetuses were female.

DNA of the three fetuses was isolated and screened by PCR to determine which RNAi constructs they carried. This was necessary since cell clones derived after transfection of each construct were pooled for the SCNT. The results of the PCR analysis are shown in Figure 57. No fetus originated from cell clones carrying the shRNA cassette of the pLenti-BLGsh489 construct, fetus F1 and F2 carried the BLG promoter directed artificial miRNA cassette from pBLG-BLGmi490 and fetus F3 the PGK promoter containing miRNA cassette from pPGK-BLGmi490.
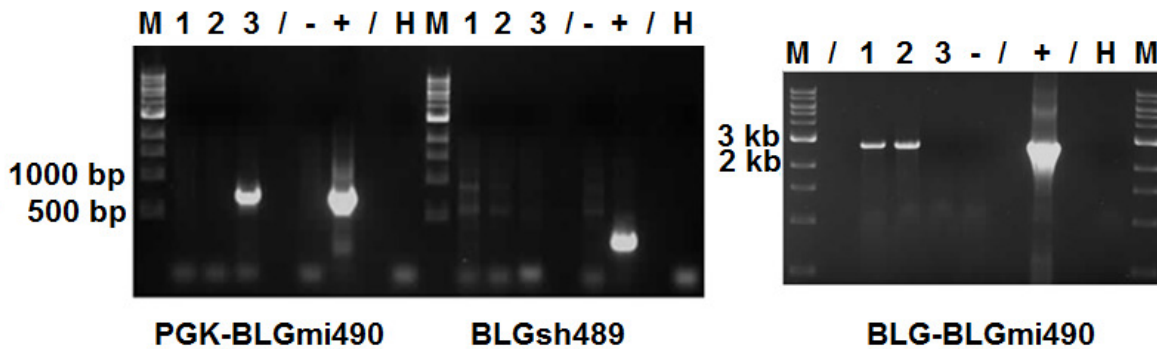
**Figure 57: PCR screening of SCNT derived fetuses for integration of RNAi cassettes.** M: 1 kb DNA Ladder. H: water control. Fragment size with primers PGK-mi_F/R: 720 bp. Fragment size with shRNA cassette f/r: 290 bp. Fragment size with primers BLG-mi_F/R: 2732 bp. 1-3: Fetus F1, F2 and F3. -: female pig genomic DNA, +: plasmid DNA pPGK-BLGmi490, pLenti-BLGsh489 or pBLG-BLGmi490. Fetus F1 and F2 were positive for the BLG promoter and fetus F3 for the PGK promoter directed BLGmi490 cassette. None of the fetuses carried the BLGsh489 cassette.

Fetal fibroblast (poFF) cultures were initiated from all three fetuses. As fetus F1 was dead and showed signs of reabsorption, the fetal fibroblasts poFF1 did not proliferate in cell culture after the first passage. The cells derived from fetus 2 and 3, poFF2 and poFF3, proliferated well in cell culture.

In total, 15 female ADMSC cell clones carrying RNAi constructs against porcine BLG were used for SCNT. Of these, six clones contained the U6 promoter directed shRNA cassette BLGsh489, five clones the PGK promoter directed artificial miRNA construct PGK-BLGmi490 and four clones the BLG promoter driven miRNA construct BLG-BLGmi490.

After SCNT, 160 reconstituted embryos were transferred into 2 synchronized recipient sows and one pregnancy established. Three fetuses were recovered, two of them showed normal morphology and one fetus was dead. The fetuses were confirmed as female by PCR analysis. The dead fetus (F1) and one other fetus (F2) were positive for the BLG promoter driven artificial miRNA cassette against porcine BLG and one fetus (F3) contained the PGK promoter driven artificial miRNA cassette. None of the fetuses were positive for the U6 promoter directed shRNA cassette.

### 3.1.17 Expression analysis of the RNAi transgenic fetuses

The next step was to evaluate, whether the PGK promoter directed artificial miRNA in the fetal fibroblasts derived from fetus F3 (poFF3) was expressed. This test was performed because the insertion of the construct into the cell genome after transfection is a random integration event and it is therefore possible, that the construct integrated into transcriptionally inactive genomic regions or is silenced (reviewed in Auerbach, 2004). Therefore RNA was obtained from poFF3 and a small RNA northern blot performed to evaluate the functionality of the PGK promoter directed miRNA construct. As controls, poFFs derived from a non-transgenic fetus and from the BLG promoter containing artificial miRNA cells poFF2 were used. The small RNA northern blot was performed as described in section

2.2.2.16 with digoxigenin (DIG) labeled locked nucleic acid (LNA) probes for the mature BLGmi490 sequence and the result is presented in Figure 58. As loading control, a DIG labeled LNA probe for the detection of the U6 snRNA was applied.
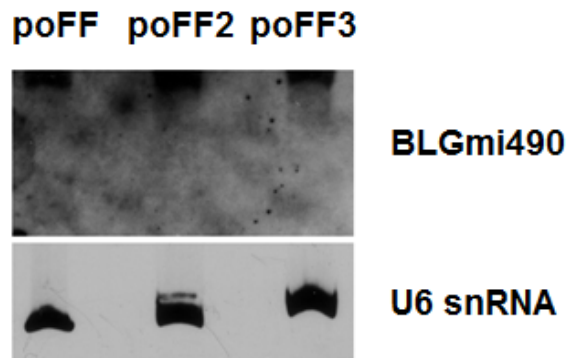


**Figure 58: Small RNA northern blot analysis for the detection of BLGmi490.** 20 µg total RNA were separated on a 12% polyacrylamide gel and electroblotted onto Hybond+ membrane. Detection of the BLGmi490 and the loading control U6 snRNA was performed with DIG labeled LNA probes. Expression of BLGmi490 was not detectable.

It was not possible to detect the expression of the BLGmi490 in poFF3 even from 20 µg total RNA. As expected, the control fetal fibroblasts (poFF) did not express BLGmi490. The loading control against U6 snRNA was detected in all samples. Therefore it can be concluded, that the PGK promoter directed artificial miRNA construct against BLG is not expressed at levels detectable by small RNA northern blot in poFF3. It was not possible to determine, whether the BLG promoter directed BLGmi490 is expressed, because the BLG promoter is lactating mammary gland specific and therefore not active in poFFs.

## 3.2    Sequence-specific gene silencing of the porcine tumor suppressor protein p53

Li-Fraumeni syndrome (LFS) is an inherited human cancer syndrome resulting from TP53 germline mutations. Patients are prone to early tumor development, multiple primary tumors and a high rate of multiple cancers (Malkin *et al.,* 1990; Evans and Lozano, 1997). So far genetically modified mice with heterozygous and homozygous deletion of TP53 have been generated as animal models for this inherited disease. These mice are susceptible to early tumor development but have certain limitations for preclinical research due to the fact that mice are considerably different in anatomy, physiology and lifespan from humans (Donehower *et al.,* 1992; Jacks *et al.,* 1994; Melo *et al.,* 2007). Pigs offer an alternative as disease models since they more closely resemble humans in physiology, size and organ development (Wernersson *et al.,* 2005; Lunney, 2007).

Sequence-specific gene silencing by RNAi is capable of creating phenotypes resembling null mutations and it has been shown that RNAi mediated downregulation of p53 in hematopoietic stem cells from Eμ-myc mice resulted in accelerated tumor development similar to knockout mice (Kunath *et al.,* 2003; Hemann *et al.,* 2003; Dickins *et al.,* 2005). In mice RNAi therefore presents an alternative to gene knockout by gene targeting. The aim of this project was to evaluate if RNAi mediated downregulation of p53 offers a faster alternative to the precise genetic modification of porcine TP53 to generate a porcine model for LFS. RNAi expression systems for short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs) were used for downregulation of porcine p53 and their efficiency in different test systems compared.

### 3.2.1    Short hairpin RNA and artificial miRNA sequences for p53 knockdown

To achieve stable downregulation of porcine p53 in various test systems and in porcine primary cells, two different types of RNAi expression constructs were employed. These were based on short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs). The first step in developing efficient shRNA and artificial miRNA silencing constructs was the selection of suitable target sequences. ShRNAs and miRNAs for knockdown of porcine p53 (NCBI Reference Sequence: NM_214145.1) were designed with the Block-iT RNAi Designer program (Invitrogen). Sequences against p53 were retrieved and a BLAST search of the known porcine database performed to exclude off-target effects as described for the design of RNAi sequences against porcine BLG (see section 3.1.1).

Three sequences for shRNA and artificial miRNAs were chosen for the downregulation of porcine p53. These sequences were directed against similar positions within the open reading frame of porcine p53. The RNAi sequences against porcine p53 were obtained as

single stranded DNA oligonucleotides. Before insertion into the respective RNAi expression plasmids, they were annealed to double stranded DNA oligonucleotides. The general structure of double stranded shRNA and miRNA oligonucleotides is illustrated in Figure 59.
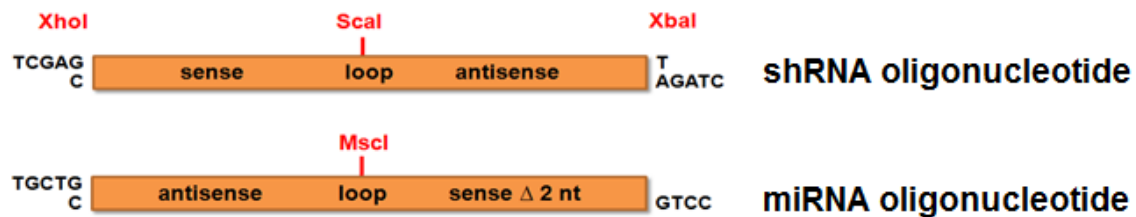


**Figure 59: General configuration of double stranded shRNA and artificial miRNA DNA oligonucleotides.** Top: Schematic diagram of a shRNA oligonucleotide. DNA oligonucleotides were designed with XhoI and XbaI restriction site overhangs. The 6 nt spacer (loop) sequence contains a ScaI restriction site. The 3' end of the oligonucleotide sequence includes a transcription terminator (TTTTT). Bottom: Schematic diagram of an artificial miRNA DNA oligonucleotide. DNA oligonucleotides were designed with BsaI restriction site compatible overhangs. The 15 nt loop sequence contains a MscI restriction site. The antisense target sequence leads to the formation of the mature miRNA sequence. The sense Δ 2 nt target sequence is 2 nucleotides shorter than the antisense target sequence, leading to the formation of an internal bulge in the resulting pre-miRNA.

The three shRNA target sequences were located at nucleotide position 312, 787 and 944 and the three artificial miRNA at position 304, 785 and 943 relative to the starting nucleotides of the porcine p53 mRNA Reference Sequence NM_214145.1. The target sequence positions are indicated in Figure 60.
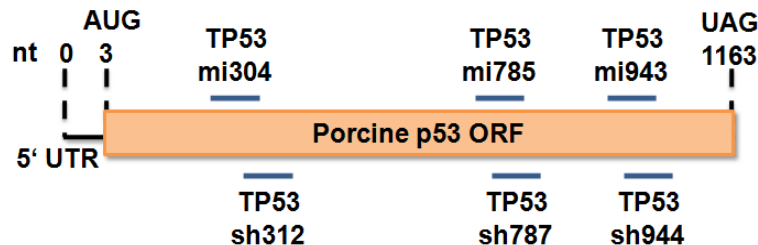


**Figure 60: Porcine p53 mRNA Reference Sequence NM_214145.1 with shRNA and artificial miRNA target positions.** Target sites were named according to their position relative to the start of NM_214145.1.

The selected sequences were inserted into shRNA and artificial miRNA expression vectors and the knockdown efficiency tested in a variety of *in vitro* test. The type of expression vectors used, their construction and the experiments to evaluate the knockdown potential will be described in the following sections.

## 3.2.2  Generation of pLenti-shRNA vectors for downregulation of porcine p53

To test the selected shRNA sequences for their knockdown ability in a variety of experiments, they were inserted into the lentiviral shRNA expression vector pLenti-shRNA (see section 3.1.4). Although it had been shown before that the lentiviral titers achieved with this vector were not suitable for the generation of transgenic pigs by lentiviral transgenesis (see section 3.1.6), this vector was chosen as shRNA expression vector. The reason behind this was that pLenti-shRNA contains a PGK promoter directed EGFP and Blasticidin

resistance cassette, allowing visual identification of transfected cells combined with a mammalian selectable marker gene. The annealed DNA oligonucleotides for the three shRNA sequences against porcine p53 (TP53sh312, TP53sh787 and TP53sh944) were inserted into the SalI and XbaI restriction sites of the lentiviral shRNA plasmid (see Figure 61). Integration of the shRNA oligonucleotide was assessed by restriction enzyme digestion of the resulting plasmids with SalI and ScaI as described for the BLG shRNA constructs (see section 3.1.1). The correct DNA sequence of constructs containing the shRNA cassette was confirmed by sequencing with primers pLenti-U6 f/r.
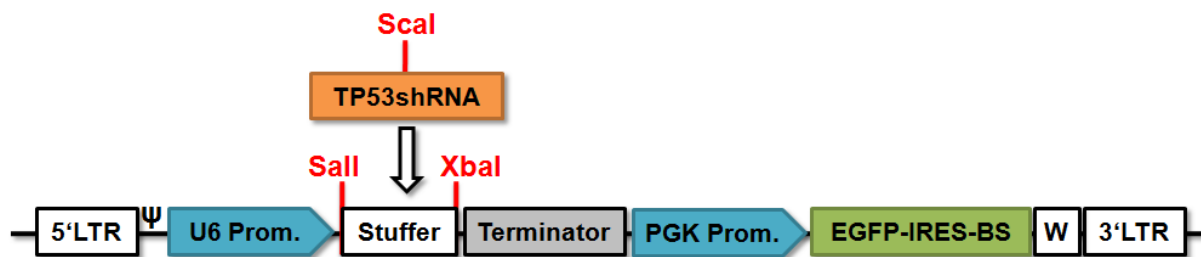


**Figure 61: Schematic diagram of the lentiviral vector pLenti-shRNA.** The plasmid contains a PGK promoter directed EGFP/Blasticidin resistance cassette (blue, green), the woodchuck hepatitis virus post-transcriptional regulatory element (W, white), 5' LTR and a delta U3 3' LTR (white). ψ: HIV packaging signal. The annealed shRNA oligonucleotides against porcine p53 were inserted into the SalI and XbaI sites of pLenti-shRNA. Thereby the SalI restriction site was eliminated. A ScaI site, introduced into the loop region of the shRNA, facilitated the identification of plasmids containing the shRNA oligonucleotides.

Three shRNA plasmids, pLenti-TP53sh312, pLenti-TP53sh787 and pLenti-TP53sh944, for the knockdown of porcine p53 were successfully constructed.

### 3.2.3  Generation of pCMV-miRNA vectors for downregulation of porcine p53

In addition to the shRNA expression plasmids against porcine p53, artificial miRNA vectors for knockdown of p53 were generated. For this purpose the commercial vector pcDNA6.2-GW/miR (pCMV-miRNA) from Invitrogen was chosen (see section 2.1.14 and 8.2). This plasmid has also been used for the downregulation of porcine BLG and the functional features have been described in section 3.1.7.

The three artificial miRNA sequences TP53mi304, TP53mi785 and TP53mi943 were inserted into BsaI digested vector pCMV-circ (see Figure 62). The loop sequence of the resulting plasmids contained a recognition site for MscI allowing insertion of the oligonucleotides to be confirmed by MscI digestion.

Furthermore a positive control plasmid against *Renilla* luciferase, pCMV-miLuc2 (target sequence starting at nt 79 relative the *Renilla* luciferase hRluc (Promega) start codon), was designed with the Block-iT RNAi Designer program. This positive control plasmid was generated because the knockdown ability of the previously used positive control plasmid pCMV-miLuc1 was only moderate (around 35% knockdown of relative *Renilla* luciferase

activity). Plasmids with correct restriction patterns were sequenced with the primers miRNA f/r to ensure correct miRNA sequences.
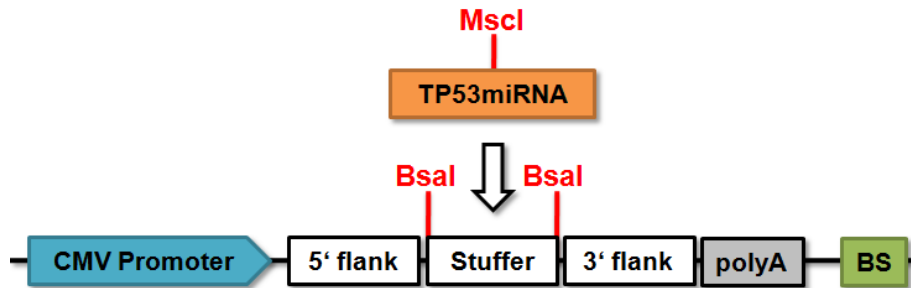


**Figure 62: Schematic diagram of the artificial miRNA vector pCMV-circ.** The artificial miRNA expression vector pCMV-circ contains a CMV promoter (blue), the 5' and 3' flanking regions of murine miR-155 (white), the stuffer sequence enabling propagation of the plasmid in bacteria (white), the HSV-TK polyadenylation sequence (grey) and a Blasticidin resistance cassette (green). Artificial miRNA against porcine p53 (orange) were inserted into the BsaI restriction sites. The artificial miRNAs contain a MscI restriction enzyme recognition site to facilitate the identification of plasmids containing miRNA sequences.

Three artificial miRNA plasmids for the downregulation of porcine p53 (pCMV-TP53mi304, pCMV-TP53mi785, pCMV-TP53mi943) and a positive control plasmid against *Renilla* luciferase (pCMV-miLuc2) were successfully generated.

### 3.2.4 Construction of the test vector psiCHECK2-TP53 for Dual-Luciferase assays

To determine the knockdown potential of the artificial miRNA and shRNA plasmids against p53, the porcine p53 cDNA had to be obtained and later inserted into the NotI restriction sites of the RNAi test vector psiCHECK2 (see section 3.1.2). In order to do so, a 1198 bp fragment of p53 was amplified with primers Ex2_1F and Ex11_2R by RT-PCR from porcine total RNA (see Figure 63). The p53 cDNA contained two nucleotides of the 5' UTR, the open reading frame and parts of the 3' UTR and was inserted into the cloning vector pGEM-T easy to generate the plasmid pGEM-TP53. The sequence of the cDNA was verified by analysis with primers SP6 and T7.
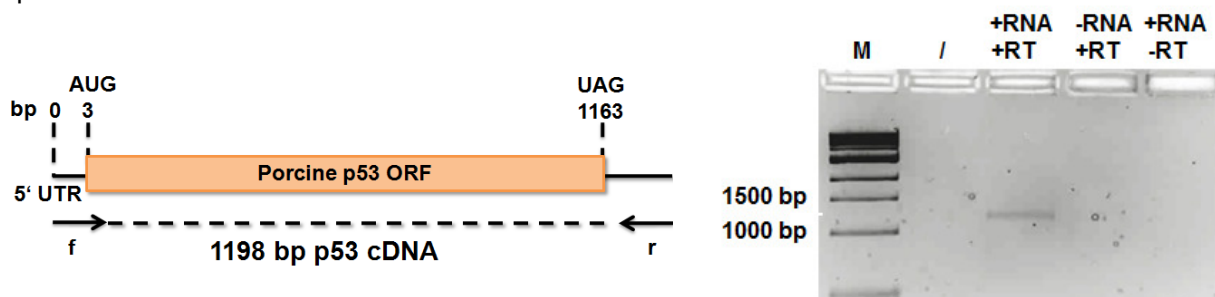


**Figure 63: Amplification of porcine p53 cDNA.** Left: Porcine p53 cDNA with primer binding sites. RT-PCR with primers Ex2_1F and Ex11_2R yielded a 1198 bp fragment containing 2 nt of the 5' UTR, the open reading frame of the p53 cDNA and parts of the 3' UTR. The start codon AUG is located on the forward primer. Right: RT-PCR of porcine p53 from porcine mesenchymal stem cells. M: 1 kb DNA ladder. +RNA +RT: p53 cDNA fragment. -RNA +RT: water control. +RNA -RT: DNA control.

Afterwards, the p53 cDNA was excised from pGEM-TP53 with NotI and inserted into the NotI sites of psiCHECK2 to generate the vector psiCHECK2-TP53 (see Figure 64).
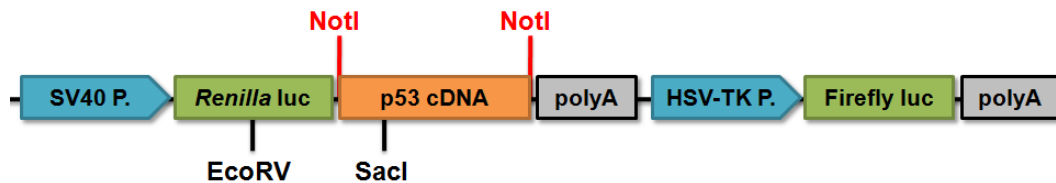


**Figure 64: Schematic diagram of the RNAi test vector psiCHECK-TP53.** The porcine p53 cDNA from pGEM-TP53 was excised with NotI and inserted into the NotI site of psiCHECK2. The correct orientation was confirmed by restriction digest with EcoRV and SacI. The RNAi test vector contains a *Renilla* and a firefly luciferase gene (green) for the expression in mammalian cells and the p53 cDNA was inserted downstream of the *Renilla* luciferase gene. This allowed the SV40 promoter (blue) directed expression of a fusion mRNA between this luciferase and the p53 cDNA. The HSV-TK promoter (blue) directed firefly luciferase served as an internal control to normalize for transfection efficiency.

PsiCHECK-TP53 clones with correct orientation of the porcine p53 cDNA were identified by restriction enzyme digestion with EcoRV and SacI and the plasmid DNA was sequenced with the primer psiCHECK Seq f to exclude mutations.

### 3.2.5  Dual-Luciferase assay to determine knockdown potential of shRNA and artificial miRNA constructs against porcine p53

The knockdown efficiency of the shRNA and artificial miRNA constructs was tested in a Dual-Luciferase assay. The RNAi test vector psiCHECK-TP53 and the shRNA expression plasmids pLenti-TP53sh312, pLenti-TP53sh787 and pLenti-TP53sh944 were cotransfected in HEK293 cells. The positive control construct pLenti-shLuc1 against *Renilla* luciferase and the negative control plasmid pLenti-shnc (see section 3.1.5) were also included in the Dual-Luciferase assay. After 48 h, cell lysates were used to determine the relative light units (RLU) generated by *Renilla* luciferase. The results of the assay are presented in Figure 65. The construct TP53sh312 showed a knockdown of 74%, TP53sh787 of 0%, TP53sh944 of 47% and shLuc1 of 63% compared to the negative control shnc.

The assay was also performed with the artificial miRNA constructs directed against porcine TP53. The plasmids pCMV-TP53mi304, pCMV-TP53mi785 and pCMV-TP53mi943 were cotransfected with psiCHECK-TP53. The newly generated positive control plasmid against *Renilla* luciferase (pCMV-miLuc2) and the negative control miRNA plasmid pCMV-minc (see section 3.1.7) were also used. The construct TP53mi304 resulted in a knockdown of 77% and TP53mi943 of 51% compared to the negative control. TP53mi785 did not show a significant knockdown of the *Renilla* luciferase-p53 fusion mRNA (see Figure 65). The positive control pCMV-miLuc2 directed against the *Renilla* luciferase mRNA resulted in a downregulation of about 31%. This knockdown was comparable to the previously used positive control plasmid against *Renilla* luciferase, pCMV-miLuc1, and therefore the new plasmid pCMV-miLuc2 showed no improvement of silencing efficiency.
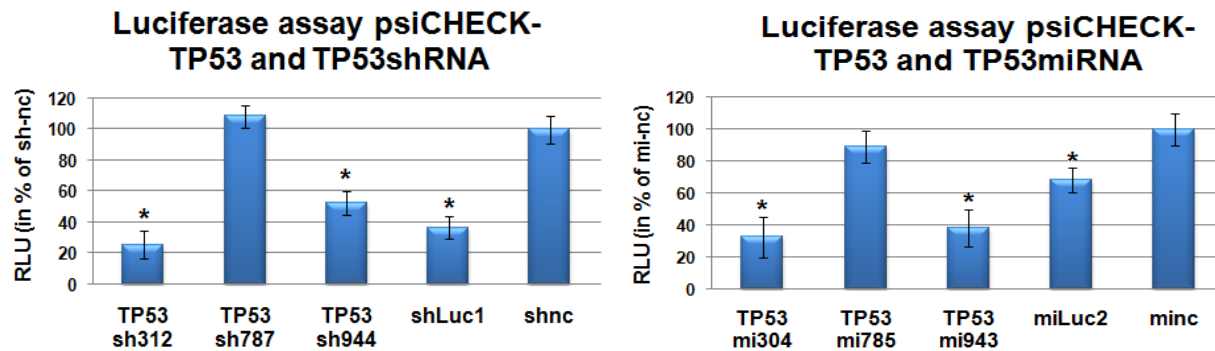
**Figure 65: Dual-Luciferase assay with psiCHECK-TP53 and lentiviral shRNA or CMV promoter directed artificial miRNA constructs against porcine p53.** Relative light units (RLU) are presented. The negative controls shnc or minc are set to 100%. Knockdown of *Renilla* is shown in comparison with the respective negative control. Measurements were performed in triplicates. (*: $p<0.05$; n=3). Left: Dual-Luciferase assay with shRNA constructs. The constructs TP53sh312, TP53sh944 and shLuc1 led to a significant knockdown of the relative *Renilla* luciferase expression. Right: Dual-Luciferase assay with miRNA constructs. The constructs TP53mi304, TP53mi943 and miLuc2 resulted in a significant knockdown of the relative *Renilla* luciferase expression.

The Dual-Luciferase assay with the U6 promoter directed shRNA against porcine p53 showed two functional shRNA sequences, TP53sh312 and TP53sh944, with a downregulation of the relative *Renilla* luciferase expression by 74% and 47% respectively. Two of the artificial miRNAs under the control of the CMV promoter achieved a significant knockdown of 77% (TP53mi304) and 51% (TP53mi943) compared to the negative control pCMV-minc.

### 3.2.6  Development of a western blot to detect porcine p53

For further experiments it was important to establish a western blot protocol for the detection of porcine p53 protein to validate the downregulation of p53. Four commercially available antibodies against murine or human p53 have been described to also detect porcine p53. Two of them (CM-1 and Ab-3) had only been tested to detect recombinant porcine p53 (Burr *et al.,* 1999) while the antibodies Pab240 and DO-1 had been used to detect endogenous p53 in porcine cells (Rodriguez-Campos *et al.,* 2001; Oh *et al.,* 2007; Qiu *et al.,* 2008b). Therefore the antibodies Pab240 and DO-1 were chosen to establish western blot conditions allowing the detection of porcine p53. The monoclonal antibody Pab240 recognizes a linear epitope in the DNA binding domain between amino acid 156 and 214 of murine p53 and the monoclonal antibody DO-1 is directed against a linear epitope between amino acid 20 and 25 at the N-terminus of human p53 (Gannon *et al.,* 1990; Stephen *et al.,* 1995).

The following experiments to assess the knockdown ability of the RNAi sequences against porcine p53 were either performed by cotransfection of a p53 expression plasmid and RNAi plasmids into a p53-deficient murine pancreatic cancer cell line (MPC) or by knockdown of endogenous p53 in porcine mesenchymal stem cells. Therefore it was important to evaluate whether the antibodies Pab240 and DO-1 could recognize recombinant p53 produced in the murine cell line MPC and endogenous porcine p53.

For this purpose a porcine p53 expression vector was constructed. The porcine p53 cDNA from pGEM-TP53 (see section 3.2.4) was inserted into EcoRI restriction site of the mammalian expression plasmid pcDNA3 to obtain the vector pcDNA-p53 (see Figure 66). This vector expresses the porcine p53 cDNA under the control of a CMV promoter and contains a neomycin resistance cassette for G418 selection in mammalian cells.
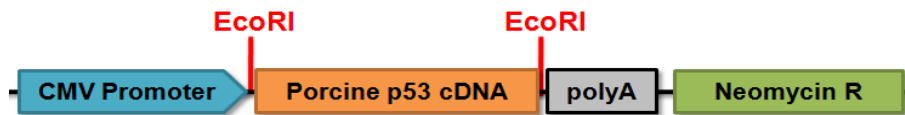


**Figure 66: Schematic diagram of the porcine p53 expression vector pcDNA-p53.** The porcine p53 cDNA was subcloned from pGEM-TP53 cDNA into the mammalian expression plasmid pcDNA3 after restriction digest with EcoRI. It is expressed from a CMV promoter (blue). The bovine growth hormone polyadenylation signal is shown in grey. The plasmid contains a neomycin resistance cassette (green) for selection with G418 in mammalian cells.

The p53 expression vector was transfected into the p53-deficient murine pancreatic cancer cell line MPC. After 48 h, cell lysates were obtained and analyzed for porcine p53 expression by western blot using the antibodies Pab240 and DO-1. In addition to the untransfected and the transfected MPC cell line, a human cell line with expression of stabilized mutant (R175H) p53, MiaPaca2, was used as positive control. The western blots are displayed in Figure 67.
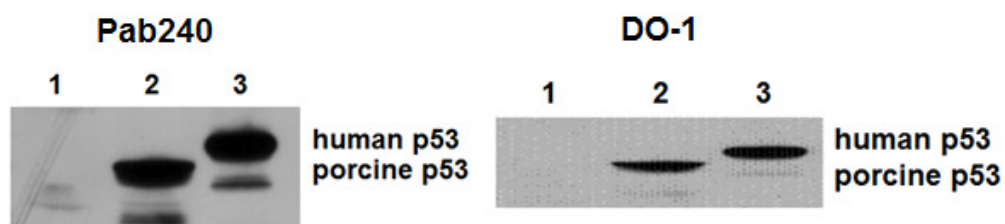


**Figure 67: Western blot analysis of p53 expression.** Western blot analysis for the detection of recombinant porcine p53 (around 46 kDa) and mutant (R175H) human p53 (around 53 kDa) protein. 20 µg total protein per lane were separated on a 12% reducing Tris/Glycine gel before transfer onto PVDF membrane. 1: p53-deficient murine pancreatic cancer cell line (MPC). 2: MPC cells transfected with pcDNA-p53. 3: Human MiaPaca2 cell line with mutant p53. Left: Porcine and human p53 were detected using the monoclonal antibody Pab240. Right: Porcine and human p53 were detected using the monoclonal antibody DO-1. Porcine p53 showed a lower molecular weight compared to human p53 protein.

Porcine p53 was detected using the antibodies Pab240 and DO-1 in the transiently transfected MPC cells and human p53 in the MiaPaca2 cell line. Porcine p53 showed a lower molecular weight compared to human p53. This is in accordance with the literature, porcine p53 has been reported to have an apparent molecular weight between 46 and 50 kDa in contrast to human p53 at 53 kDa (Burr *et al.,* 1999; Qiu *et al.,* 2008b). This molecular weight difference can not only be due to the shorter amino acid sequence of porcine compared to human p53 (386 and 393 amino acids respectively) as this would account for a size difference of only 0.77 kDa (Rettenberger *et al.,* 1993; Shu *et al.,* 2007). It might therefore reflect variations of posttranslational modifications between the two species. However, no information regarding these modifications of porcine p53 is available.

For detection of endogenous p53 in porcine primary mesenchymal stem cells it was necessary to evaluate whether both antibodies were suitable. To this end, cell lysates of

bone marrow mesenchymal stem cells (BMMSC) or adipose tissue derived mesenchymal stem cells (ADMSC) were prepared. The antibody Pab240 led to the detection of many unspecific bands in BMMSCs and ADMSCs, rendering the identification of porcine p53 impossible (data not shown). The antibody DO-1 was tested with BMMSCs cell lysates and specifically detected porcine p53 (see Figure 68).
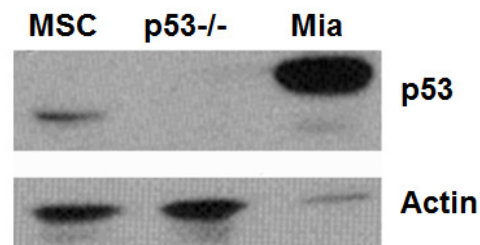


**Figure 68: Western blot analysis of endogenous p53 in porcine mesenchymal stem cell (BMMSC) lysates with the antibody DO-1.** 50 µg of total protein from bone marrow derived mesenchymal stem cells (MSC) were separated on a 12% reducing Tris/Glycine gel before transfer onto PVDF membrane. Porcine p53 (around 46 kDa) in MSCs was detected with the monoclonal antibody DO-1. As a negative control, the p53-deficient MPC cell line and as a positive control, 20 µg protein from human MiaPaca2 (Mia) cells with mutant p53 were used. Actin (42 kDa) served as loading control.

These results showed that both monoclonal antibodies, Pab240 and DO-1, were able to detect recombinant porcine p53 expressed in murine cell line lysates. However, despite previous reports with primary porcine cells (Rodriguez-Campos *et al.,* 2001), the antibody Pab240 was not suitable for the detection of endogenous porcine p53 cell lysates. In accordance with the publication of Qiu *et al.* (2008b), the antibody DO-1 specifically detected endogenous and recombinant porcine p53 and was therefore used for the following experiments.

### 3.2.7 Validation of RNAi constructs in a p53-deficient cell line

The knockdown potential of the p53 shRNA and miRNA constructs was further evaluated by cotransfecting a p53 expression vector and the RNAi plasmids into a cell line followed by western blot analysis. This test was performed to validate the knockdown ability of the constructs as determined by the Dual-Luciferase assay and to reduce the number of RNAi constructs to be tested in porcine primary cells. The murine pancreatic cancer cell line MPC was chosen because it does not express p53 and handling and transfection of this cell line is simple compared to porcine primary cells. A similar assay has also been used by Hemann *et al.* (2003) to evaluate the silencing effects of shRNAs against murine p53.

The porcine p53 expression plasmid pcDNA-p53 (see section 3.2.6) was cotransfected with the shRNA or artificial miRNA plasmids into the p53-deficient MPC cell line at a molar ratio of 1:2 or 1:3 respectively. The plasmids pLenti-shnc and pCMV-minc were included as negative controls. Cell lysates were analyzed 48 h after transfection by western blot to identify downregulation of porcine p53. Glyceraldehyd-3-phosphate dehydrogenase (GAPDH) was

used as a loading control and the western blot is shown in Figure 69. The construct pLenti-TP53sh312 showed an estimated downregulation of porcine p53 by about 70% whereas the other two shRNAs did not downregulate p53. In contrast to these results, all three artificial miRNA constructs against porcine p53 (TP53mi304, TP53mi785 and TP53mi943) led to a knockdown estimated as between 60 to 80%.
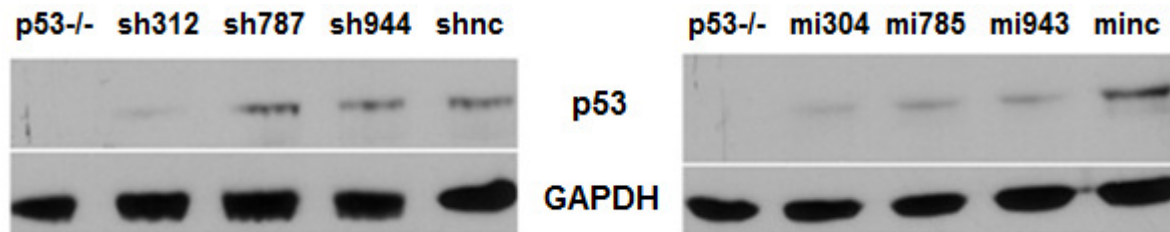


**Figure 69: Western blot analysis of the experiment with shRNA and artificial miRNA plasmids in the MPC cell line.** 20 µg of total protein were separated on a 12% reducing Tris/Glycine gel followed by western blotting onto PVDF membrane. Detection of porcine p53 was performed with the antibody DO-1 and detection of GAPDH was used as a loading control. The porcine p53 expression plasmid pcDNA-p53 and the pLenti-TP53shRNA plasmids (TP53sh312, TP53sh787, TP53sh944) and the negative control shRNA plasmid (shnc) or the pCMV-TP53miRNA plasmids (TP53mi304, TP53mi785, TP53mi943) and the negative control miRNA plasmid (minc) were transfected into the MPC cell line. One of the shRNAs (TP53sh312) and all of the miRNA plasmids (TP53mi304, TP53mi785, TP53mi943) showed an estimated downregulation of porcine p53 by 60 to 80% compared to the respective negative control plasmids. In the untransfected MPC cell line (p53-/-) no expression of p53 was detectable.

The results of the assay in MPC cells differed from those obtained in the Dual-Luciferase assay. In the Dual-Luciferase experiment, two shRNAs (TP53sh312, TP53sh943) and two artificial miRNAs (TP53mi304, TP53mi943) against porcine p53 resulted in a significant downregulation whereas in the experiment in MPC cells only one shRNA (TP53sh312) and all three miRNAs (TP53mi304, TP53mi785, TP53mi943) demonstrated knockdown of between 60 to 80%.

### 3.2.8 Artificial miRNA vectors for knockdown in porcine primary adipose tissue derived mesenchymal stem cells

Before creating stable porcine primary cell pools to determine the knockdown efficiency of the RNAi constructs in these cells, the artificial miRNA expression vector pCMV-circ was modified. The viral CMV promoter of the pCMV-circ plasmid was exchanged with a cellular PGK promoter. This step was necessary, because it has been reported that the CMV promoter is not suitable for stable gene expression from plasmid DNA in mesenchymal stem cells and leads to mosaic gene expression in transgenic pigs (Byun *et al.,* 2005; Bosch *et al.,* 2006; Deppenmeier *et al.,* 2006). The functionality of the PGK promoter for stable transgene expression in porcine MSCs had previously been tested in our laboratory (Landmann, unpublished results). In addition to the promoter exchange, a cassette containing a CAGGS promoter (chicken beta-actin promoter, CMV IE enhancer and beta-actin intron, Niwa *et al.,* 1991) directing the expression of EGFP was added to visualize transfected cells. A diagram of the resulting plasmid, pPGK-miRNA, is presented in Figure 70.
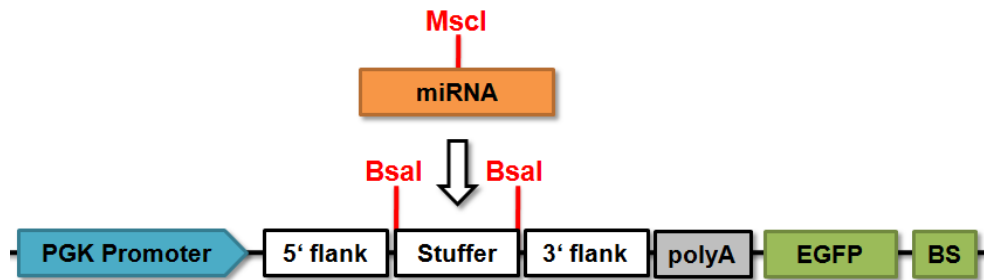
**Figure 70: Schematic diagram of the artificial miRNA vector pPGK-miRNA.** The CMV promoter of pCMV-circ was removed by restriction digest. The PGK promoter (blue) was isolated from pPGK-EGFP1 and inserted into the linearized vector pCMV-circ. In addition, the CAGGS promoter EGFP cassette was excised from pCAGGS-EGFP and the cassette (CAGGS promoter, EGFP, rabbit beta-globin polyadenylation signal; green) inserted into the FspI site of pCMV-circ to obtain the artificial miRNA vector pPGK-miRNA. This vector contains an artificial miRNA expression cassette containing flanking regions of murine miR-155 (white), a stuffer sequence for propagating the plasmid in bacteria (white) under the control of the PGK promoter.

The functionality of the EGFP expression cassette was tested in HEK293 cells. Strong expression of EGFP was detectable at 48 h after transient transfection (see Figure 71).
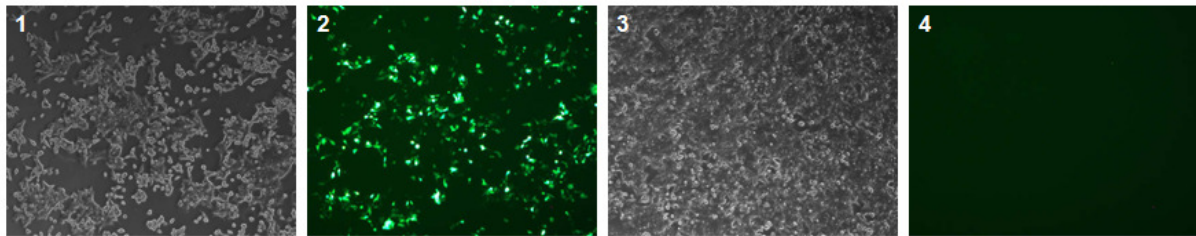


**Figure 71: EGFP expression from pPGK-miRNA in HEK293 cells.** HEK293 cells were transiently transfected with pPGK-miRNA. 48 h after transfection, strong EGFP expression was detectable after excitation at 488 nm (2). The corresponding phase contrast image is shown in 1. Untransfected HEK293 cells (3: phase contrast, 4: fluorescence image) showed no EGFP expression.

The miRNA sequences TP53mi304, TP53mi785, TP53mi943 against porcine p53, the control sequence miLuc2 against *Renilla* luciferase and the negative control sequence minc were inserted into the BsaI sites of pPGK-miRNA to generate pPGK-TP53mi304, pPGK-TP53mi785, pPGK-TP53mi943, pPGK-miLuc2 and pPGK-minc. The Dual-Luciferase assay was performed with these constructs to ensure functionality of the miRNA expression cassette under the control of the PGK promoter (see Figure 72).
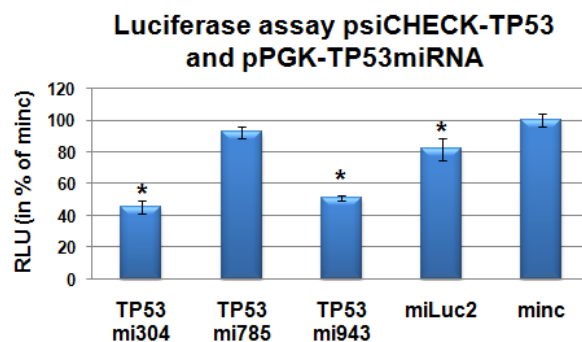


**Figure 72: Dual-Luciferase assay with pPGK-TP53miRNA and psiCHECK-TP53.** The modified artificial miRNA constructs with the PGK promoter and the EGFP expression cassette were tested in the Dual-Luciferase assay. TP53mi304, TP53mi943 and miLuc2 led to a significant knockdown of the relative *Renilla* luciferase expression compared to the negative control plasmid (minc). ($p < 0.05$; n=3)

The construct pPGK-TP53mi304 resulted in a significant downregulation of relative *Renilla* luciferase expression by 54%, pPGK-TP53mi943 by 49% and the miLuc2 by 18% compared to the negative control minc. Cotransfection of the construct pPGK-TP53mi785 did not result in a significant knockdown.

These results were compared to the downregulation achieved with the CMV promoter containing TP53miRNA constructs (see section 3.2.5). The construct TP53mi943 showed comparable knockdown efficiencies independent of the promoter (CMV: 51%; PGK: 49%). whereas the CMV promoter directed constructs TP53mi304 and miLuc2 were more efficient than the same miRNAs expressed from the PGK promoter (TP53mi304: 77% and 54%; miLuc2: 31% and 18% respectively). The constructs carrying TP53mi785 with the two different promoters did not achieve a significant downregulation of the relative *Renilla* luciferase activity. It is however not possible to draw firm conclusions regarding the observed differences because in contrast to the other luciferase experiments, the assay with PGK promoter directed artificial miRNAs was only performed once.

### 3.2.9 Generation of stable pools of primary porcine adipose tissue derived mesenchymal stem cells with RNAi constructs against porcine p53

The RNAi constructs, which had been evaluated in the Dual-Luciferase assay and in MPC cells, were then also tested in primary mesenchymal stem cells derived from porcine adipose tissue (ADMSCs). These previous assays had either been performed in human or murine cells and with p53 fusion mRNAs or expression constructs and the results varied between the assays. Therefore all six RNAi constructs against porcine p53 were analyzed in porcine primary cells to assess knockdown in the porcine cell environment with endogenous p53 mRNA. ADMSCs were chosen as it had been shown before that this cell type supports fetal development after somatic cell nuclear transfer (see section 3.1.16.2) and would therefore be suitable to directly generate transgenic pigs with p53 knockdown after *in vitro* analysis.

ADMSCs were transfected by electroporation with the lentiviral shRNA and artificial miRNA constructs under the control of the PGK promoter against p53 and the respective negative control plasmids. Stable pools of ADMSC transfectants were established by selection with Blasticidin for 10 days. The reason to establish cell pools instead of single colonies was to reduce the time in culture, which is important for the generation of transgenic pigs with p53 knockdown by somatic cell nuclear transfer. Moreover, cells in cell pools provide varying expression levels of introduced constructs which better reflect the overall knockdown ability independent of the integration site in individual clones. After the selection with Blasticidin, all cells expressed the EGFP reporter gene included on the RNAi plasmids albeit with different intensities depending on the construct type used. In accordance with the literature, the CAGGS promoter on the artificial miRNA constructs resulted in higher expression of EGFP

than the PGK promoter directed EGFP on the lentiviral shRNA plasmids. This was most likely due to the strong CAGGS promoter (Niwa *et al.,* 1991).

The porcine ADMSC pools were tested for downregulation of p53 protein by western blot analysis at passage 11 with the p53 antibody DO-1. Two of three shRNA constructs (TP53sh787, TP53sh944) and two of three artificial miRNA constructs (TP53mi785, TP53mi943) showed silencing of p53 estimated between 50 and 85% in the stable primary ADMSC pools. The negative control RNAi sequences shnc and miLuc2 did not downregulate p53 expression. The p53-deficient MPC cell line was used as a control and showed no p53 expression whereas MPC cells transfected with the porcine p53 expression construct pcDNA-p53 were used as a positive control and p53 was detectable. The result of the western blot is shown in Figure 73.
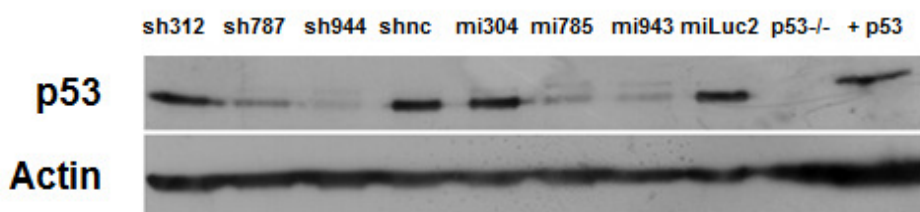


**Figure 73: Western blot analysis of stable primary porcine ADMSC pools with RNAi constructs.** The knockdown of endogenous p53 protein (around 46 kDa) in ADMSCs with RNAi constructs was evaluated by western blot analysis. The shRNA constructs TP53sh787 (sh787) and TP53sh944 (sh944) and the artificial miRNA constructs TP53mi785 (mi785) and TP53mi943 (mi943) resulted in an estimated downregulation of porcine p53 by 50 to 85%. The constructs TP53sh312 (sh312) and TP53mi304 (mi304) did not downregulate p53 expression. All results were compared to the negative control shRNA (shnc) or miRNA (miLuc2). As an additional control, protein extract from the p53-deficient MPC cell line (p53-/-) and as a positive control MPC transfected with the porcine p53 expression construct pcDNA-p53 (+p53) were evaluated for p53 expression. Actin (around 42 kDa) served as loading control.

These results indicate knockdown potential of two shRNA and two artificial miRNA constructs in primary porcine ADMSCs. The sequences TP53sh787, TP53sh944, TP53mi785 and TP53mi943 were effective in downregulating endogenous p53 in the porcine cells. The effective shRNA and miRNA sequences were directed against overlapping target sequence positions of the porcine p53 mRNA.

### 3.2.10 Evaluation of p53 knockdown with the reporter plasmid pRGC-Luc

Further evaluation of p53 knockdown in ADMSCs was performed with a p53 reporter plasmid. For this purpose the p53 response reporter vector pRGC-Luc was used. This plasmid contains a firefly luciferase cDNA under the control of a minimal CMV promoter fused to 17 repeats of the p53 consensus DNA recognition sequence from the human ribosomal gene cluster (RGC). This sequence represents a binding site for p53 (Kern *et al.,* 1991). Binding of p53 to the RGC sequence induces the expression of firefly luciferase. When TP53 is mutated or absent, firefly luciferase expression is not initiated. A schematic overview over the system is given in Figure 74.
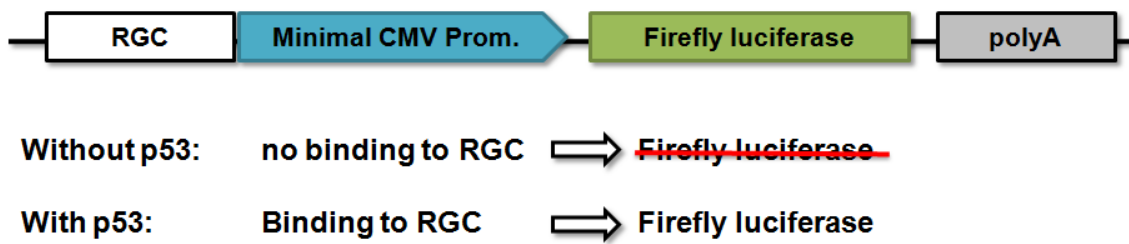
**Figure 74: Schematic overview of the p53 reporter assay with plasmid pRGC-Luc.** The reporter plasmid pRGC-Luc consists of a minimal CMV promoter (blue) under the control of 17 repeats of the p53 consensus DNA recognition sequence of the human ribosomal gene cluster (RGC; white) and a polyadenylation signal (grey). The composite promoter directs the expression of firefly luciferase (green) after binding of p53 to the RGC sequence. In case of a downregulation or lack of p53, the firefly luciferase expression is initiated at a lower level or completely abolished.

The RGC sequence is of human origin and it was therefore necessary to determine whether porcine p53 could bind and transcriptionally activate firefly luciferase expression from the plasmid pRGC-Luc. To this end, the murine p53-deficient MPC cell line was transfected with pcDNA-p53 and pRGC-Luc. The plasmid pRenilla was cotransfected as an internal control to normalize for transfection efficiency. This plasmid contains a *Renilla* luciferase cDNA under the control of a SV40 promoter and was generated by eliminating the firefly luciferase expression cassette from the RNAi test vector psiCHECK2. A schematic diagram of the vector pRenilla is presented in Figure 75.
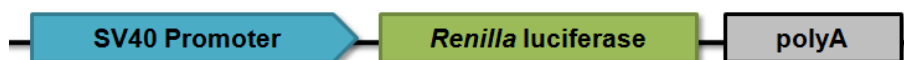


**Figure 75: Schematic diagram of the plasmid pRenilla.** The plasmid pRenilla was created by removing the HSV-TK promoter and the firefly luciferase coding region of psiCHECK2 by restriction enzyme digestion followed by religation of the plasmid.

Cell lysates were analyzed for luciferase expression 48 h after transfection. A control experiment was performed in parallel with pRGC-Luc, pRenilla and pcDNA-BLG. The plasmid pcDNA-BLG directs expression of porcine Beta-Lactoglobulin, a protein which cannot bind to the p53 recognition sequence and therefore served as a negative control. The results of the luciferase assay are illustrated in Figure 76 A. Porcine p53 expression in the p53-deficient MPC cell line led to strong transcriptional activation of the firefly luciferase on pRGC-Luc. This confirmed that porcine p53 can bind to the RGC consensus DNA sequence of the plasmid.

The firefly luciferase induction by endogenous porcine p53 was also tested. For this experiment, pRGC-Luc and pRenilla were transfected either into primary porcine ADMSCs or into the murine p53-deficient pancreatic cancer cell line. Luciferase expression was measured after 48 h. Endogenous porcine p53 led to an activation of firefly luciferase expression compared to the p53-deficient cell line (see Figure 76 B).
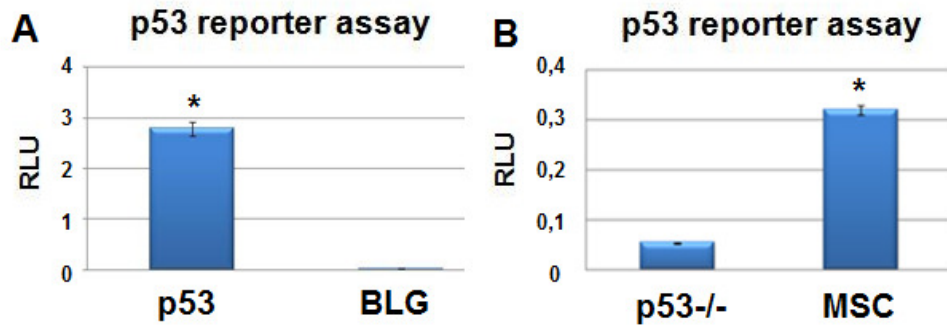
**Figure 76: p53 reporter assay with pRGC-Luc in MPC cells and in porcine ADMSCs.** A: The plasmids pRGC-Luc, pRenilla and pcDNA-p53 or pcDNA-BLG were cotransfected into the murine p53-deficient cell line MPC. 48 h after transfection, expression of *Renilla* and firefly luciferase were determined and relative light units calculated (RLU). Porcine p53 expression induced the transcription of the firefly luciferase on pRGC-Luc. B: The plasmids pRGC-Luc and pRenilla were cotransfected in the MPC cell line and in porcine ADMSCs. 48 h after transfection, expression of *Renilla* and firefly luciferase was determined and relative light units calculated (RLU). Endogenous porcine p53 induced transcription of the firefly luciferase of pRGC-Luc. (n=3; *: p < 0.05)

Because these preliminary tests confirmed the ability of porcine p53 to induce expression of firefly luciferase from pRGC-Luc, ADMSC cell pools that had shown the highest knockdown of p53 by western blot analysis (pools containing TP53mi785, TP53mi943, TP53sh944) were then tested with the reporter plasmid (see Figure 77).
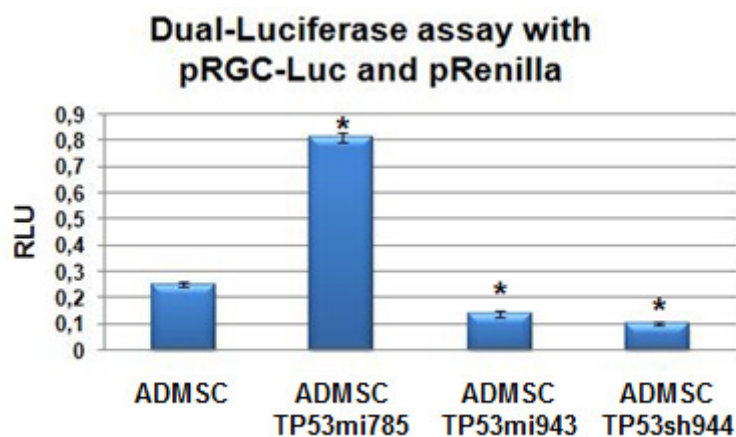


**Figure 77: Dual-Luciferase assay with pRGC-Luc and pRenilla in stable ADMSC pools with shRNA or miRNA constructs.** pRGC-Luc and pRenilla were cotransfected into stable porcine ADMSC pools containing the artificial miRNA constructs TP53mi785, TP53mi943 and the shRNA construct TP53sh944. The expression of firefly luciferase was normalized to the cotransfected *Renilla* luciferase. Compared to wildtype ADMSC, cells with TP53mi785 showed about 325% increased relative firefly luciferase expression. Cells with TP53mi943 and TP53sh944 showed a significantly lower level of relative firefly luciferase expression (45% and 60%). (p<0.05; n=3)

Cells containing the artificial miRNA construct TP53mi943 and the shRNA construct TP53sh944 showed a significantly lower level of relative firefly luciferase expression compared to wildtype ADMSC (45% and 60% downregulation). Unexpectedly, the ADMSCs containing TP53mi785 showed an approximately 325% increase of relative firefly luciferase expression compared to wildtype ADMSC cells. This result was surprising, but there is evidence that the p53 reporter assays do not always represent the actual p53 status in cells, since especially in the absence of wildtype p53, the p53 family members p63 and p73 can

also bind to the RCG recognition sequence and activate luciferase transcription (Wischhusen *et al.,* 2004).

## 3.2.11 Functional test of p53 knockdown in ADMSCs

A functional assay was performed to evaluate the silencing potential of shRNA and artificial miRNA constructs against porcine p53. In this assay, the resistance of ADMSC pools carrying RNAi constructs to the chemotherapeutic drug doxorubicin, also known as adriamycin, was tested. This assay is based on incubation of cells with doxorubicin followed by assessment of colony forming units after the treatment. Doxorubicin induces p53 activity leading to apoptosis in cells, although the exact mechanism is unclear (Dunkern *et al.,* 2003). Cells deficient in p53 or cells with p53 knockdown show higher resistance to the chemotherapeutic treatment with doxorubicin (Yamamoto *et al.,* 1999; Dunkern *et al.,* 2003; Dickins *et al.,* 2005; Stern *et al.,* 2008). A schematic overview of the doxorubicin response is depicted in Figure 78.
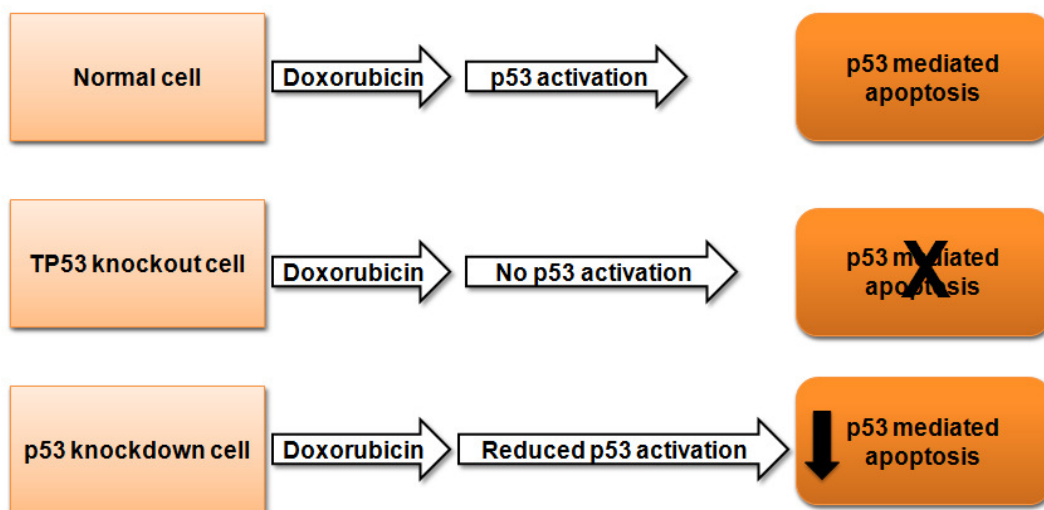


**Figure 78: Schematic overview of the response of different cells to doxorubicin.** In normal cells, doxorubicin causes activation of p53 and leads to p53 mediated apoptosis. In cell which lack p53 (e.g. cells with knockout or knockdown of p53), doxorubicin cannot activate p53 mediated apoptosis.

In a first experiment, the response of porcine ADMSCs to doxorubicin was assessed. Two different concentrations, 1 µg/ml and 2 µg/ml, doxorubicin were used to inhibit colony formation of wildtype ADMSC. As determined by this assay, 1 µg/ml doxorubicin completely inhibited colony formation (see Figure 79 A).

Once the conditions for this functional assay had been established, the ADMSC pools with highest knockdown efficiency in the western blot analysis as determined in section 3.2.9 (TP53mi785, TP53mi943 and TP53sh944) and wildtype cells were treated with doxorubicin. After 21 days, colony formation was visible in the cell pool with the artificial miRNA TP53mi785 (see Figure 79 B).
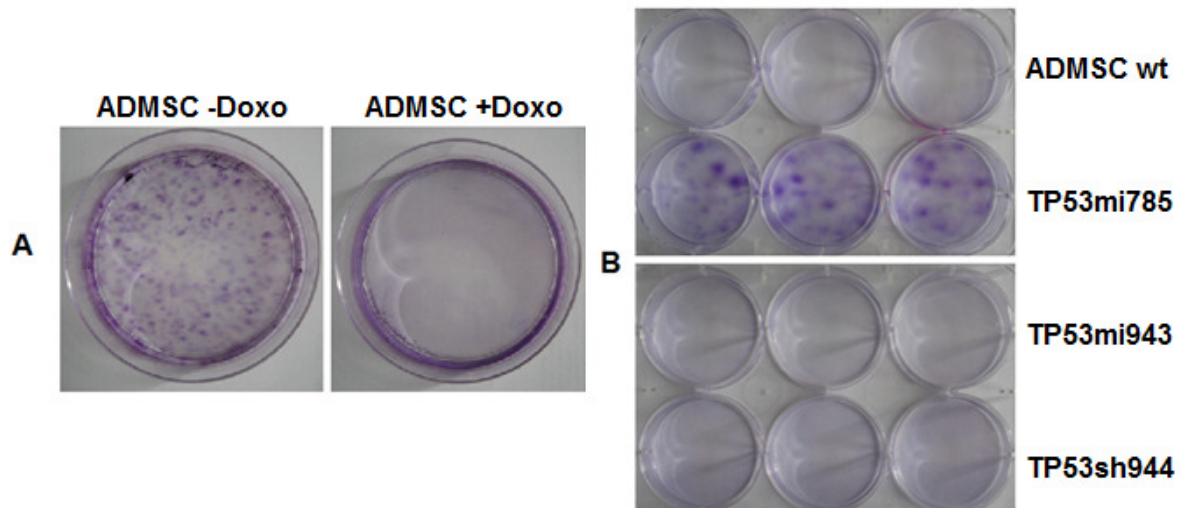
**Figure 79: Doxorubicin assay with wildtype and RNAi transgenic porcine ADMSCs.** A: Response of ADMSCs to doxorubicin (Doxo). Wildtype ADMSCs were incubated with or without 1 µg/ml doxorubicin for 24 h and cultured for 14 d. Untreated cells formed colonies whereas the treated cell did not proliferate. B: Doxorubicin assay in ADMSCs and ADMSC pools containing artificial miRNA or shRNA constructs. Stable porcine primary ADMSC cell pools with artificial miRNAs (TP53mi785, TP53mi943) or shRNAs (TP53sh944) against porcine p53 were treated with 1 µg/ml doxorubicin for 24 h and cultured for 21 d after the treatment. Cells containing the artificial miRNA construct TP53mi785 showed resistance to the chemotherapeutic drug.

These results indicate the RNAi constructs TP53mi943 and TP53sh944 were not efficient enough to confer resistance to doxorubicin although they significantly downregulated the expression of porcine p53 as shown by western blot analysis. In contrast, the ADMSC pool with the TP53mi785 construct resulted in colony formation, indicating that this pool contains cells with knockdown of p53 sufficient to survive the assay.

Therefore the artificial miRNA TP53mi785 might be the most promising sequence to generate a porcine cancer model since the sequence showed a functional reduction of porcine p53. Moreover, the doxorubicin assay might serve as a selection method to determine single cell clones with high knockdown efficiencies suitable for the generation of transgenic pigs with p53 downregulation by somatic cell nuclear transfer.

# 4    Discussion

Animals with modified genome sequences play an important role in studying the function of genes, development of therapeutic drugs, the generation of animal models for human diseases and to produce recombinant proteins. Up to now, most studies evaluating the function of genes and the generation of animal models for human diseases are performed in mice but pigs offer a valuable alternative because they are more similar to humans in size, development and physiology and are genetically closer to humans than mice (Wernersson *et al.,* 2005; Lunney, 2007).

Gene knockout by gene targeting is the method of choice to inactivate endogenous genes. This approach is based on the replacement of endogenous with exogenous DNA sequences by homologous recombination (Thomas *et al.,* 1986). Because no definitive ES cells are available for livestock species, the precise genetic modification has to be performed in somatic cells which show a lower frequency of homologous recombination compared to murine ES cells and a limited lifespan *in vitro* (Wang and Zhou, 2003). This leads to a large number of cell clones which have to be analyzed and many colonies cannot be expanded sufficiently. After *in vitro* manipulation, the cells are used for somatic cell nuclear transfer (Wilmut *et al.,* 1997) to generate gene-targeted animals, a technique which is difficult and only few research groups are able to produce viable offspring.

In the past years, an alternative to conventional gene knockout has emerged. This method, RNA Interference (RNAi), is initiated by double stranded RNA within cells and leads to sequence-specific downregulation of gene expression (Fire *et al.,* 1998). A stable, long-term downregulation of the expression of genes can be achieved by introducing RNAi expression constructs into cells or animals. The two most common types of RNAi constructs include short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs). ShRNAs can be used for knockdown of gene expression in the whole animal since they are expressed by ubiquitous RNA polymerase III promoters. Artificial miRNAs are derived from endogenous miRNAs and are generally controlled by RNA polymerase II promoters, allowing ubiquitous but more importantly also tissue-specific expression of artificial miRNAs, depending on the choice of promoter (Hasuwa *et al.,* 2002; Zamore, 2002; Paddison *et al.,* 2002; Yu *et al.,* 2002; Miyagishi and Taira, 2002; Paul *et al.,* 2002; Brummelkamp *et al.,* 2002; McManus *et al.,* 2002; Lee *et al.,* 2002; Xia *et al.,* 2006; Rao *et al.,* 2006).

It has been shown that the downregulation of gene expression by RNAi can generate phenotypes similar to loss-of-function mutations even without breeding the animals to homozygosity (Kunath *et al.,* 2003). Since RNAi mediated downregulation of genes can be achieved by addition of RNAi constructs to the genome of animals instead of replacing endogenous DNA sequences, the method offers a faster alternative to gene knockout by

gene targeting. Therefore the application of RNAi would be especially valuable in livestock species with longer generation times than mice and no ES cells, such as the pig or the cow. In contrast to gene knockout with promoter-trap vectors, the successful generation of RNAi transgenic animals is independent of the expression of the target gene because RNAi sequences can be randomly integrated into the cell genome. This greatly enhances the possibilities to study gene function and the generation of livestock animals with reduced gene expression.

The goal of the project was to establish RNA Interference technology in pigs for the downregulation of a ubiquitously transcribed gene, the tumor suppressor protein TP53, and a gene which is only transcribed in a specific organ at a specific time, Beta-Lactoglobulin (BLG) in the lactating mammary gland. This project tried to evaluate if RNAi can serve as an alternative to the precise genetic modification of endogenous genes in pigs. Moreover, the two most commonly used vector systems for RNAi, shRNAs and artificial miRNAs, were compared.

## 4.1 General aspects of RNAi sequence design

In general, RNAi sequences for the downregulation of genes are designed with available online programs. There are major differences between these programs in the algorithm used for the identification of functional RNAi sequences. Some programs select RNAi sequences based on empirical data, while others use rational RNAi sequence design (Ui-Tei *et al.,* 2004; Reynolds *et al.,* 2004). This *in silico* prediction is not always accurate and the retrieval of functional sequences is limited (Kumar *et al.,* 2003). Despite advances in this field, the factors determining the efficiency of RNAi sequences in living cells are still largely unknown. Therefore, screening of multiple sequences per target is an essential procedure.

RNAi sequences can have unwanted effects on the expression of other genes or on global gene expression, so-called specific and non-specific off-target effects. The specific effects are mediated by partial complementarity of the RNAi sequences (guide or passenger strand) with mRNAs other than the target mRNA. This results in unwanted silencing of these mRNAs (Jackson *et al.,* 2003). These effects have been shown with regions of nucleotide complementarity as small as 7 to 11 bases between the guide strand and mRNA (Jackson *et al.,* 2003; Lin *et al.,* 2005). To minimize these effects, it is essential to perform a BLAST search to exclude binding to other mRNAs. However, finding RNAi sequences with less than 7 nucleotides complementarity to mRNAs other than the target is very difficult and often no sequences matching these strict criteria can be identified. As a compromise, the threshold for specific sequences in this project was set at 15 nucleotides. RNAi sequences with more than 15 nucleotides complementarity to mRNAs other than porcine p53 or porcine BLG were excluded. However, the porcine genome is not yet fully sequenced and the RNAi sequences

could only be compared to the existing porcine database. Therefore, off-target effects of the chosen sequences cannot be excluded.

In addition, some RNAi sequences are known to activate the cellular interferon response leading to global reduction of protein expression within the cell rather than to sequence-specific gene silencing (Brigde *et al.,* 2003; Sledz *et al.,* 2003). This phenomenon is referred to as non-specific off-target effects. Two shRNA sequence motifs (5'-UGUGU-3' and 5'-GUCCUUCAA-3') have been found to activate the interferon pathway (Judge *et al.,* 2005; Hornung *et al.,* 2005). These two sequence motifs are not the only factors responsible for the induction of the interferon response but care was taken to avoid RNAi sequences containing those two motifs.

## 4.2  Sequence-specific gene silencing of porcine Beta-Lactoglobulin

### 4.2.1  Background

The first target gene was the porcine milk whey protein Beta-Lactoglobulin (BLG). This protein is one of the main components of whey in pigs and ruminants (Kontopidis *et al.,* 2004; Farrell *et al.,* 2004) but is not expressed in humans, rodents and lagomorphs (Monti *et al.,* 1989). The function of the porcine protein is unknown and no definitive function has been assigned to bovine BLG.

Functional analysis can be achieved by gene inactivation (for example by gene targeting) or by overexpression of the respective protein. However, gene knockout by gene targeting has not yet been achieved for BLG. This is mainly because the gene is expressed only during lactation in the mammary gland and not in cells commonly used for somatic cell nuclear transfer, limiting the use of promoter-trap vectors (Thomson *et al.,* 2003). RNA mediated gene silencing might therefore offer an alternative to inactivate BLG expression as RNAi transgenic animals can be generated independent of the transcription of the target gene.

The aim of this part of the project was to evaluate whether RNAi offers an alternative for the elimination of this whey protein instead of gene knockout. Moreover shRNA and artificial miRNAs expression vectors should be compared.

### 4.2.2  Evaluation of RNAi sequences with a Dual-Luciferase assay

As discussed above, RNAi sequences are generally designed by computer algorithms which only give indications concerning the knockdown obtained in living cells (Kumar *et al.,* 2003). Therefore RNAi sequences have to be evaluated for their knockdown potential *in vitro*. Six shRNA sequences against porcine BLG were designed and their knockdown efficiency determined in a Dual-Luciferase assay in HEK293 cells. Of the 6 sequences, four showed a significant knockdown in this test. The two best candidate sequences, BLGsh334 and

BLGsh489 with a knockdown of 74% and 55% respectively, were inserted into the lentiviral shRNA plasmid pLenti-shRNA and showed comparable knockdown efficiencies in the Dual-Luciferase assay.

Based on these two shRNAs, two artificial miRNAs were designed to target similar sequence regions of porcine BLG. They were then also assessed in the Dual-Luciferase assay and the artificial miRNA construct BLGmi490 resulted in a comparable knockdown to the shRNA (60% and 55%). This was not true for BLGsh334 and BLGmi334, the shRNA was more effective in the downregulation of the *Renilla*-BLG fusion mRNA than the artificial miRNA construct (74% and 30%). Interestingly, the same was observed for the positive control sequences shLuc1 and miLuc1 against the *Renilla* luciferase.

The finding that shRNA constructs can result in a higher level of downregulation than artificial miRNAs in HEK293 cells has been shown before and this was found to be related to a higher expression level of the shRNA compared to the artificial miRNAs (Boudreau *et al.,* 2008). However, the higher knockdown ability is not a general property of shRNAs, artificial miRNA constructs have also been shown to be highly effective (Zeng *et al.,* 2002, Xia *et al.,* 2006).

As BLGsh489 and BLGmi490 were equally effective, this might indicate a higher knockdown potential of this target sequence compared to BLGsh334 and BLGmi334 or shLuc1 and miLuc1 independent of the construct used.

### 4.2.3 Porcine mammary epithelial cells as *in vitro* test system for BLG knockdown

After the RNAi constructs against BLG were tested in the Dual-Luciferase assay in HEK293 cells, the intention was to evaluate their efficiency in a cell culture system closely resembling the mammary gland. Two groups had reported the successful culture of porcine mammary epithelial cells (PMEC) and their functional differentiation (Kumura *et al.,* 2001; Sun *et al.,* 2005).

This led to an attempt to establish an *in vitro* system for RNAi mediated downregulation of endogenous porcine BLG. For this purpose, primary porcine mammary epithelial cells were isolated according to the method described by Sun *et al.* (2005) and culture conditions for PMEC were investigated. In the culture medium used by Sun *et al.* (2005), termed MEC medium, cells showed epithelial morphology, reached passage 8 after 56 d in culture but could not be cultured further. This was in contrast to the description of Sun *et al.* (2005), who reported 15 to 20 passages before the cells reached senescence.

Several reports have indicated that milk protein expression in primary MEC is possible *in vitro* if the *in vivo* organization of mammary cells can be imitated (Emerman and Pitelka,

1977; Li *et al.,* 1987; Barcellos-Hoff *et al.,* 1989). To mimic the *in vivo* organization, the cells can be cultured on floating collagen gels or on/in extracellular basement matrix such as Matrigel, a soluble basement membrane extract from Engelbrecht Holm-Swarm sarcoma mouse model (reviewed in LeBleu *et al.,* 2007; Kleinman and Martin, 2005). Matrigel has been used by many groups for the functional differentiation of primary MEC and MEC cells lines of different origin (for example: Medina *et al.,* 1987; Li *et al.,* 1987; Barcellos-Hoff *et al.,* 1989; Ip and Darcy, 1996; Talhouk *et al.,* 1998; Debnath *et al.,* 2003).

3D culture on Matrigel for the induction of milk protein expression in PMEC was used for this project. The PMEC cells from non-lactating pigs were cultured on top of Matrigel with prolactin supplementation. They formed alveolar-like 3D structures and at higher cell density duct-like structures but did not express porcine milk genes as determined by RT-PCR analysis. Moreover, serum-free conditions were examined to exclude effects of the serum supplementation on the functional differentiation of PMEC, but these conditions did not result in milk gene expression either.

The first series of experiments was performed with primary MEC from non-lactating sows as described by Kumura *et al.* (2001), which in our hands could not be induced to express milk genes. Because Sun *et al.* (2005) had isolated functional PMEC from lactating sows, these cells were also isolated for this project. They were cultured in MEC medium and showed epithelial morphology. At high density, these cells formed spontaneous hollow 3D dome structures, indicating a high differentiation ability of the PMECs (McGrath, 1975; Pantschenko *et al.,* 2000). Culturing these PMECs on top of or embedded in Matrigel in the presence and absence of prolactin resulted in the formation of alveolar-like structures but again no milk gene expression. MEC from other species have been shown to express milk proteins for limited time *in vitro* (Baumrucker *et al.,* 1988; Talhouk *et al.,* 1993; Pantschenko *et al.,* 2000; German and Barash, 2002). However, not all established primary cultures showed this ability. Ovine MEC isolated by Düchler *et al.* (1998) formed domes in postconfluent monolayers but the expression of BLG was not detectable.

In contrast to published work concerning porcine mammary gland epithelial cells (Kumura *et al.,* 2001; Sun *et al.,* 2005), none of the conditions used in this project led to the expression of milk genes. It has to be taken into consideration, that Sun *et al.* (2005) are the only group that have reported long-term culture of PMEC. Cells were requested from the authors but the appeal remained unanswered. Kumura *et al.* (2001) succeeded in the induction of milk protein expression but did not show long term culture of PMEC. This, together with the fact that only two groups have reported the culture of functional primary PMEC supports the view, that the culture and differentiation of these cells is not easily achieved and it remains unclear why the isolated PMECs did not express milk proteins.

### 4.2.4  Stable knockdown of BLG in cell lines

Due to the lack of milk protein expression in PMEC, cell lines stably expressing porcine BLG were generated. The clonal cell lines CB1 and HpB9, derived from CHO-K1 and HEK293 respectively, produced porcine BLG as determined by RT-PCR and western blot analysis. Stable pools with the RNAi constructs of these clonal cell lines were analyzed by western blot to evaluate the knockdown of porcine BLG. Surprisingly, the most effective construct from the Dual-Luciferase assay, BLGsh334 (74% downregulation), had no effect on the expression level of porcine BLG in CB1 and HpB9. The same was true for the artificial miRNA construct BLGmi334. The differences observed between the Dual-Luciferase assay and the knockdown in the stable cells pools might be related to the secondary structure of the target mRNA. The *Renilla* luciferase-BLG fusion mRNA might show higher accessibility for the RNAi sequences BLGsh334 and BLGmi334 compared to the BLG mRNA itself (Bohula *et al.,* 2003; Vickers *et al.,* 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Overhoff *et al.,* 2005). To test this in future work, cell lines stably expressing the *Renilla*-BLG fusion mRNA would have to be generated and transfected with the RNAi constructs. The influence of the target secondary structure will be discussed in more detail for the second target gene, porcine p53.

The constructs BLGsh489 and BLGmi490, with a silencing efficiency of 55% and 60% in the Dual-Luciferase assay, resulted in a downregulation of BLG. However, this effect was cell line specific. The RNA polymerase III promoter directed shRNA construct led to a downregulation of BLG by 67% in HpB9 whereas the CMV promoter directed artificial miRNA resulted in 74% knockdown in CB1. These results were surprising, because cell type specific functionality of shRNAs and artificial miRNAs has not been reported. Both construct types have previously been shown to result in gene knockdown in HEK293 and CHO-K1 cells. The use of shRNAs for downregulation in CHO-K1 cells has been described for a variety of mRNAs such as cytosolic sialidase, α1,6-fucosyltransferase, lactate dehydrogenase A subunit and dihydrofolate reductase (Wu, 2009b). This indicates that shRNA mediated downregulation of genes in CHO-K1 cells is feasible. The artificial miRNA vector used in this project, pcDNA6.2-GW/miR, has been shown capable of silencing the cyclin-dependent kinase inhibitor p21 in HEK293 cells (Idogawa *et al.,* 2009). In a direct comparison of different cell types a similar degree of downregulation of firefly luciferase by siRNAs was achieved in Hela, HEK293, CHO-K1 and colo205 cells, independent of the cell type (Ui-Tei *et al.,* 2004; Ui-Tei *et al.,* 2006). Furthermore, the silencing of TIP5, a component of the nucleolar remodeling complex, by artificial miRNAs in HEK293 and CHO-K1 cells resulted in a similar level of gene silencing (Santoro *et al.,* 2009).

A possible explanation for the differences in BLG knockdown in HpB9 might be that in HEK293 cells, shRNA constructs are often expressed at higher levels than artificial miRNA constructs, leading to increased knockdown efficiency by this construct type (Boudreau *et al.,* 2008). Since both construct types have previously been used in both cell types for gene silencing, the reason for the cell line specific activity of the constructs remains unclear without further investigation of the expression level. To test this in future experiments, the level of mature miRNAs and siRNAs in the respective cell pools will be determined by small RNA northern blot. The same experiment will be performed with CB1 cells containing the RNAi constructs to determine why the miRNA but not the shRNA resulted in a downregulation of BLG in these cells.

### 4.2.5  Generation of RNAi transgenic pigs

#### 4.2.5.1  Lentiviral transgenesis

The original plan was to produce pigs with RNAi constructs by lentiviral transduction of porcine zygotes according to Hofmann *et al.* (2003). However, the lentiviral shRNA vector pLenti-shRNA led to the production of insufficient lentiviral titers for this method (Lois *et al.,* 2002). The low titer of shRNA containing lentiviruses was most likely due to the shRNA hairpin within the construct which can result in Dicer mediated cleavage of the viral mRNA (Poluri and Sutton, 2008). Because of these results, the artificial miRNAs were not inserted into a lentiviral vector and an alternative approach, somatic cell nuclear transfer, was used to generate RNAi transgenic pigs with shRNA and artificial miRNA constructs.

#### 4.2.5.2  Donor cells for somatic cell nuclear transfer (SCNT)

In pigs, different cell types have been used as donor cells for SCNT. These include granulosa cells, de-differentiated adipocytes, adult fibroblasts and the most commonly used cell type, fetal fibroblasts (Polejaeva *et al.,* 2000; Tomii *et al.,* 2005; Tomii *et al.,* 2009; Brunetti *et al.,* 2008; Onishi *et al.,* 2000; Betthauser *et al.,* 2000; Hyun *et al.,* 2003). To evaluate whether a less differentiated cell type can increase the efficiency of SCNT, several groups have also employed porcine bone marrow derived mesenchymal stem cells (BMMSCs) for *in vitro* analysis. Faast *et al.* (2006) and Jin *et al.* (2007) compared SCNT embryos derived from BMMSCs and porcine fetal fibroblasts (poFFs). BMMSCs as nuclear donors led to a higher rate of blastocyst development compared to poFFs. Similar results were obtained by Kumar *et al.* (2007) and a comparison of the expression of key embryonic genes such as Oct4 and Nanog showed that BMMSC derived embryos were more similar to *in vivo* embryos than were poFF derived embryos (Kumar *et al.,* 2007). Porcine BMMSCs have also been successfully used to generate piglets by SCNT in our laboratory (Landmann, unpublished data) and in addition to our experiments, SCNT using this cell type from cattle resulted in healthy offspring (Kato *et al.,* 2004). These results demonstrate the suitability of

BMMSCs to generate live offspring after SCNT. This, together with the enhanced rate of blastocyst development *in vitro* compared to poFF and also the ease of isolation of BMMSCs from slaughterhouse material, led to the choice of BMMSCs as a donor cell type.

Besides from bone marrow, MSCs can also be isolated from adipose tissue. Previously a cell type derived from adipose tissue has been successfully used by Tomii *et al.* (2005; 2009) to clone pigs by SCNT. They isolated mature adipocytes from pigs and de-differentiated these cells into preadipocytes which were then used for SCNT. For the generation of RNAi transgenic pigs with BLG knockdown, a more undifferentiated cell type, adipose tissue derived mesenchymal stem cells (ADMSCs), was used and evaluated as donor cells for SCNT.

### 4.2.5.3  Comparison of porcine BMMSCs and ADMSCs

Positive effects of FGF-2 on the proliferation rate of bone marrow MSCs (BMMSCs) have been reported (Van den Bos *et al.,* 1997; Tsutsumi *et al.,* 2001; Bianchi *et al.,* 2003) and in our hands the addition of FGF-2 to the culture medium led to faster proliferation of porcine BMMSCs compared to cultures without FGF-2. Chiou *et al.* (2006) and Lee *et al.* (2009) reported enhanced proliferation rates of murine or human adipose tissue derived MSCs (ADMSCs) after supplementation with FGF-2 but no report concerning porcine ADMSCs had been published. Due to the results with porcine BMMSCs, the porcine ADMSCs were also cultured in the presence of FGF-2 which led to fast cell proliferation.

The porcine BMMSCs as well as ADMSCs showed differentiation potential towards osteoblasts, chondroblasts and adipocytes. The ability to differentiate into the three mesenchymal lineages together with the plastic adherence of the cells fulfilled two of the three criteria for the characterization of mesenchymal stem cells (Dominici *et al.,* 2006). The third criterion, expression of cell surfaces markers, was not examined because of the lack of suitable antibodies.

Although BMMSCs and ADMSCs isolated from the same female pig differentiated into osteoblasts, chondroblasts and adipocytes, their differentiation potential varied. While BMMSCs showed extensive differentiation into all three cell types as shown previously by Ringe *et al.* (2002), ADMSCs differentiated only marginally towards osteoblasts and favorably into adipocytes. This finding differed to published descriptions of porcine ADMSCs as Qu *et al.* (2007) and Wang *et al.* (2008) showed that porcine ADMSCs have a strong potential to differentiate towards osteoblasts. However, unlike Qu *et al.* (2007) and Wang *et al.* (2008), the ADMSCs in this project were cultured with FGF-2. The addition of FGF-2 has been shown to reduce osteogenic differentiation of murine ADMSCs and increase adipogenic differentiation of human ADMSCs (Quarto and Longaker, 2006; Kakudo *et al.,* 2007).

Moreover, the osteogenic and adipogenic differentiation medium contained dexamethasone which has been reported to inhibit osteoblast differentiation and increase the expression level of peroxisome proliferator activated receptor gamma (PPARγ), an early indicator of adipogenesis, in murine ADMSCs (Malladi *et al.,* 2006). It is therefore possible that the supplementation with FGF-2 and dexamethasone reduced osteoblast differentiation and increased adipogenic differentiation of porcine ADMSCs.

The chondrogenic differentiation of ADMSCs and BMMSCs was similar regarding the amount of glycosaminoglycanes produced although cell pellets formed by ADMSCs were smaller than pellets from BMMSCs. These results were in accordance with the literature (Wang *et al.,* 2008).

### 4.2.5.4  Generation of RNAi transgenic pigs by somatic cell nuclear transfer

Due to the previously discussed advantages of mesenchymal stem cells as donor cells for somatic cell nuclear transfer (SCNT), characterized ADMSCs and BMMSCs from the same female pig were chosen for the generation of transgenic pigs with RNAi constructs against porcine BLG. The SCNT approach had an advantage compared to the initially intended lentiviral transgenesis because the sex of the animal could be pre-determined. This would allow analysis directly in the F0 generation.

Results of western blot analysis in HpB9 and CB1 cells did not provide an indication of which constructs, the shRNA or the artificial miRNA, would lead to a downregulation of BLG in the pig. Therefore, the shRNA construct BLGsh489 and the artificial miRNA construct BLGmi490 under the control of the ubiquitous PGK or the mammary gland specific BLG promoter, were used. After selection, many cell clones were obtained from ADMSCs but only a low number from BMMSCs. This result was surprising because BMMSCs have previously been successfully applied in our laboratory to generate cell clones and even gene targeted animals. However, these results were obtained with cells from different animals and variability regarding colony formation between cells isolations has previously been shown in our laboratory (Wiskow, unpublished data).

A total of 160 embryos were generated by SCNT, transferred into two synchronized recipients and one pregnancy was established. A 50% pregnancy rate after SCNT using single cell clones was encouraging and an indication that ADMSCs are competent nuclear donors. ADMSCs have practical benefits for future work because they can be easily isolated in large cell numbers from porcine adipose tissue.

Three fetuses were recovered at day 32 of pregnancy. Two of those showed an integration of the BLG promoter, one of the PGK promoter directed miRNA cassette whereas no fetus was derived from ADMSCs containing the U6 promoter directed shRNA construct against porcine

BLG. Although three fetuses represent too low a number to draw firm conclusions about the influence of the RNAi construct on fetus development, this was consistent with the observed growth characteristics of the respective cell pools derived from single clones containing one type of construct. The pool with the PGK promoter directed artificial miRNA proliferated well but the cell pool containing the artificial miRNA under the control of the BLG promoter grew faster. This might be because the BLG promoter is not active in ADMSCs. In contrast, the cell pool with the U6 promoter directed shRNA proliferated very slowly.

These differences in proliferation rate might be due to toxic effects of the shRNA construct. Potential toxicity of shRNAs has been reported in mice. Grimm *et al.* (2006) showed adverse effects of shRNAs after intravenous infusion in the liver of mice. More than half of the constructs tested led to the death of the animals. This toxicity has been attributed to abundant expression of U6 promoter directed shRNA, interfering with the endogenous miRNA pathway by inhibition of endogenous small RNAs processing (Grimm *et al.,* 2006). Significantly, the expression of one of these shRNA as an artificial miRNA from a liver specific promoter reduced target gene expression without any signs of liver damage (Giering *et al.,* 2008). A comparative study of the toxic effects of shRNA and artificial miRNA after injection into mouse brain revealed similar effects. Two out of three shRNAs resulted in neurotoxicity, whereas the same sequences expressed as artificial miRNA did not (McBride *et al.,* 2008). Moreover, toxic effects of shRNA expression have also been shown in primary porcine cells. Stewart *et al.* (2008) showed an induction of the interferon response by shRNAs in porcine fetal fibroblasts and similar to Grimm *et al.* (2006) and McBride *et al.* (2008) they found that shRNA expression interfered with the endogenous miRNA pathway.

It is therefore possible that the shRNA construct induced the cellular interferon response or lead to negative effects on the processing of natural miRNAs, resulting in growth inhibition of this ADMSC pool and lack of fetuses after SCNT. However, in contrast to the report by Grimm *et al.* (2006) and McBride *et al.* (2008), the expression of miRNAs against BLG from the PGK promoter also resulted in a growth retardation compared to the pool with the BLG promoter artificial miRNA construct. Nevertheless, it was possible to obtain one fetus with the PGK promoter construct, indicating no apparent negative effect of the artificial miRNA construct on embryo development.

Despite reports of shRNA mediated toxicity, the successful generation of mice with RNA polymerase III promoter directed shRNA constructs has been described, indicating that not all shRNAs are toxic. One example is the generation of transgenic mice with a shRNA cassette for the systemic downregulation of superoxide dismutase 1 (SOD1). These animals recapitulated a SOD1 *null* phenotype and showed 80 to 95% downregulation of SOD1 in all tissues without interfering with the endogenous miRNA pathway (Sasaguri *et al.,* 2009). In

the report by Grimm *et al.* (2006), only 36 out of 49 tested sequences resulted in toxicity and RNAi transgenic pigs have also been produced with shRNA constructs (Dieckhoff *et al.,* 2008). This indicates that not only the high expression level of shRNAs but also the target sequence itself has an influence on toxicity. The small number of fetuses generated in this project clearly makes it difficult to draw definitive conclusions. Further SCNT with the RNAi cell pools would be necessary to investigate the validity of these findings.

### 4.2.5.5 Expression analysis of artificial miRNA constructs in transgenic fetuses

To evaluate whether the porcine fetal fibroblast (poFF) derived from the RNAi transgenic fetuses express the artificial miRNA construct against porcine BLG, small RNA northern blot analysis was performed. No expression of the PGK or BLG promoter directed miRNA construct against porcine BLG could be detected. This result was not surprising for the construct with the BLG promoter because this promoter is lactating mammary gland specific (Schnieke *et al.,* 1997) and therefore not active in poFFs. The lack of expression of the PGK promoter directed miRNA construct can be due to different reasons. One reason could be an inactivation of the PGK promoter by methylation or the integration into a transcriptionally inactive genomic region (Palmiter *et al.,* 1982; Sutherland *et al.,* 2000; Mehta *et al.,* 2009). Another reason might be that only few copies of the transgene integrated and therefore the expression level of the miRNA is not sufficient to be detected. To investigate this possibility, Southern blot analysis will be performed in future work to determine the copy number of the PGK promoter directed miRNA.

An additional factor is the unknown detection limit of the small RNA northern blot analysis. The U6 probe used as a loading control is commercially available and optimized for the detection of the U6 snRNA. In contrast, the probe used for BLGmi490 has been designed for this project and therefore the detection limit is unknown. The use of digoxigenin-labeled locked nucleic acid (LNA) probes for small RNA northern blot has previously been described, however no general protocol has been established (Wienholds *et al.,* 2005; Weston *et al.,* 2006; Ramkissoon *et al.,* 2006; Mead and Tu, 2008; Yan *et al.,* 2009). For this project, up to 20 µg of total RNA were analyzed for the expression of BLGmi490 but it is possible that the amount of RNA was not sufficient due to low expression of the construct.

As the results of the small RNA northern blot analysis showed, that BLGmi490 is not or maybe weakly expressed, it is unlikely that a second round of SCNT would result in pigs with knockdown of porcine BLG. Therefore the original ADMSC clones should be analyzed to select a clone with high expression level of the artificial miRNA prior to the repeated generation of transgenic pigs. However, this approach is technically difficult to conduct

because single clones of ADMSCs have to be expanded sufficiently to obtain the amount of RNA necessary for small RNA northern blot which is not trivial.

Because it is not possible to evaluate if the BLG promoter directed artificial miRNA in poFF2 is active, the transgene copy number will now be determined by Southern blot followed by the use of these cells for a second round of SCNT.

For future work a modification of the artificial miRNA vector might facilitate the evaluation of RNAi construct expression. It has been shown that a reporter gene can be inserted upstream of the artificial miRNA sequence or the miRNA sequence can be placed into an intron of a reporter gene such as red fluorescent protein (Qiu *et al.,* 2008a). This would generate a construct where the reporter is co-transcribed with the RNAi construct from the same promoter, greatly facilitating the expression analysis of the RNAi construct.

## 4.3  Sequence-specific gene silencing of porcine p53

### 4.3.1  Background

The second target gene chosen for RNAi mediated downregulation was the porcine tumor suppressor protein p53. This gene is ubiquitously expressed in all tissues and plays an important role in the cellular stress response. Stress signals such as expression of oncogenes, hypoxia, DNA damage and others lead to the activation of p53 stress response, leading to growth arrest and consequently to DNA repair, senescence or apoptosis (Vousden and Lu, 2002; Shu *et al.,* 2007). The fact that p53 is mutated in about 50% of human cancers shows the importance of this protein for normal cell function (Greenblatt *et al.,* 1994). An inherited cancer syndrome, the Li-Fraumeni syndrome (LFS), is a result of TP53 germline mutations leading to a loss of p53 function and patients develop tumors before the age of 50. The syndrome is characterized by multiple primary tumors and a high rate of multiple cancers (Malkin *et al.,* 1990; Evans and Lozano, 1997). Up to now, murine models of LFS with heterozygous and homozygous deletion of TP53 have been generated and are susceptible to early tumor development (Donehower *et al.,* 1992; Jacks *et al.,* 1994).

In mice, it has also been shown that RNAi mediated downregulation of p53 is possible. Artificial miRNA and shRNA constructs against murine p53 were introduced into hematopoietic stem cells derived from mice expressing the oncogene c-myc. After transplantation into irradiated mice, the downregulation of p53 led to enhanced tumor development similar to a loss-of-function mutation or knockout of p53 (Hemann *et al.,* 2003; Dickins *et al.,* 2005). In addition, mice with ubiquitous knockdown of p53 have been reported (Rubinson *et al.,* 2003). This confirms that RNAi can offer an alternative to gene knockout in mice.

Because pigs resemble humans more than mice regarding physiology, size and organ development, they would be useful as disease models (Wernersson *et al.,* 2005; Lunney, 2007). The goal of this project was therefore to establish RNAi in pigs and to examine if RNAi mediated downregulation of p53 can be used instead of gene knockout by gene targeting to generate a porcine model for LFS. For this purpose, expression systems for the two most common experimental RNAi constructs, short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs), were generated and evaluated regarding their ability to silence porcine p53 *in vitro* in different test systems. The three main parts of the project were: first the design of RNAi sequences against porcine p53, second the evaluation of these sequences in mammalian cell lines and third evaluation in primary porcine cells suitable for somatic cell nuclear transfer.

### 4.3.2  Knockdown of porcine p53 by different RNAi constructs

The potential of shRNA and artificial miRNAs for the downregulation of porcine p53 was evaluated in three different assays. ShRNA and artificial miRNA constructs were evaluated in a Dual-Luciferase assay, in a cotransfection experiment in the p53-deficient murine MPC cell line and in stable pools of primary porcine adipose tissue derived mesenchymal stem cells (ADMSCs). A summary of the knockdown efficiencies of the constructs in these three assays is presented in Table 11.

**Table 11: Summary of knockdown efficiencies of RNAi constructs against porcine p53.**

| Assay (cell type) | downregulation by shRNA | | | downregulation by miRNA | | |
|---|---|---|---|---|---|---|
| | sh312 | sh787 | sh944 | mi304 | mi785 | mi943 |
| Dual-Luciferase assay (HEK293) | 74% | 0% | 47% | 77% | 10% | 51% |
| Western blot cotransfection (MPC) | 70% | 5% | 5% | 80% | 60% | 70% |
| Western blot (ADMSC) | <5% | 50% | 85% | <5% | 75% | 80% |

MPC: murine p53-deficient pancreatic cancer cell line; ADMSC: primary porcine adipose tissue derived mesenchymal stem cells

### 4.3.3  Transient assays: Dual-Luciferase assay and transfection in MPC cells

There have been many reports describing RNAi mediated downregulation of human p53 *in vitro* and murine p53 *in vitro* and *in vivo* (e.g. Brummelkamp *et al.,* 2002; Hemann *et al.,* 2003; Rubinson *et al.,* 2003; Dickins *et al.,* 2005). However, the sequences used for the knockdown of human and murine p53 did not show 100% homology with the porcine p53 mRNA. Therefore knockdown sequences for porcine p53 were designed using an online RNAi design program (Block-iT, Invitrogen). Because these sequences had not been previously validated, they were evaluated for their knockdown efficiencies in two transient assays to minimize the number of sequences which had to be analyzed in porcine primary

cells. To this end, six RNAi sequences against porcine p53 were designed. Of these, three were used as shRNAs transcribed from a U6 promoter and three as artificial miRNAs expressed from a CMV promoter. Pairs of shRNA and miRNA sequences were chosen to target overlapping sequence positions of the porcine mRNA to allow a comparison of silencing efficiencies.

The first test, a Dual-Luciferase RNAi reporter assay, was based on a commercial plasmid, psiCHECK2. In the Dual-Luciferase assay, two shRNAs and two artificial miRNA showed a significant knockdown of the relative *Renilla* luciferase expression (see Table 11). The finding that these sequences targeted similar sequence positions of the porcine p53 mRNA with comparable knockdown efficiencies was encouraging and supported further examination in other tests. The frequency of 2 out of 3 computer generated RNAi sequences resulting in a significant knockdown was higher than has been reported in the literature (Kumar *et al.,* 2003).

The knockdown ability of all six RNAi constructs was also tested by cotransfecting a porcine p53 expression plasmid and the RNAi vectors into a murine p53 deficient cell line (MPC) to investigate the knockdown efficiencies in a second independent assay. Western blot analysis was performed to prove expression of p53 and to evaluate the knockdown ability of the RNAi constructs. A similar test has previously been used to determine knockdown efficiencies of shRNAs against murine p53. Hemann *et al.* (2003) performed a cotransfection experiment with a murine p53 expression vector and shRNAs plasmids against murine p53 in the p53-deficient human lung adenocarcinoma cell line H1299. They found three different shRNA sequences with varying abilities to downregulate murine p53. Importantly these results correlated with the *in vivo* knockdown.

Here, the test was employed to validate the knockdown obtained from the Dual-Luciferase assay. However, the results of the experiment in MPC cells were only partially consistent with the Dual-Luciferase assay. Two shRNA and two miRNA constructs showed a downregulation or lack of knockdown of porcine p53 as determined by the Dual-Luciferase assay (see Table 11). In contrast one shRNA (TP53sh944), which had shown significant knockdown in the Dual-Luciferase assay, had no effect on porcine p53 expression in MPC cells and one artificial miRNA construct (TP53mi785) was only effective in MPC cells.

These results make clear, that pre-screening of RNAi sequences in different test systems does not necessarily reflect the real knockdown ability of RNAi sequences. It is possible that the use of transient expression and the necessity to cotransfect two plasmids in both assays may increase the variability of the results. In the Dual-Luciferase assay, a normalization of the resulting substrate conversion of the RNAi regulated *Renilla* luciferase against the firefly

luciferase can be performed. This results in less variability than transient cotransfection into the p53-deficient cell line MPC. In the second system, no evaluation of the transfection efficiency was performed. This would have been possible for the lentiviral shRNA constructs, because they contain an EGFP expression cassette, but not for the artificial miRNA constructs. Cotransfection of a third plasmid encoding EGFP along with the miRNA constructs or the introduction of a visible marker gene into the miRNA plasmids might allow normalization for transfection efficiency. In this case, the detection of the marker gene, a housekeeping gene and p53 by western blot analysis would result in more reliable information from this experiment. A similar approach was performed by Ramsoondar *et al.* (2009). They cotransfected a shRNA expression plasmid, a second plasmid containing the target gene and a third plasmid encoding another protein as a transfection control into CHO and PK15 cells to evaluate the knockdown potential of shRNAs against porcine endogenous retroviruses (PERV). However, it is also possible that the observed results are not due to the transient nature of this assay but represent an inherent difference in the cell types used.

Another approach which might reduce variability in analyzing the knockdown potential of RNAi sequences would be the generation of stable double transgenic cell lines with the porcine p53 expression vector and RNAi constructs in the MPC cell line. This is however not possible because stable overexpression of p53 results in apoptosis of cells. Therefore an inducible system for p53 expression would have to be used but this would likely create another source of variability (Yamada *et al.,* 2007).

### 4.3.4  Porcine primary cell pools with RNAi mediated p53 knockdown

The first two experiments to evaluate the knockdown potential of the six RNAi sequences against porcine p53 were performed either in human cells (HEK293) or in murine cells (MPC). These transient assays were applied to limit the number of sequences which had to be tested in porcine primary cells. As they did not result in an identification of ideal candidate sequences, all six RNAi constructs were tested in porcine primary cells. These cells are more likely to mimic the *in vivo* effect of the RNAi constructs in the pig than human or murine cell lines. This view is supported by Dieckhoff *et al.* (2007; 2008) who found a correlation between the knockdown achieved in primary porcine fibroblasts and piglets expressing the same shRNA sequence against porcine endogenous retroviruses (PERV).

For RNAi mediated downregulation of porcine p53, the RNAi sequences were introduced into primary porcine adipose tissue derived mesenchymal stem cells (ADMSCs). This cell type was chosen since our laboratory has extensive experience in the culture of porcine mesenchymal stem cells, the isolation of cells from porcine fat was convenient, resulted in a large number of fast proliferating cells and it had be shown before that this cell type supports fetal development after SCNT (see section 3.1.16.2). All used RNAi constructs contained a

selectable marker gene and stable cell pools with the RNAi constructs against porcine p53 were generated. Western blot analysis showed that four out of six RNAi constructs resulted in efficient downregulation of endogenous porcine p53 (see Table 11). Two of those were shRNA and two artificial miRNA constructs directed against overlapping regions of porcine p53 mRNA. Again, the results were different from the two previously discussed transient assays. The only construct which was consistently effective in all three tests was TP53mi943.

### 4.3.5   Comparison of RNAi mediated knockdown of p53 in different assays

The results obtained from the various assays regarding the knockdown of porcine p53 by shRNA and artificial miRNA based RNAi constructs varied according to the test and the target position on the porcine mRNA.

The first observation was that the knockdown by shRNAs and artificial miRNAs directed against similar sequence positions was equally effective within the Dual-Luciferase assay or in porcine primary cells independent of the type of construct. In contrast, the miRNA constructs were more efficient than the shRNA constructs in the cotransfection experiment in the MPC cell line. However, as discussed above, this experiment might not be the ideal test to determine knockdown efficiencies because the data cannot be normalized to cover variations arising from transfection.

The second finding was that the knockdown efficiencies were highly dependent on the type of experiment. The main differences between the three tests were first the use of different cell types, second the downregulation of p53 in a transient or stable manner and third the RNAi mediated knockdown of endogenous porcine p53 versus recombinant p53 or a fusion mRNA.

Regarding the use of different cells types, it can be presumed that analysis of knockdown effects in porcine primary cells is likely to better reflect the outcome in transgenic animals than assays performed in human and murine cancer cell lines. This hypothesis is supported by Dieckhoff *et al.* (2007; 2008) who found a correlation of RNAi mediated gene knockdown in porcine primary cells and thereafter in RNAi transgenic pigs.

Concerning the difference between transient and stable knockdown of porcine p53, it is likely that the generation of stable primary cell pools compared to transient cotransfection experiment results in less variable information about the knockdown efficiencies. As the EGFP transgene was encoded on all RNAi vectors used in porcine primary cells and almost all of the cells expressed EGFP at comparable levels, it provided an indication about the amount of construct present in the cells. This was important to ensure that different knockdown rates in the cell pools were not due to varying amounts of RNAi constructs within

the cells but a sequence-specific effect. The same stable primary cell pools were used for western blot analysis to detect the knockdown effect over time (data not shown) and resulted in stable downregulation of porcine p53 over generations, demonstrating the benefit of stable cell pools compared to transient experiments.

The third and most important difference between the assays was that the endogenous p53 mRNA serves as a target in porcine cells. The cDNA generated for the Dual-Luciferase assay and the recombinant porcine p53 expression plasmid did not contain all nucleotides of the endogenous mRNA. The 5' end of the porcine p53 mRNA was not known at the time of the experiments. Therefore the p53 cDNA only included several bases upstream of the ATG start codon of porcine p53. In addition, the cDNA did not contain the full length 3' UTR, resulting in a shorter cDNA compared to the endogenous p53 mRNA. It has been shown that the secondary structure of the target mRNA and thereby the accessibility of the target sequences by RNAi constructs or antisense oligonucleotides has an effect on silencing efficiency (Bohula *et al.,* 2003; Vickers *et al.,* 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Overhoff *et al.,* 2005). Sun and Rossi (2009) reported that downregulation of the *Renilla* luciferase in the RNAi test plasmid psiCHECK2 was strongly dependent on the length of target sequence inserted downstream of the luciferase. The resulting knockdown efficiencies varied between 10 and 80% against the same target sequence depending on the total size of the inserted DNA, most likely because of the altered secondary structure of the fusion mRNA. It is therefore possible that the use of a fusion mRNA between the *Renilla* luciferase and the porcine p53 cDNA altered the mRNA secondary structure in a way that influenced the binding of the RNAi constructs. This might explain the differences in knockdown potential between the Dual-Luciferase assay and in porcine cells. This hypothesis is supported by the fact that shRNA and artificial miRNA sequences directed against overlapping regions of the porcine p53 mRNA showed similar knockdown efficiencies, depending on the assay. In the Dual-Luciferase assay TP53sh312 and TP53mi304 downregulated the relative *Renilla* luciferase expression whereas both sequences were not effective against the endogenous p53. TP53sh787 and TP53mi785 did not result in a knockdown of the relative *Renilla* luciferase expression, but both showed a reduction of endogenous porcine p53. The sequences TP53sh944 and TP53mi943 were effective in both assays. The differences between the Dual-Luciferase assay and the western blot in porcine primary cells could therefore be explained by the formation of different mRNA secondary structures in the two tests, leading to higher or lower accessibility of the respective target regions resulting in a different degree of silencing. However this correlation between shRNA and artificial miRNA constructs was not observed in the transient experiment in the MPC cell line, further indicating that this test might not suitable for evaluating RNAi constructs.

The general conclusion after comparison of different test systems to screen the knockdown of porcine p53 is that the evaluation of silencing efficiencies in primary cells of the species of choice might be more suitable than artificial test systems in mammalian cell lines. This approach is however only possible for genes expressed in cell types which can be cultured *in vitro*. Furthermore, the knockdown potential of RNAi sequences for the downregulation of porcine p53 seems to be mainly related to the target sequence position on the mRNA and not the type of construct, shRNA or artificial miRNA, used.

## 4.3.6 Functional analysis of p53 knockdown: p53 reporter assay and doxorubicin resistance

In addition to three previously discussed experiments, a functional analysis of p53 knockdown in ADMSCs was performed. To this end, the three primary porcine ADMSCs cell pools with the highest knockdown efficiencies according to the western blot analysis (see Table 11) were used for further evaluation with a p53 reporter assay and resistance to the chemotherapeutic drug doxorubicin was determined.

The p53 reporter assay is based on a plasmid encoding firefly luciferase under the control of a minimal CMV promoter fused to 17 repeats of a p53 consensus DNA binding sequence from the human ribosomal gene cluster (RGC) (Kern *et al.,* 1991). Reduced levels of p53 in the cells result in a lower expression of the firefly luciferase. Initial tests showed that recombinant and endogenous porcine p53 can induce the luciferase expression from the p53 reporter plasmid. The plasmid was then transfected into porcine primary ADMSC pools and compared to wildtype ADMSCs. Two of the tested cell pools, containing TP53sh944 and TP53mi943, showed a significant downregulation of relative firefly luciferase expression compared to wildtype cells. This result was consistent with the western blot analysis, however the knockdown efficiency as determined by western blot was higher than in the reporter assay. In contrast, TP53mi785 resulted in a strong upregulation of the relative firefly luciferase expression. This result was surprising but it has been shown that the p53 reporter assay does not always accurately reflect p53 activity in cells. Wischhusen *et al.* (2004) demonstrated that in p53-deficient cells, the p53 family members p63 and p73 can initiate the expression from a reporter plasmid similar to pRGC-Luc used in this project. Therefore the downregulation of p53 by TP53mi785 and the resulting increase in firefly luciferase expression from the p53 reporter plasmid might reflect enhanced expression of p63 or p73 rather than an actual increase of p53 in this cell pool. This might also be the reason why the knockdown efficiencies of the p53 reporter assay are lower than the downregulation observed in the western blot analysis, since the reporter assay results in information about the whole p53 family. It can therefore be concluded that this test is not ideal for evaluating the knockdown ability of RNAi constructs against porcine p53.

A functional test of p53 knockdown was also performed in stable porcine primary ADMSC pools with RNAi constructs. For this, the cell pools with lowest expression level of p53 were treated with the chemotherapeutic drug doxorubicin, an inducer of p53 mediated apoptosis. It has been shown that p53-deficient cells or cells with reduced levels of p53 show higher resistance to treatment with doxorubicin than cells with wildtype level of p53 (Yamamoto *et al.,* 1999; Dunkern *et al.,* 2003; Dickins *et al.,* 2005; Stern *et al.,* 2008). However, how doxorubicin activates p53 and apoptosis is still unclear. One hypothesis is based on the direct involvement of p53 in the endogenous miRNA pathway. The pri-miRNA processing enzyme Drosha interacts with p53 and other proteins after treatment of cells with doxorubicin. p53 then mediates the association of growth suppressive pri-miRNAs with Drosha, leading to an increased amount of the corresponding mature miRNAs which inhibit cell proliferation. Decreased levels of p53 reduce the processing of these pri-miRNAs after doxorubicin treatment and enhance cell survival (Suzuki *et al.,* 2009).

The doxorubicin treatment of ADMSC pools was performed to examine whether the downregulation of p53 was sufficient to confer resistance to this chemotherapeutic drug. Colonies were formed by one pool that contained the artificial miRNA construct TP53mi785 whereas wildtype cells and the other two analyzed cell pools did not form colonies. The increased chemoresistance has also been shown after p53 knockdown in c-myc expressing hematopoietic stem cells *in vivo,* resulting in enhanced tumor development (Dickins *et al.,* 2005). It is unknown, why the other two RNAi sequences with similar knockdown efficiencies in the western blot analysis did not result in resistance. A possible explanation is that the cell pool with TP53mi785 contained single cells with sufficient downregulation of p53 to exert a biological effect whereas the other cell pools did not.

These data indicate that TP53mi785 is the most promising candidate for the downregulation of porcine p53. Since all the results were obtained with ADMSC pools, analysis of single cell clones might lead to the identification of clones with high knockdown efficiencies and possibly an even stronger resistance to doxorubicin. Moreover, the doxorubicin assay provides a method to directly identify cell clones with functional p53 knockdown which can then be used to generated RNAi transgenic pigs by SCNT.

### 4.3.7 Model for Li-Fraumeni syndrome – knockout or knockdown of p53?

The main aim of the porcine p53 knockdown project was to evaluate different test systems for the examination of RNAi sequences and to investigate whether a knockdown of porcine p53 was an effective alternative to gene knockout. Heterozygous germline TP53 mutations in LFS patients result in early tumor onset and multiple primary tumors. However, these cancers arise only after the second TP53 allele is mutated or deleted by somatic mutations (Malkin *et al.,* 1990; Evans and Lozano, 1997). In contrast, RNAi mediated downregulation of

p53 can lead to varying degrees of loss-of-function. This variation is mainly due to the efficiency of the RNAi sequences used for the knockdown (Hemann *et al.,* 2003; Dickins *et al.,* 2005).

To imitate the conditions of LFS, a highly functional RNAi sequence has to be used to mimic loss-of-function mutations in both alleles of TP53. It is as yet unclear, what degree of gene knockdown would be necessary for the generation of a phenotype resembling a homozygous TP53 knockout by gene targeting and if the achieved knockdown of porcine p53 by 50 to 85% would be sufficient.

Enhanced cancer formation in transgenic mice has been reported by RNAi mediated knockdown of p53 using shRNA or artificial miRNA constructs. The mice expressed the c-myc oncogene under the control of the immunoglobulin heavy-chain enhancer in hematopoietic stem cells. Additional knockdown of p53 in these cells resulted in enhanced formation of lymphomas and reduced survival after transplantation of double transgenic cells into irradiated mice (Hemann *et al.,* 2003; Dickins *et al.,* 2005). Mice with shRNA mediated knockdown of p53 alone have also been generated, but no further reports regarding tumor formation in these animals were published (Rubinson *et al.,* 2003). It is therefore possible that the level of p53 downregulation achieved was insufficient to result in tumor formation. Stern *et al.* (2008) have generated mice with Cre-recombinase initiated tissue-specific knockdown of p53 in endothelial and hematopoietic lineages or ubiquitously in all embryonic tissues. Although the level of p53 was significantly reduced and led to an increased resistance to doxorubicin, these mice did not show an increase in tumor formation. The authors subsequently combined the downregulation of p53 in B-cells with the expression of the c-myc oncogene. This combination led to transformation of the cells *in vivo* after injection into lethally irradiated mice and the animals developed lymphomas within 100 days (Stern *et al.,* 2008).

These findings suggest that RNAi might not be the method of choice for the generation of a porcine animal model for the Li-Fraumeni syndrome, especially since a conventional knockout of TP53 by gene targeting would more closely resemble the cause of the human disease. This is supported by the finding that even the enhanced *in vitro* resistance to doxorubicin after knockdown of p53 alone by Stern *et al.* (2008) was not associated with enhanced tumor formation.

Nevertheless, RNAi mediated p53 knockdown has been shown to increase the incident of tumor formation when combined with the expression of the c-myc oncogene (Hemann *et al.,* 2003; Dickins *et al.,* 2005; Stern *et al.,* 2008). It has also been shown that the transformation of primary cells by oncogenic H-ras is dependent on the expression of another oncogene or

the inactivation of p53 or p16. This is due to the fact that expression of oncogenic H-ras by itself induces a cell cycle arrest in G1. In combination with a lack of p53, this cell cycle arrest is prevented, leading to transformation of primary cells (Serrano *et al.,* 1997). The same effect was observed after RNAi mediated downregulation of p53 in murine primary cells expressing oncogenic K-ras (Dickins *et al.,* 2005).

These reports emphasize the importance of p53 in controlling cell transformation. Therefore the downregulation of porcine p53 together with the expression of genes such as oncogenic c-myc or H-/K-ras in pigs might accelerate tumor formation. This approach could also be applied to enhance the tumorigenic effects of specific mutations of endogenous porcine genes like K-ras or adenomatous polyposis coli (APC) introduced by gene targeting. Our group has previously generated piglets with a heterozygous mutation in the tumor suppressor gene APC (Landmann, unpublished data). This mutation mimics one of the most common alterations responsible for an inherited cancer syndrome, the familial adenomatous polyposis (FAP). As tumors in FAP patients arise only after a series of mutations in oncogenes and tumor suppressor genes such as p53 (Fodde, 2002), RNAi mediated knockdown of p53 in pigs with targeted APC mutation might accelerate tumor formation and increase tumor progression.

In future work another application for RNAi in the study of cancer development would be the combination of a heterozygous precise modification of endogenous porcine TP53 by gene targeting resembling common mutations involved in human cancer development combined with RNAi mediated knockdown of wildtype p53. The downregulation of only mutant but not of wildtype p53 has been shown by I-Tsuen Chen *et al.* (2009) by designing a shRNA around the mutation site containing one mismatch to wildtype p53 mRNA. This approach could be inverted to downregulate wildtype p53 in pigs with a heterozygous targeted mutation of TP53 to enhance tumorigenesis. This would possibly avoid the need to generate animals with a homozygous mutation in TP53, reducing the time for the generation of a porcine cancer model. However, it is not trivial to design sequences with such high specificity.

Therefore another approach could be applied. Willis *et al.* (2004) showed that the ectopic expression of mutant p53 can decrease the binding of wildtype p53 to p53 responsive elements, inhibit the p53 induced growth repression and cell cycle arrest, exerting a dominant-negative effect. It is therefore possible that the overexpression of mutant p53 instead of a targeted mutation in pigs is sufficient to generate a porcine cancer model. This effect might be enhanced by reducing the amount of wildtype p53 which could be achieved by designing an overexpression construct for mutant p53 with a different 3' UTR compared to wildtype p53 and using RNAi constructs against the wildtype 3' UTR sequence.

### 4.3.8 An alternative use for porcine cells with p53 knockdown – generation of porcine iPS cells

Induced pluripotent stem (iPS) cells are pluripotent cells generated from somatic cells by the introduction of certain transcription factors. iPS were first produced by Takahashi and Yamanaka in 2006 after infecting murine fibroblasts with retroviruses encoding Oct3/4, Sox2, Klf4 and c-myc. The introduction of these four factors combined with ES cell culture conditions resulted in the generation of cells resembling ES cells in morphology, growth characteristics, ES cell specific marker expression and teratoma formation in nude mice (Takahashi and Yamanaka, 2006). Since then, various iPS cell lines have been produced by different methods including plasmid transfection, transduction with viruses containing stem cell factors, protein transfection and many more (Okita *et al.,* 2008; Carey *et al.,* 2009; Zhou *et al.,* 2009). These cells can be used to generate chimeric mice and even whole animals by tetraploid complementation (Okita *et al.,* 2007; Zhao *et al.,* 2009). iPS cells have also been obtained from human (Takahashi *et al.,* 2007; Yu *et al.,* 2007), rat (Liao *et al.,* 2009; Li *et al.,* 2009), rhesus monkey (Liu *et al.,* 2008), pig and minipig (Wu *et al.,* 2009a; Ezashi *et al.,* 2009; Esteban *et al.,* 2009).

However, the efficiency of iPS cell generation is generally very low. It has recently been shown that siRNA mediated downregulation of p53 in combination with the expression of Oct4, Sox2, Klf4 and c-myc enhanced the generation of iPS cells from human fibroblasts (Zhao *et al.,* 2008). Several other reports showed that decreased p53 protein level enhances the formation of iPS cells. Hong *et al.* (2009) showed a significant increase in the iPS generation efficiency in murine embryonic fibroblasts with heterozygous or homozygous deletion of TP53 compared to wildtype cells even without c-myc. This increase in colony formation was mainly due to reduced silencing of the introduced transgenes in p53-deficient cells. The generation of iPS cells from terminally differentiated T lymphocytes from p53-deficient mice was possible whereas this was not achieved with cells from wildtype p53 mice. Moreover, Hong *et al.* (2009) reported improved iPS cell derivation from human fibroblasts expressing a dominant-negative mutant p53 and also with cells expressing a shRNA against human p53. Similar results were obtained in a comparison of murine and human p53 deficient cells or cells expressing a shRNA against p53 by Marión *et al.* (2009). They showed enhanced development of iPS cells from these cells and the iPS cells were generated faster than in cells with p53. This was even true for cells with short telomeres or after γ-irradiation which were not efficiently reprogrammed in the presence of p53. They concluded that p53 inhibits the reprogramming of cells with DNA damage by inducing apoptosis (Marión *et al.,* 2009). In a study by Kawamura *et al.* (2009) it was possible to obtain murine iPS cells from cells with decreased levels of p53 by shRNA expression and in p53-deficient cells by supplying only two factors, Oct4 and Sox2, which were suitable for the generation of chimeric

mice (Kawamura *et al.,* 2009). A fourth report demonstrated that RNAi mediated downregulation of p53 at any time point during the reprogramming process resulted in a higher efficiency of iPS cell generation and even senescent cells could be reprogrammed (Utikal *et al.,* 2009).

The enhanced frequency of iPS cell generation in a p53 *null* background did however have serious drawbacks. First, the lack of inactivation of the c-myc expression together with the p53 deficiency led to tumor formation in chimeric mice generated from these iPS cells and resulted in rapid death after seven weeks (Hong *et al.,* 2009). Permanent deletion or downregulation of p53 can cause genomic instability leading to iPS cells of lower quality (Hong *et al.,* 2009). This problem is even more apparent after the successful generation of iPS cells from cells with short telomeres and other types of DNA damage in the absence of p53 which cannot be reprogrammed when p53 is present. These cells also showed an increase in chromosomal damage such as chromosomal breaks and fragmentation lowering the quality of iPS cells (Marión *et al.,* 2009).

However, RNAi mediated downregulation of p53 might still play a useful role in facilitating the generation of iPS cells from species other than human and mouse. Because embryonic stem cells have already been obtained from these two species, the appropriate culture conditions for the maintenance of pluripotent cells have been established (Evans and Kaufman, 1981: Martin, 1981; Thomson *et al.,* 1998). This is not true for many important mammalian species such as the pig. Up to now, no definitive ES cells have been isolated from pigs and one reason is that the culture conditions necessary to maintain these cells in an undifferentiated state are not known. Recently, the generation of porcine iPS cells has been reported. However these cells did not retain their pluripotent potential after the expression of the doxycyclin induced exogenous stem cell factors was terminated. In another study it has been shown that the expression of the exogenous stem cell factors was not completely silenced (Wu *et al.,* 2009a; Ezashi *et al.,* 2009; Esteban *et al.,* 2009). This indicates that fully reprogrammed porcine iPS cells have not yet been generated.

RNAi mediated downregulation of p53 could therefore be useful to enhance the generation of porcine iPS cells that could be used to determine suitable culture conditions for this cell type. This would likely aid the subsequent generation of normal iPS and ES cells from pig. In the future, a permanent downregulation of p53 is not desirable for the generation of pigs from these porcine iPS cells due to the negative effects reported in mice. If the findings are confirmed that the downregulation of p53 is essential for enhancing iPS colony formation, porcine cells with either an inducible RNAi construct or the transient addition of siRNAs against p53 might be used to circumvent the problems associated with permanent downregulation of p53 for the generation of porcine iPS cells.

Porcine iPS cells would be very useful for the generation of gene targeted pigs. It has been reported that the frequency of gene targeting by homologous recombination in murine ES cells is about 20 times higher than in murine somatic myoblasts (Arbonés *et al.,* 1994) and ES cells also have a longer lifespan in culture. If the same is true for iPS cells, the generation of porcine iPS cells with an enhanced frequency of homologous recombination would greatly facilitate the precise genetic modification of the porcine genome. These modified cells could then be used as donor cells for SCNT or to generate chimeric pigs by blastocyst or laser-assisted cell injection. In mice, the laser-assisted method offers the advantage that injection of cells in 8 cell stage embryos can generate founder animals almost exclusively derived from the injected cells (Poueymirou *et al.,* 2007).

## 4.4 Improved RNAi constructs and alternative application of RNAi

### 4.4.1 Bifunctional RNAi constructs

The RNAi pathway can be divided into cleavage-dependent and cleavage-independent parts. RNAi sequences with perfect complementarity to their target mRNA lead to mRNA cleavage, whereas sequences with internal mismatches, especially in the center of the RNAi sequence, lead to translational repression (Pasquinelli, 2002; Agrawal *et al.,* 2003, Carrington and Ambros, 2003). It has been proposed that this feature could be employed to enhance the RNA mediated silencing effect by generating constructs with two hairpin structures. The first hairpin would show full complementarity to the target mRNA whereas the second hairpin within the same construct would have internal mismatches. These hairpins are then loaded onto cleavage-dependent and cleavage-independent RISCs and would silence the target mRNA by cleavage and translational repression at the same time (Rao *et al.,* 2009). Although this approach has not yet been used, it might enhance the post-transcriptional gene silencing by RNAi constructs and could provide a basis for further work on porcine p53 or BLG.

### 4.4.2 Use of endogenous miRNAs to control transgene expression

In addition to the downregulation of endogenous genes by artificial miRNA, the use of endogenous miRNAs to control transgene expression has been described. For this, Brown *et al.* (2007) fused the reporter gene GFP to target sites of the human miRNA miR-302a. This miRNA is highly expressed in human embryonic stem cells but not in differentiated cells. The introduction of the GFP fusion construct into human ES cells led to a downregulation of GFP in undifferentiated ES cells which was reversed during differentiation of the cells. In another experiment it was shown that if GFP was fused to target sites of a neuron specific miRNA, GFP fluorescence was diminished during differentiation in embryoid bodies (Brown *et al.,* 2007).

Potentially, this system might be useful for the identification and monitoring of porcine iPS cells by introducing a reporter gene fused to a pluripotent cell specific miRNA target site into cells before the generation of iPS cells. In this case, the de-differentiation of the cells towards iPS cells could be identified by reduction of the expression of the reporter gene due to the expression of the endogenous miRNA. However, not many porcine endogenous miRNAs are known which currently hampers this approach (Kim *et al.,* 2008; Reddy *et al.,* 2009).

## 4.5 Emerging technologies for gene inactivation and gene targeting

### 4.5.1 Adeno-associated virus-mediated gene targeting

Recently the targeting of a gene which is not expressed in porcine fetal fibroblasts, the cystic fibrosis transmembrane conductance regulator (CFTR) has been achieved by virus-mediated gene targeting. Rogers *et al.* (2008) reported the disruption and mutation of the CFTR gene in pigs with a vector based on adeno-associated virus (AAV). The targeting vector contained a selection cassette and flanking regions homologous to the genomic CFTR sequence. Due to the size limitation of AAV vectors (4.5 kb), the targeting vector was composed of short stretches of homology to the CFTR sequence. Targeting efficiencies of up to 10% of all G418 resistant colonies were obtained. This finding is in stark contrast to the failure of targeting CFTR using a conventional vector with positive selection (Rogers *et al.,* 2008). Viable piglets with the disruption or mutation of CFTR were produced by SCNT. In addition to the experiments in pigs, Sun *et al.* (2008) have used AAV-mediated gene targeting to disrupt the CFTR gene with a targeting frequency of 0.5 to 2% in ferret fetal fibroblasts. These reports indicate that AAV-mediated gene targeting can be successfully used to target a non-transcribed gene in fetal fibroblasts.

The high targeting efficiencies of AAV-mediated gene targeting has been attributed to various factors. These include the fact that AAV delivers single stranded DNA which might enhance homologous recombination, the inverted terminal repeats of the vector which might facilitate the binding of recombination or repair proteins or an effect of the delivery of viral proteins on homologous recombination (Hendrie and Russell, 2005; Vasileva and Jessberger, 2005). However, the exact mechanism of AAV-mediated gene targeting remains elusive.

The enhanced efficiency of gene targeting of genes not transcribed in primary cells such as fetal fibroblasts together with the finding that only short stretches of homology between the targeting vector and the genomic sequence are necessary, facilitates gene targeting in livestock species. AAV-mediated gene targeting would provide an alternative and feasible option to RNAi for the inactivation of BLG.

Furthermore, it has been reported that AAV-mediated gene targeting in human cells is enhanced up to 100fold by double strand breaks in the gene of interest (Miller *et al.,* 2003; Porteus *et al.,* 2003). This presents an opportunity to combine specific double strand breaks by engineered ZFNs (see below) with AAV-mediated gene targeting which would possibly enhance targeting efficiencies even more.

### 4.5.2  Zinc-finger nucleases

Recently, an alternative approach to gene knockout or gene knockdown in mammals has emerged. This approach employs the ability of zinc-finger nucleases (ZFNs) to introduce double strand breaks at selected DNA sequences. ZFNs are engineered fusion proteins between zinc-finger DNA binding domains and the nuclease domain of the restriction endonuclease FokI (Porteus and Carroll, 2005). They can be designed to recognize specific DNA sequences and after dimerization, the FokI domain introduces DNA double strand breaks (DSBs) at the chosen position. This leads to an activation of the cellular double strand break repair mechanism, either by the non homologous end joining (NHEJ) pathway or by homologous recombination (Sonoda *et al.,* 2006). During NHEJ, the free DNA ends are joined often leading to small deletions or insertions at the break site. This can result in mutations or inactivation of the gene. Frequently, both alleles are affected after transient expression of ZFN (Santiago *et al.,* 2008).

In addition, DSBs can be repaired by homologous recombination. In this case the break is repaired using either the sister chromatide as a template or by homologous recombination with introduced donor DNA, such as plasmid DNA. The result is a replacement of the target gene with exogenous DNA sequences, leading to ZFN mediated gene targeting (Porteus and Carroll, 2005). The technology has been used to perform gene knockout and gene targeting in a variety of species including zebrafish (Meng *et al.,* 2008; Doyon *et al.,* 2008; Foley *et al.,* 2009), *Drosophila melanogaster* (Bibikova *et al.,* 2003; Beumer *et al.,* 2006), plants (Wright *et al.,* 2005; Shukla *et al.,* 2009; Townsend *et al.,* 2009) and in mammalian cells (Santiago *et al.,* 2008; Pruett-Miller *et al.,* 2008; Zou *et al.,* 2009). Recently, targeted gene disruption of two endogenous genes and a reporter gene in rats by injection of ZFN mRNA into zygotes has been reported (Geurts *et al.,* 2009).

The use of ZFNs is independent of the transcriptional activity of the gene in a particular cell type and therefore offers a valuable alternative to gene targeting especially for genes not transcribed in fetal fibroblast or MSCs (Hockemeyer *et al.,* 2009). Moreover, it has been shown that the transient expression of plasmids encoding ZFNs or injection of ZFN mRNA into zygotes or embryos results in efficient gene-targeting or knockout (Beumer *et al.,* 2008; Geurts *et al.,* 2009). In the rat, the frequency of gene knockout by ZFN was reported to be 12% of all tested founder animals, a high efficiency sufficient to avoid the need to screen

embryos before embryo transfer. This feature would be beneficial for the genome modification of livestock species by injecting ZFNs with or without targeting vectors into zygotes. It would allow the generation of knockout or gene targeted animals without the use of SCNT.

Therefore, ZFN mediated knockout would be an alternative to conventional gene knockout or RNAi mediated gene silencing, especially in the case of genes not transcribed in fetal fibroblasts or MSCs such as the porcine or the bovine BLG. This approach would be useful for the elimination of BLG from cows' milk instead of RNAi mediated downregulation to produce milk for children suffering from cow's milk allergy or intolerance and provide information about the function of the protein.

## 4.6  Concluding remarks

In this work, a comparison of shRNA and artificial miRNA constructs for the knockdown of porcine genes has been provided. Several *in vitro* assays have been performed to select the most effective RNAi sequences for the downregulation of porcine p53 and BLG. It could be shown that the different test systems to determine the knockdown potential of shRNA and artificial miRNA sequences showed variability regarding the knockdown achieved. This indicates that RNAi sequences have to be tested in more than one assay to predict their effectiveness. The best estimation of the *in vivo* functionality of RNAi sequences seems to be screening in primary cells. Furthermore, this evaluation in primary cells allows the direct use of these cells to generate animals by SCNT.

Downregulation of recombinant BLG in cell lines was achieved by shRNA and artificial miRNA constructs targeting similar sequence position. The effect of shRNA or artificial miRNA constructs was cell line specific, a finding which has not previously been reported in the literature. The knockdown of porcine BLG in primary cells was not possible because isolated mammary epithelial cells could not be induced to express milk genes.

In the course of this project, RNAi transgenic fetuses with artificial miRNA constructs were generated by SCNT of porcine adipose tissue derived mesenchymal stem cells whereas this was not achieved for the shRNA against porcine BLG. These results show that this cell type can be genetically manipulated, generate single cell clones *in vitro* and support fetal development after SCNT.

Expression analysis of the fetuses revealed that the PGK promoter directed miRNA construct against porcine BLG was not expressed at detectable levels and the construct with the BLG promoter could not be tested in fetal fibroblasts. It is therefore unclear, whether the BLG promoter construct will be expressed, unless transgenic pigs are generated and analyzed during lactation. Therefore the cells with the BLG promoter miRNA will be used for a second

round of cloning to generate the first RNAi transgenic pigs with a tissue-specific artificial miRNA construct.

This work provides the first report regarding the downregulation of endogenous porcine p53. For porcine p53 mainly the RNAi sequences and not the type of construct determined the knockdown potential. One miRNA construct even led to a functional reduction of p53 as shown by enhanced resistance to doxorubicin. Therefore this construct seems to be the most promising candidate for RNAi mediated downregulation of porcine p53 *in vivo*. It can however be assumed that RNAi mediated downregulation of porcine p53 is not sufficient to create an animal model for the Li-Fraumeni syndrome. Therefore the combination of p53 knockdown with overexpression or precise genetic modification of porcine oncogenes seems to be the most promising approach to generate a porcine cancer model

# 5   Abbreviations

| | |
|---|---|
| % | Percent |
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microliter |
| µM | Micromolar |
| µm | Micrometer |
| 3D | Three dimensional |
| ADMSC | Adipose tissue derived mesenchymal stem cell |
| APC | Adenomatous polyposis coli |
| AMD | Age-related macular degeneration |
| APS | Ammonium persulfate |
| Arg | Argenine |
| bFGF | Basic fibroblast growth factor |
| BLG | Beta-Lactoglobulin |
| bp | Base pair |
| BSA | Bovine serum albumin |
| CB1 | CHO-K1 cell clone expressing porcine BLG |
| cDNA | Complementary DNA |
| CFTR | Cystic fibrosis transmembrane conductance receptor |
| CIP | Calf Intestine Phosphatase |
| CMA | Cows' milk allergy |
| CMI | Cows' milk intolerance |
| CMV | Cytomegalovirus |
| $CO_2$ | Carbone dioxide |
| Cys | Cysteine |
| d | Day |
| Da | Dalton |
| $ddH_2O$ | Double distilled water |
| $dH_2O$ | Distilled water |

| | |
|---|---|
| DMSO | Dimethylsulfoxide |
| DNA | Desoxyribonucleic acid |
| ds | double stranded |
| dsRNA | Double stranded RNA |
| DTT | 1,4-Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | recombinant human epidermal growth factor |
| EGFP | Enhanced green fluorescent protein |
| EHS | Engelbrecht Holm-Swarm |
| EIAV | Equine Infectious Anemia Virus |
| ES cell | Embryonic stem cell |
| FAP | Familial adenomatous polyposis |
| FCS | Fetal calf serum |
| FGF-2 | Basic fibroblast growth factor |
| g | gram |
| g | gravitational acceleration |
| GAG | Glycosaminoglycan |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| h | Hour |
| HBSS | Hanks' balanced salt solution |
| HBV | Hepatitis B Virus |
| HCl | Hydrochloric acid |
| hDAF | Human decay-accelerating factor |
| hEPO | Human erythropoietin |
| HIV-1 | Human Immunodeficiency Virus 1 |
| HpB9 | HEK293 cell clone expressing porcine BLG |
| HR | Homologous recombination |
| IBMX | 3-Isobutyl-1-methyl xanthin |
| ICSI | Intracytoplasmatic sperm injection |
| ICSI-GT | Intracytoplasmatic sperm injection gene transfer |

| | |
|---|---|
| IRES | Internal ribosome entry site |
| kb | Kilobase |
| kDa | Kilo Dalton |
| Klenow | DNA Polymerase I Large (Klenow) Fragment |
| KO | Knockout |
| l | Liter |
| LB | Luria-Bertani |
| LFS | Li-Fraumeni syndrome |
| LNA | Locked nucleic acid |
| LTR | Long terminal repeat |
| Luc | Luciferase |
| M | Molar |
| MEC | Mammary epithelial cell |
| MH | Malignant hyperthermia |
| min | Minute |
| miRNA | MicroRNA |
| ml | milliliter |
| mm | millimeter |
| MMLV | Moloney murine leukemia virus |
| mRNA | Messenger RNA |
| ms | millisecond |
| MSC | Bone marrow mesenchymal stem cell |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| ng | Nanogram |
| nm | Nanometer |
| nt | Nucleotide |
| O/N | Overnight |
| ORF | Open reading frame |
| p53 | Tumor suppressor protein p53 |
| P-body | Cytoplasmatic processing body |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PERV | Porcine endogenous retrovirus |
| PGK | Phosphoglycerate kinase |
| pmol | Picomol |
| poFF | Porcine fetal fibroblast |
| polyA | polyadenylation site |
| PrP | Prion protein |
| PS | Porcine serum |
| PTGS | Post-transcriptional gene silencing |
| PVDF | Polyvinylidene fluoride |
| Q286 | Quantum 286 |
| RGC | Ribosomal gene cluster |
| rhEGF | Recombinant human epidermal growth factor |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | RNA Interference |
| rpm | Rounds per minute |
| RSV | Rous sarcoma virus |
| RT | Room temperature |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| RYR1 | Ryanodine receptor 1 |
| s | Second |
| SCNT | Somatic cell nuclear transfer |
| SDS | Sodiumdodecylsulfate |
| SDS-PAGE | Sodiumdodecylsulfate polyacrylamide gel electrophoresis |
| shRNA | Short hairpin RNA |
| siRNA | Short interfering RNA |
| SMGT | Sperm-mediated gene transfer |
| snRNA | small nuclear RNA |
| SOD1 | Superoxide dismutase 1 |

| | |
|---|---|
| Sod2 | Superoxide dismutase 2 |
| ssRNA | single stranded RNA |
| SV40 | Simian virus 40 |
| TGF-β | Transforming growth factor beta |
| TP53 | Tumor suppressor protein p53 gene |
| TU | Transducing units |
| U | Unit |
| UTR | Untranslated region |
| UV | Ultraviolet |
| V | Volt |
| WPRE | Woodchuck hepatitis virus post-transcriptional regulatory element |
| wt | Wildtype |
| WT1 | Wilms' Tumor 1 |

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# 8   Appendix

## 8.1   Cloning of the lentiviral shRNA vector pLenti-shRNA

To generate a lentiviral RNAi plasmid, a shRNA cassette was inserted into a lentiviral backbone. Furthermore a visual marker (EGFP) and a selectable marker for mammalian cells (Blasticidin resistance) were inserted.

The first step was to clone a stuffer sequence into the shRNA plasmid IMG-800. This stuffer sequence was cloned downstream of the U6 promoter as a substitute for shRNA oligonucleotides. Therefore the plasmid pSL1180 was digested with XbaI and SalI. The 118 bp fragment was purified and ligated into the 3407 bp IMG-800 backbone cut with XbaI and SalI. The resulting plasmid was named IMG-Stuffer (see Figure 80).
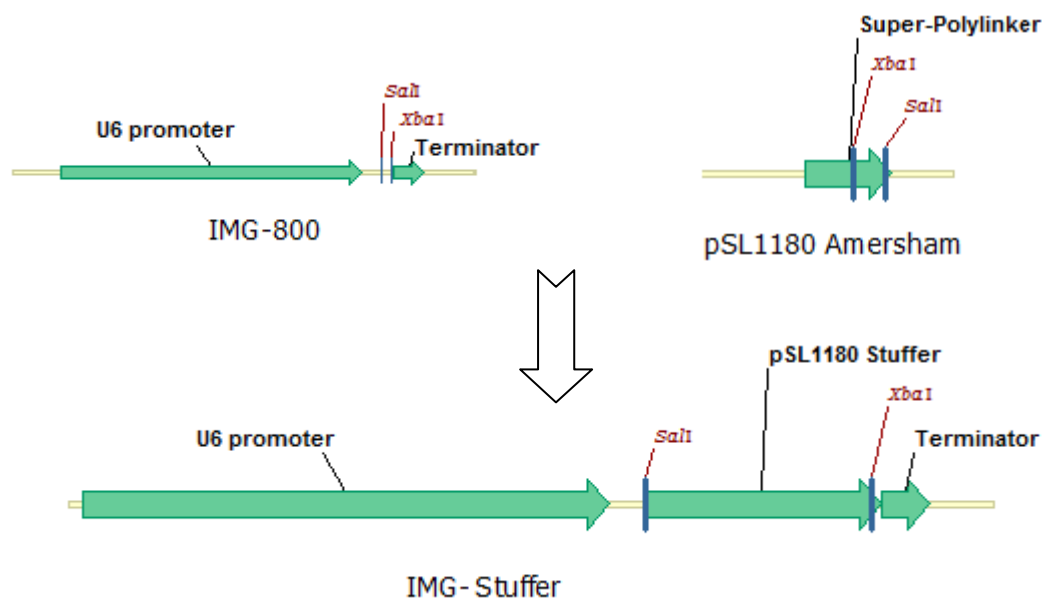


**Figure 80: Cloning of the plasmid IMG-Stuffer.** A 118 bp stuffer sequence from pSL1180 Amersham was cloned into the SalI and XbaI restrictions sites of IMG-800.

To obtain the U6-shRNA cassette with the stuffer region, IMG-800 pSL1180 Stuffer was digested with MluI, overhanging ends blunted with Klenow and then the plasmid was digested with PstI to generate a fragment of 461 bp. This fragment contained the U6 promoter, the shRNA insertion site with the stuffer region and the shRNA terminator.

The destination vector for the shRNA cassette was pLenti4s, a lentiviral vector. Because it was necessary for later cloning steps to eliminate the XbaI restriction site of the plasmid, pLenti4s was cut with XbaI, blunted with Klenow and religated to the 6346 bp plasmid pLenti4s-XbaI. To insert the shRNA cassette downstream of the lentiviral packaging signal, pLenti4s-XbaI was digested with AfeI and PstI. The 6295 bp backbone was purified. The 461 bp shRNA cassette from IMG-800 pSL118 Stuffer was ligated with the backbone to produce the plasmid 6756 bp plasmid pLenti4s-U6-Stuffer (see Figure 81).
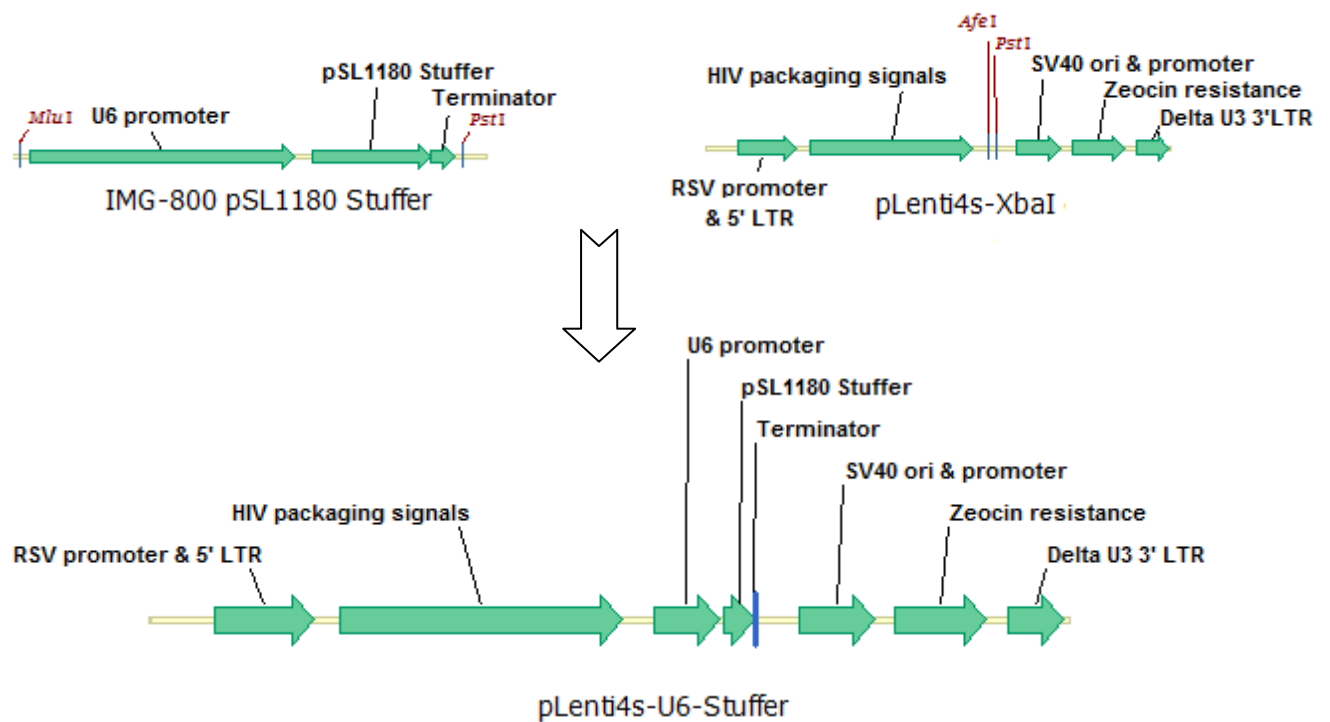
**Figure 81: Cloning of the plasmid pLenti4s-U6-Stuffer.** The shRNA cassette containing the pSL1180 Stuffer was inserted into the AfeI and PstI sites of the lentiviral plasmid pLenti4s-XbaI.

To replace the Zeocin resistance, a PGK promoter-EGFP-IRES-Blasticidin resistance cassette for selection and visual determination of transfected cells was generated. pIRES2-EGFP was cut with SmaI and XhoI to obtain a 5263 bp backbone fragment. The plasmid pPGK-EGFP1 was digested with SalI, blunted and religated to eliminate the SalI restriction site which was important for further cloning steps. pPGK-EGFP1-SalI was digested with NotI, blunted with Klenow and cut with XhoI to obtain a 1303 bp fragment containing the PGK promoter and EGFP. This fragment was ligated with pIRES2-EGFP to generate the 6566 bp plasmid pPGK-EGFP-IRES-EGFP1 (see Figure 82).
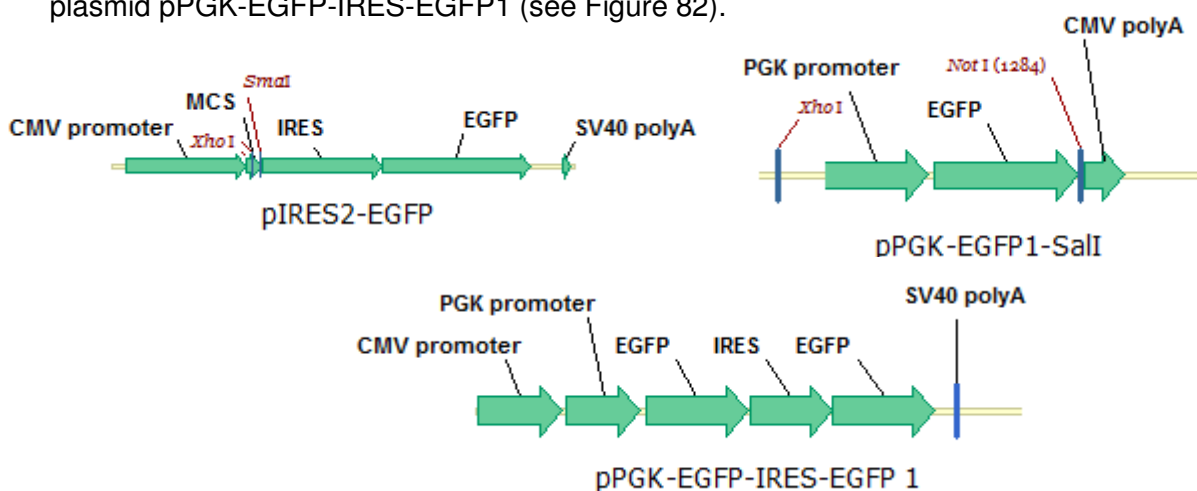


**Figure 82: Cloning of the plasmid pPGK-EGFP-IRES-EGFP1.** The PGK promoter and IRES were inserted into the vector pIRES2-EGFP.

The PGK promoter-EGFP-IRES cassette was obtained via PCR with the primers IRESPGKEGFP f/r containing EcoRV restriction sites on both primers. The PCR product (2300 bp) was digested with XhoI and EcoRV to generate a 1900 bp fragment (see Figure 83). For the Blasticidin resistance, the vector pLenti6/TR-woodchuck was cut with PmlI and SfiI upstream of the Blasticidin resistance and downstream of the SV40 polyA signal to generate a fragment of 1798 bp. To remove the Zeocin resistance cassette, the plasmid pLenti4-U6-Stuffer was cut with XhoI and SfiI and the 5213 bp backbone purified. The PGK promoter-EGFP-IRES cassette with XhoI and EcoRV ends, the Blasticidin-Woodchuck-SV40 polyA fragment with PmlI and SfiI ends were ligated with pLenti4-U6-Stuffer in a three fragment ligation to generate the lentiviral shRNA vector pLenti-shRNA (see Figure 83).
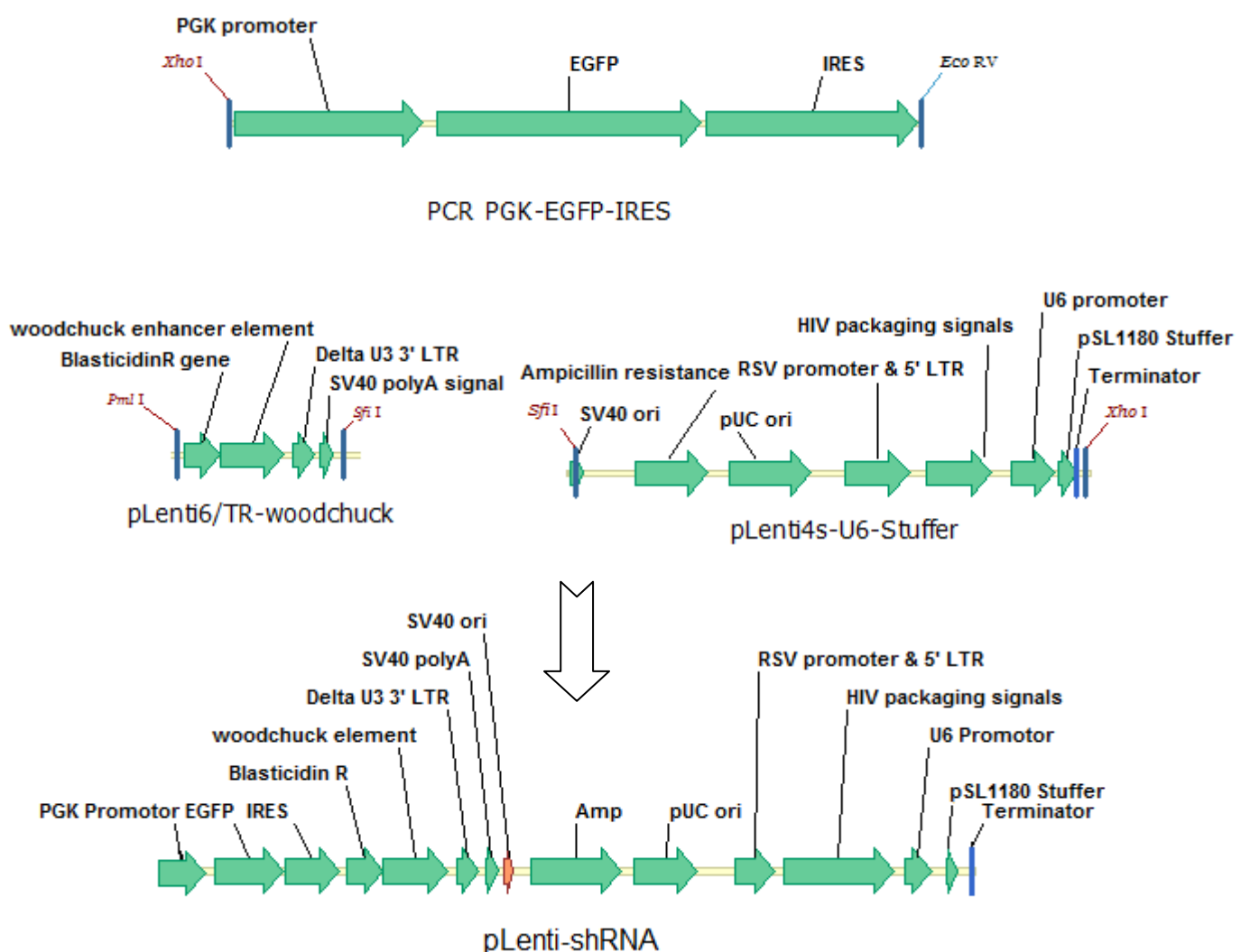


**Figure 83: Cloning of the plasmid pLenti-shRNA.** The PCR fragment containing PGK-EGFP-IRES, the cassette from pLenti6/TR-woodchuck with Blasticidin resistance, the woodchuck enhancer element, delta U3 3' LTR and SV40 polyA were ligated with the pLenti4s-U6-Stuffer backbone to create pLenti-shRNA.

## 8.2   Vector maps of plasmids for cloning or transfection

### pcDNA-BLG — 6090 bp

porcine Beta-Lactoglobulin
BGH polyA
CMV promoter
SV40 promoter/ori
NeoR
SV40 polyA
AmpR

### pSL1180 Amersham — 3422 bp

Superpolylinker
bla promoter
AmpR

### psiCHECK2 — 6273 bp

SV40 early enhancer/promoter
Chimeric intron
Renilla luciferase
Sequencing primer f
polyA
HSV-TK promoter
firefly luciferase
SV40 late polyA
AmpR

### pJET1.2-blunt — 2974 bp

AmpR
Insertion site
eco47IR
Ori

### pcDNA6.2-GW/miR — 4944 bp

Spectinomycin promoter
CMV promoter
SpectinomycinR
5' miR flanking region
3' miR flanking region
TK polyA
pUC ori
SV40 early promoter/ori
SV40 polyA
BlasticidinR

### pPGK-EGFP1 — 4658 bp

PGK promoter
EGFP
CMV polyA
Neo/KanR

pLenti4s
6342 bp

- SV40 promoter/ori
- HIV packaging signals
- Zeocin
- Delta U3 3' LTR
- RSV promoter + 5' LTR
- SV40 polyA
- pUC ori
- AmpR

pMIX-FIX
9063 bp

- pUC18
- BLG promoter
- BLG 3' UTR and polyA

IMG-800
(pSuppressorNeo)
3416 bp

- U6 promoter
- U6 snRNA +27
- Terminator
- F1 ori
- bla promoter
- pUC ori
- SV40 early promoter
- Neo/KanR

pLP1
8889 bp

- P CMV
- β-globin intron
- gag/pol
- RRE
- β-globin pA
- pUC ori
- Ampicillin

pLP2
4180 bp

- P RSV
- Rev
- pUC ori
- HIV-1 pA
- Ampicillin

pLP/VSVG
5821 bp

- P CMV
- β-globin intron
- VSV-G
- β-globin pA
- pUC ori
- Ampicillin

pLenti6/TR-woodchuck
9045 bp

- RSV promoter + 5' LTR
- 5' splice donor
- HIV packaging signals
- HIV-1 REV response element
- 3' splice acceptor
- second 3' splice acceptor
- CMV promoter
- pUC ori
- AmpR
- bla promoter
- beta-globin intron II
- tetR
- SV40 early promoter/ori
- SV40 polyA
- Delta U3 3' LTR
- WPRE
- BlasticidinR
- EM7 promoter

# 9 Literature

Aasen E. and Medrano J.F. Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats. Biotechnology. **1990** 8: 1279-1281

Agrawal N., Dasaradhi P.V., Mohmmed A., Malhotra P., Bhatnagar R.K., Mukherjee S.K. RNA Interference: Biology, mechanism, and applications. Microbiol Mol Biol Rev. **2003** 67: 657-685

Al Yacoub N., Romanowska M., Haritonova N., Foerster J. Optimized production and concentration of lentiviral vectors containing large inserts. J Gene Med. **2007** 9: 579-584

Arbonés M.L., Austin H.A., Capon D.J., Greenburg G. Gene targeting in normal somatic cells: inactivation of the interferon-gamma receptor in myoblasts. Nat Genet. **1994** 6: 90-97

Auerbach A.B. Production of functional transgenic mice by DNA pronuclear microinjection. Acta Biochim Pol. **2004** 51: 9-31

Ballester M., Sánchez A., Folch J.M. Assignment of the beta-lactoglobulin (BLG) gene to porcine chromosome 1. Anim Genet. **2005** 36: 356-358

Barcellos-Hoff M.H., Aggeler J., Ram T.G., Bissell M.J. Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. Development. **1989** 105: 223-235

Barnett S.W. Manual of Animal Technology, 1st edition **2007**, Blackwell Publishing. Pages 151-154

Baulcombe D.C. RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol Biol. **1996** 32: 79-88

Baumrucker C.R., Deemer K.P., Walsh R., Riss T.L. Akers R.M. Primary culture of bovine mammary acini on a collagen matrix. Tissue Cell. **1988** 20: 541-554

Behm-Ansmant I., Rehwinkel J., Doerks T., Stark A., Bork P., Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. **2006** 20: 1885-1898

Berezikov E., Chung W.J., Willis J., Cuppen E., Lai E.C. Mammalian mirtron genes. Mol Cell. **2007** 28: 328-336

Berns K., Hijmans E.M., Mullenders J., Brummelkamp T.R., Velds A., Heimerikx M., Kerkhoven R.M., Madiredjo M., Nijkamp W., Weigelt B., Agami R., Ge W., Cavet G., Linsley P.S., Beijersbergen R.L., Bernards R. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature. **2004** 428: 431-437

Bernstein E., Caudy A.A., Hammond S.M., Hannon G.J. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. **2001** 409: 363-366

Betthauser J., Forsberg E., Augenstein M., Childs L., Eilertsen K., Enos J., Forsythe T., Golueke P., Jurgella G., Koppang R., Lesmeister T., Mallon K., Mell G., Misica P., Pace M., Pfister-Genskow M., Strelchenko N., Voelker G., Watt S., Thompson S., Bishop M. Production of cloned pigs from in vitro systems. Nat Biotechnol. **2000** 18: 1055-1059

Beumer K., Bhattacharyya G., Bibikova M., Trautman J.K., Carroll D. Efficient gene targeting in Drosophila with zinc-finger nucleases. Genetics. **2006** 172: 2391-2403

Bianchi G., Banfi A., Mastrogiacomo M., Notaro R., Luzzatto L., Cancedda R., Quarto R. Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp Cell Res. **2003** 287: 98-105

Bibikova M., Beumer K., Trautman J.K., Carroll D. Enhancing gene targeting with designed zinc finger nucleases. Science. **2003** 300: 764

Billy E., Brondani V., Zhang H., Müller U., Filipowicz W. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc Natl Acad Sci. **2001** 98: 14428-14433

Bode A.M., Dong Z. Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer. **2004** 4: 793-805

Bogenhagen D.F., Brown D.D. Nucleotide sequences in Xenopus 5S DNA required for transcription termination. Cell. **1981** 24: 261-270

Bohula E.A., Salisbury A.J., Sohail M., Playford M.P., Riedemann J., Southern E.M., Macaulay V.M. The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGF1R transcript. J Biol Chem. **2003** 278: 15991-15997

Boquest A.C., Noer A., Collas P. Epigenetic programming of mesenchymal stem cells from human adipose tissue. Stem Cell Rev. **2006** 2: 319-329

Bosch P., Pratt S.L., Stice S.L. Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. Biol Reprod. **2006** 74: 46-57

Boudreau R.L., Monteys A.M., Davidson B.L. Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs. RNA. **2008** 14: 1834-1844

Brackett B.G., Baranska W., Sawicki W., Koprowski H. Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. Proc Natl Acad Sci. **1971** 68: 353-357

Bradley A., Evans M., Kaufman M.H., Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature. **1984** 309: 255-256

Bridge A.J., Pebernard S., Ducraux A., Nicoulaz A.L., Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet. **2003** 34: 263-264

Brown B.D., Gentner B., Cantore A., Colleoni S., Amendola M., Zingale A., Baccarini A., Lazzari G., Galli C., Naldini L. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol. **2007** 25: 1457-1467

Brummelkamp T.R., Bernards R., Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science. **2002** 296: 550-553

Brunetti D., Perota A., Lagutina I., Colleoni S., Duchi R., Calabrese F., Seveso M., Cozzi E., Lazzari G., Lucchini F., Galli C. Transgene expression of green fluorescent protein and germ line transmission in cloned pigs derived from in vitro transfected adult fibroblasts. Cloning Stem Cells. **2008** 10: 409-419

Burr P.D., Argyle D.J., Reid S.W., Nasir L. Nucleotide sequence of the porcine p53 cDNA, and the detection of recombinant porcine p53 expressed in vitro with a variety of anti-p53 antibodies. Oncogene. **1999** 18: 5005-5009

Byun H.M., Suh D., Jeong Y., Wee H.S., Kim J.M., Kim W.K., Ko J.J., Kim J.S., Lee Y.B., Oh Y.K. Plasmid vectors harboring cellular promoters can induce prolonged gene expression in hematopoietic and mesenchymal progenitor cells. Biochem Biophys Res Commun. **2005** 332: 518-523

Cabot R.A., Kühholzer B., Chan A.W., Lai L., Park K.W., Chong K.Y., Schatten G., Murphy C.N., Abeydeera L.R., Day B.N., Prather R.S. Transgenic pigs produced using in vitro matured oocytes infected with a retroviral vector. Anim Biotechnol. **2001** 12: 205-214

Cao W., Hunter R., Strnatka D., McQueen C.A., Erickson R.P. DNA constructs designed to produce short hairpin, interfering RNAs in transgenic mice sometimes show early lethality and an interferon response. J Appl Genet. **2005** 46: 217-225

Capecchi M.R. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet. **2005** 6: 507-512

Caplen N.J., Fleenor J., Fire A., Morgan R.A. dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. Gene. **2000** 252: 95-105

Caplen N.J., Parrish S., Imani F., Fire A., Morgan R.A. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci. **2001** 98: 9742-9747

Carey B.W., Markoulaki S., Hanna J., Saha K., Gao Q., Mitalipova M., Jaenisch R. Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc Natl Acad Sci. **2009** 106: 157-162

Carrington J.C, Ambros V. Role of microRNAs in plant and animal development. Science. **2003** 301: 336-338

Carthew R.W, and Sontheimer E.J. Origins and mechanisms of miRNAs and siRNAs. Cell. **2009** 136: 642-655

Chan A.W., Homan E.J., Ballou L.U., Burns J.C., Bremel R.D. Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. Proc Natl Acad Sci. **1998** 95: 14028-14033

Chari N.S., Pinaire N.L., Thorpe L., Medeiros L.J., Routbort M.J., McDonnell T.J. The p53 tumor suppressor network in cancer and the therapeutic modulation of cell death. Apoptosis. **2009** 14: 336-347

Chen J., Lin J., Levine A.J. Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. Mol Med. **1995** 1: 142-152

Chiou M., Xu Y., Longaker M.T. Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose-derived mesenchymal cells. Biochem Biophys Res Commun. **2006** 343: 644-652

Cho S.K., Hwang K.C., Choi Y.J., Bui H.T., Nguyen V.T., Park C., Kim J.H., Kim J.H. Production of Transgenic Pigs Harboring the Human Erythropoietin (hEPO) Gene Using Somatic Cell Nuclear Transfer. J Reprod Dev. **2009** 55: 128-136

Chu C.Y., Rana T.M. Small RNAs: regulators and guardians of the genome. J Cell Physiol. **2007** 213: 412-419

Cifrian E., Guidry A.J., O'Brien C.N., Nickerson S.C., Marquardt W.W. Adherence of Staphylococcus aureus to cultured bovine mammary epithelial cells. J Dairy Sci. **1994** 77: 970-983

Colleoni S., Donofrio G., Lagutina I., Duchi R., Galli C., Lazzari G. Establishment, differentiation, electroporation, viral transduction, and nuclear transfer of bovine and porcine mesenchymal stem cells. Cloning Stem Cells. **2005** 7: 154-166

Conti A., Giuffrida M.G., Napolitano L., Quaranta S., Bertino E., Coscia A., Costa S., Fabris C. Identification of the human beta-casein C-terminal fragments that specifically bind to purified antibodies to bovine beta-lactoglobulin. J Nutr Biochem. **2000** 11: 332-337

D'Alfonso L., Collini M., Ragona L., Ugolini R., Baldini G., Molinari H. Porcine beta-lactoglobulin chemical unfolding: Identification of a non-native α-helical intermediate. Proteins. **2005** 58: 70-79

Dai Y., Vaught T.D., Boone J., Chen S.H., Phelps C.J., Ball S., Monahan J.A., Jobst P.M., McCreath K.J., Lamborn A.E., Cowell-Lucero J.L., Wells K.D., Colman A., Polejaeva I.A., Ayares D.L. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol. **2002** 20: 251-255

Das G., Henning D., Wright D., Reddy R. Upstream regulatory elements are necessary and sufficient for transcription of a U6 RNA gene by RNA polymerase III. EMBO J. **1988** 7: 503-512

Debnath J., Brugge J.S. Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer. **2005** 5: 675-688

Debnath J., Muthuswamy S.K., Brugge J.S. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods. **2003** 30: 256-268

Denli A.M., Tops B.B., Plasterk R.H., Ketting R.F., Hannon G.J. Processing of primary microRNAs by the Microprocessor complex. Nature. **2004** 432: 231-235

Denning C., Priddle H. New frontiers in gene targeting and cloning: success, application and challenges in domestic animals and human embryonic stem cells. Reproduction. **2003** 126: 1-11

Deppenmeier S., Bock O., Mengel M., Niemann H., Kues W., Lemme E., Wirth D., Wonigeit K., Kreipe H. Health status of transgenic pigs expressing the human complement regulatory protein CD59. Xenotransplantation. **2006** 13: 345-356

Dickins R.A., Hemann M.T., Zilfou J.T., Simpson D.R., Ibarra I., Hannon G.J., Lowe S.W. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet. **2005** 37: 1289-1295

Dieckhoff B., Karlas A., Hofmann A., Kues W.A., Petersen B., Pfeifer A., Niemann H., Kurth R., Denner J. Inhibition of porcine endogenous retroviruses (PERVs) in primary porcine cells by RNA interference using lentiviral vectors. Arch Virol. **2007** 152: 629-634

Dieckhoff B., Petersen B., Kues W.A., Kurth R., Niemann H., Denner J. Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs. Xenotransplantation. **2008** 15: 36-45

Doench J.G., Petersen C.P., Sharp P.A. siRNAs can function as miRNAs. Genes Dev. **2003** 17: 438-442

Dominici M., Le Blanc K., Mueller I., Slaper-Cortenbach I., Marini F., Krause D., Deans R., Keating A., Prockop D.J., Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. **2006** 8: 315-317

Donehower L.A., Harvey M., Slagle B.L., McArthur M.J., Montgomery C.A. Jr, Butel J.S., Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. **1992** 356: 215-221

Doyon Y., McCammon J.M., Miller J.C., Faraji F., Ngo C., Katibah G.E., Amora R., Hocking T.D., Zhang L., Rebar E.J., Gregory P.D., Urnov F.D., Amacher S.L. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol. **2008** 26: 702-708

Düchler M., Schmoll F., Pfneisl F., Brem G., Schellander K. OMEC II: a new ovine mammary epithelial cell line. Biol Cell. **1998** 90: 199-205

Dunkern T.R., Wedemeyer I., Baumgärtner M., Fritz G., Kaina B. Resistance of p53 knockout cells to doxorubicin is related to reduced formation of DNA strand breaks rather than impaired apoptotic signaling. DNA Repair (Amst). **2003** 2: 49-60

Elbashir S.M., Lendeckel W., Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **2001a** 15: 188-200

Elbashir S.M., Harborth J., Lendeckel W., Yalcin A., Weber K., Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **2001b** 411: 494-498

Eliyahu D., Michalovitz D., Eliyahu S., Pinhasi-Kimhi O., Oren M. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci. **1989** 86: 8763-8767

Emerman J.T., Pitelka D.R. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro. **1977** 13: 316-328

Emerman J.T., Fiedler E.E., Tolcher A.W., Rebbeck P.M. Effects of defined medium, fetal bovine serum, and human serum on growth and chemosensitivities of human breast cancer cells in primary culture: inference for in vitro assays. In Vitro Cell Dev Biol. **1987** 23: 134-140

Esteban M.A., Xu J., Yang J., Peng M., Qin D., Li W., Jiang Z., Chen J., Deng K., Zhong M., Cai J., Lai L., Pei D. Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J Biol Chem. **2009** 284: 17634-17640

Evans M.J., Kaufman M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature. **1981** 292: 154-156

Evans S.C., Lozano G. The Li-Fraumeni syndrome: an inherited susceptibility to cancer. Mol Med Today. **1997** 3: 390-395

Ezashi T., Telugu B.P., Alexenko A.P., Sachdev S., Sinha S., Roberts R.M. Derivation of induced pluripotent stem cells from pig somatic cells. Proc Natl Acad Sci. **2009** 106: 10993-10998

Faast R., Harrison S.J., Beebe L.F., McIlfatrick S.M., Ashman R.J., Nottle M.B. Use of adult mesenchymal stem cells isolated from bone marrow and blood for somatic cell nuclear transfer in pigs. Cloning Stem Cells. **2006** 8: 166-173

Farrell H.M. Jr, Jimenez-Flores R., Bleck G.T., Brown E.M., Butler J.E., Creamer L.K., Hicks C.L., Hollar C.M., Ng-Kwai-Hang K.F., Swaisgood H.E. Nomenclature of the proteins of cows' milk--sixth revision. J Dairy Sci. **2004** 87: 1641-1674

Fässler R. Lentiviral transgene vectors. EMBO Rep. **2004** 5: 28-29

Fields S., Jang S.K. Presence of a potent transcription activating sequence in the p53 protein. Science. **1990** 249: 1046-1049

Finlay C.A., Hinds P.W., Levine A.J. The p53 proto-oncogene can act as a suppressor of transformation. Cell. **1989** 57: 1083-1093

Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. **1998** 391: 806-811

Fodde R. The APC gene in colorectal cancer. Eur J Cancer. **2002** 38: 867-871

Foley J.E., Yeh J.R., Maeder M.L., Reyon D., Sander J.D., Peterson R.T., Joung J.K. Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). PLoS One. **2009** 4: e4348

Foord O., Navot N., Rotter V. Isolation and characterization of DNA sequences that are specifically bound by wild-type p53 protein. Mol Cell Biol. **1993** 13: 1378-1384

Francolini M., Lavitrano M., Lamia C.L., French D., Frati L., Cotelli F., Spadafora C. Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Mol Reprod Dev. **1993** 34: 133-139

Fujii J., Otsu K., Zorzato F., de Leon S., Khanna V.K., Weiler J.E., O'Brien P.J., MacLennan D.H. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. Science. **1991** 253: 448-451

Fujimura T., Murakami H., Kurome M., Takahagi Y., Shigehisa T., Nagashima H. Effects of recloning on the efficiency of production of α1,3-galactosyltransferase knockout pigs. J Reprod Dev. **2008** 54: 58-62

Furuta Y, Behringer RR. Recent innovations in tissue-specific gene modifications in the mouse. Birth Defects Res C Embryo Today. **2005** 75: 43-57

Gallagher D.P., Cotter P.F., Mulvihill D.M. Porcine milk proteins: A review. Int Dairy Journal. **1997** 7: 99-118

Gannon J.V., Greaves R., Iggo R., Lane D.P. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J. **1990** 9: 1595-1602

German T., Barash I. Characterization of an epithelial cell line from bovine mammary gland. In Vitro Cell Dev Biol Anim. **2002** 38: 282-292

Geurts A.M., Cost G.J., Freyvert Y., Zeitler B., Miller J.C., Choi V.M., Jenkins S.S., Wood A., Cui X., Meng X., Vincent A., Lam S., Michalkiewicz M., Schilling R., Foeckler J., Kalloway S., Weiler H., Ménoret S., Anegon I., Davis G.D., Zhang L., Rebar E.J., Gregory P.D., Urnov F.D., Jacob H.J., Buelow R. Knockout rats via embryo microinjection of zinc-finger nucleases. Science. **2009** 325: 433

Giering J.C., Grimm D., Storm T.A., Kay M.A. Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic Mol Ther. **2008** 16: 1630-1636

Godovac-Zimmermann J., Conti A., Liberatori J., Braunitzer G. Homology between the primary structures of beta-lactoglobulins and human retinol-binding protein: evidence for a similar biological function? Biol Chem Hoppe Seyler. **1985** 366: 431-434

Golding M.C., Long C.R., Carmell M.A., Hannon G.J., Westhusin M.E. Suppression of prion protein in livestock by RNA interference. Proc Natl Acad Sci. **2006** 103: 5285-5290

Gonzalez-Alegre P., Paulson H.L. Technology insight: therapeutic RNA interference--how far from the neurology clinic? Nat Clin Pract Neurol. **2007** 3: 394-404

Gordon J.W., Scangos G.A., Plotkin D.J., Barbosa J.A., Ruddle F.H. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci. **1980** 77: 7380-7384

Greenblatt M.S., Bennett W.P., Hollstein M., Harris C.C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. **1994** 54: 4855-4878

Gregory R.I., Chendrimada T.P., Cooch N., Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. **2005** 123: 631-640

Gregory R.I., Yan K.P., Amuthan G., Chendrimada T., Doratotaj B., Cooch N., Shiekhattar R. The Microprocessor complex mediates the genesis of microRNAs. Nature. **2004** 432: 235-240

Grimm D., Streetz K.L., Jopling C.L., Storm T.A., Pandey K., Davis C.R., Marion P., Salazar F., Kay M.A. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature. **2006** 441: 537-541

Hajeri P.B., Singh S.K. siRNAs: their potential as therapeutic agents - Part I. Designing of siRNAs. Drug Discov Today. **2009** 14: 851-858

Halliday J.A., Bell K., McKenzie H.A., Shaw D.C. Feline whey proteins: identification, isolation and initial characterization of alpha-lactalbumin, beta-lactoglobulin and lysozyme. Comp Biochem Physiol B. **1990** 95: 773-779

Hammer R.E., Pursel V.G., Rexroad C.E. Jr, Wall R.J., Bolt D.J., Ebert K.M., Palmiter R.D., Brinster R.L. Production of transgenic rabbits, sheep and pigs by microinjection. Nature. **1985** 315: 680-683

Hammond S.L., Ham R.G., Stampfer M.R. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc Natl Acad Sci. **1984** 81: 5435-5439

Hammond S.M., Bernstein E., Beach D., Hannon G.J. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature. **2000** 404: 293-296

Hasuwa H., Kaseda K., Einarsdottier T., Okabe M. Small interfering RNA and gene silencing in transgenic mice and rats. FEBS Lett. **2002** 532: 227-230

Hemann M.T., Fridman J.S., Zilfou J.T., Hernando E., Paddison P.J., Cordon-Cardo C., Hannon G.J., Lowe S.W. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nat Genet. **2003** 33: 396-400

Hendrie P.C., Russell D.W. Gene targeting with viral vectors. Mol Ther. **2005** 12: 9-17

Hirabayashi M., Takahashi R., Ito K., Kashiwazaki N., Hirao M., Hirasawa K., Hochi S., Ueda M. A comparative study on the integration of exogenous DNA into mouse, rat, rabbit, and pig genomes. Exp Anim. **2001** 50: 125-131

Hockemeyer D., Soldner F., Beard C., Gao Q., Mitalipova M., Dekelver R.C., Katibah G.E., Amora R., Boydston E.A., Zeitler B., Meng X., Miller J.C., Zhang L., Rebar E.J., Gregory P.D., Urnov F.D., Jaenisch R. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol. **2009** 27: 851-857

Hoedemaeker F.J., Visschers R.W., Alting A.C., De Kruif K.G., Kuil M.E., Abrahams J.P. A novel pH-dependent dimerization motif in β-lactoglobulin from pig (sus scrofa). Acta Cryst. **2002** D58: 480-486

Hofmann A., Kessler B., Ewerling S., Weppert M., Vogg B., Ludwig H., Stojkovic M., Boelhauve M., Brem G., Wolf E., Pfeifer A. Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep. **2003** 4: 1054-1060

Hong H., Takahashi K., Ichisaka T., Aoi T., Kanagawa O., Nakagawa M., Okita K., Yamanaka S. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature. **2009** 460: 1132-1135

Hong J., Wei N., Chalk A., Wang J., Song Y., Yi F., Qiao R.P., Sonnhammer E.L., Wahlestedt C., Liang Z., Du Q. Focusing on RISC assembly in mammalian cells. Biochem Biophys Res Commun. **2008** 368: 703-708

Hornung V., Guenthner-Biller M., Bourquin C., Ablasser A., Schlee M., Uematsu S., Noronha A., Manoharan M., Akira S., de Fougerolles A., Endres S., Hartmann G. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med. **2005** 11: 263-270

Humphreys D.T., Westman B.J., Martin D.I., Preiss T. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci. **2005** 102: 16961-16966

Huynh H.T., Robitaille G., Turner J.D. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res. **1991** 197: 191-199

Hyun S., Lee G., Kim D., Kim H., Lee S., Nam D., Jeong Y., Kim S., Yeom S., Kang S., Han J., Lee B., Hwang W. Production of nuclear transfer-derived piglets using porcine fetal fibroblasts transfected with the enhanced green fluorescent protein. Biol Reprod. **2003** 69: 1060-1068

Idogawa M., Sasaki Y., Suzuki H., Mita H., Imai K., Shinomura Y., Tokino T. A single recombinant adenovirus expressing p53 and p21-targeting artificial microRNAs efficiently induces apoptosis in human cancer cells. Clin Cancer Res. **2009** 15: 3725-3732

Ilan N., Barash I., Gootwine E., Shani M. Establishment and initial characterization of the ovine mammary epithelial cell line NISH. In Vitro Cell Dev Biol Anim. **1998** 34: 326-332

Invitrogen, Manual BLOCK-iT Pol II miR RNAi Expression Vector Kits, Version E, **2007**

Ip M.M., Darcy K.M. Three-dimensional mammary primary culture model systems. J Mammary Gland Biol Neoplasia. **1996** 1: 91-110

I-Tsuen Chen E.,Wu J.R., Su W.H., Wang T.H., Au L.C. Selective Inhibition of p53 Dominant Negative Mutation by shRNA Resulting in Partial Restoration of p53 Activity. J Med Sci. **2009** 29: 179-185

Jacks T., Remington L., Williams B.O., Schmitt E.M., Halachmi S., Bronson R.T., Weinberg R.A. Tumor spectrum analysis in p53-mutant mice. Curr Biol. **1994** 4: 1-7

Jackson A.L., Bartz S.R., Schelter J., Kobayashi S.V., Burchard J., Mao M., Li B., Cavet G., Linsley P.S. Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol. **2003** 21: 635-637

Jaenisch R. Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. Proc Natl Acad Sci. **1976** 73: 1260-1264

Jakobsson I., Lindberg T., Benediktsson B., Hansson B.G. Dietary bovine beta-lactoglobulin is transferred to human milk. Acta Paediatr Scand. **1985** 74: 342-345

Jin H.F., Kumar B.M., Kim J.G., Song H.J., Jeong Y.J., Cho S.K., Balasubramanian S., Choe S.Y., Rho G.J. Enhanced development of porcine embryos cloned from bone marrow mesenchymal stem cells. Int J Dev Biol. **2007** 51: 85-90

Jones S.B., Kalan E.B. Modified procedure for isolation of a major swine whey protein. J Dairy Sci. **1971** 54: 288-291

Judge A.D., Sood V., Shaw J.R., Fang D., McClintock K., MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol. **2005** 23: 457-462

Kagan R.S. Food allergy: an overview. Environ Health Perspect. **2003** 111: 223-225

Kakudo N., Shimotsuma A., Kusumoto K. Fibroblast growth factor-2 stimulates adipogenic differentiation of human adipose-derived stem cells. Biochem Biophys Res Commun. **2007** 359: 239-244

Kato Y., Imabayashi H., Mori T., Tani T., Taniguchi M., Higashi M., Matsumoto M., Umezawa A., Tsunoda Y. Nuclear transfer of adult bone marrow mesenchymal stem cells: developmental totipotency of tissue-specific stem cells from an adult mammal. Biol Reprod. **2004** 70: 415-418

Kawamura T., Suzuki J., Wang Y.V., Menendez S., Morera L.B., Raya A., Wahl G.M., Belmonte J.C. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature. **2009** 460: 1140-1144

Kern S.E., Kinzler K.W., Bruskin A., Jarosz D., Friedman P., Prives C., Vogelstein B. Identification of p53 as a sequence-specific DNA-binding protein. Science. **1991** 252: 1708-1711.

Kessler E., Brew K. The whey proteins of pig's milk. Isolation and characterization of a β-lactoglobulin. Biochim Biophys Acta. **1970** 200: 449-458

Ketting R.F., Fischer S.E., Bernstein E., Sijen T., Hannon G.J., Plasterk R.H. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. **2001** 15: 2654-2659

Khvorova A., Reynolds A., Jayasena S.D. Functional siRNAs and miRNAs exhibit strand bias. Cell. **2003** 115: 209-216

Kim J., Cho I.S., Hong J.S., Choi Y.K., Kim H., Lee Y.S. Identification and characterization of new microRNAs from pig. Mamm Genome. **2008** 19: 570-580

Kleinman H.K., Martin G.R. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol. **2005** 15: 378-386

Kong Y.W., Cannell I.G., de Moor C.H., Hill K., Garside P.G., Hamilton T.L., Meijer H.A., Dobbyn H.C., Stoneley M., Spriggs K.A., Willis A.E., Bushell M. The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. Proc Natl Acad Sci. **2008** 105: 8866-8871

Kontopidis G,. Holt C., Sawyer L. The ligand-binding site of bovine beta-lactoglobulin: evidence for a function? J Mol Biol. **2002** 318: 1043-1055

Kontopidis G., Holt C., Sawyer L. Invited review: beta-lactoglobulin: binding properties, structure, and function. J Dairy Sci. **2004** 87: 785-796

Kotnik K., Popova E., Todiras M., Mori M.A., Alenina N., Seibler J., Bader M. Inducible transgenic rat model for diabetes mellitus based on shRNA-mediated gene knockdown. PLoS ONE. **2009** 4: e5124

Kretschmer-Kazemi Far R., Sczakiel G. The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. Nucleic Acids Res. **2003** 31: 4417-4424

Kumar B.M., Jin H.F., Kim J.G., Ock S.A., Hong Y., Balasubramanian S., Choe S.Y., Rho G.J. Differential gene expression patterns in porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. Dev Dyn. **2007** 236: 435-446

Kumar R., Conklin D.S., Mittal V. High-throughput selection of effective RNAi probes for gene silencing. Genome Res. **2003** 13: 2333-2340

Kumura H., Tanaka A., Abo Y., Yui S., Shimazaki K., Kobayashi E., Sayama K. Primary culture of porcine mammary epithelial cells as a model system for evaluation of milk protein expression. Biosci Biotechnol Biochem. **2001** 65: 2098-2101

Kunath T., Gish G., Lickert H., Jones N,. Pawson T., Rossant J. Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. Nat Biotechnol. **2003** 21: 559-561

Kunkel G.R., Maser R.L., Calvet J.P., Pederson T. U6 small nuclear RNA is transcribed by RNA polymerase III. Proc Natl Acad Sci. **1986** 83: 8575-8579

Kurome M., Ueda H., Tomii R., Naruse K., Nagashima H. Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. Transgenic Res. **2006** 15: 229-240

Kushibiki S., Hodate K., Kurisaki J., Shingu H., Ueda Y., Watanabe A., Shinoda M. Effect of beta-lactoglobulin on plasma retinol and triglyceride concentrations, and fatty acid composition in calves. J Dairy Res. **2001** 68: 579-586

Lagos-Quintana M., Rauhut R., Lendeckel W., Tuschl T. Identification of novel genes coding for small expressed RNAs. Science. **2001** 294: 853-858

Lai L., Kolber-Simonds D., Park K.W., Cheong H.T., Greenstein J.L., Im G.S., Samuel M., Bonk A., Rieke A., Day B.N., Murphy C.N., Carter D.B., Hawley R.J., Prather R.S. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science. **2002** 295: 1089-1092

Lane D.P. Cancer. p53, guardian of the genome. Nature. **1992** 358: 15-16

Lane D.P., Crawford L.V. T antigen is bound to a host protein in SV40-transformed cells. Nature. **1979** 278: 261-263

Lau N.C., Lim L.P., Weinstein E.G., Bartel D.P. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science. **2001** 294: 858-862

Lavitrano M., Busnelli M., Cerrito M.G., Giovannoni R., Manzini S., Vargiolu A. Sperm-mediated gene transfer. Reprod Fertil Dev. **2006** 18: 19-23

Lavitrano M., Camaioni A., Fazio V.M., Dolci S., Farace M.G., Spadafora C. Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell. **1989** 57: 717-723

LeBleu V.S., Macdonald B., Kalluri R. Structure and function of basement membranes. Exp Biol Med. **2007** 232: 1121-1129

Lee G.S., Kim H.S., Hyun S.H., Lee S.H., Jeon H.Y., Nam D.H., Jeong Y.W., Kim S., Kim J.H., Han J.Y., Ahn C., Kang S.K., Lee B.C., Hwang W.S. Production of transgenic cloned piglets from genetically transformed fetal fibroblasts selected by green fluorescent protein. Theriogenology. **2005** 63: 973-991

Lee R.C. and Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. **2001** 294: 862-864

Lee R.C., Feinbaum R.L., Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. **1993** 75: 843-854

Lee S.Y., Lim J., Khang G., Son Y., Choung P.H., Kang S.S., Chun S.Y., Shin H.I., Kim S.Y., Park E.K. Enhanced ex vivo expansion of human adipose tissue-derived mesenchymal stromal cells by fibroblast growth factor-2 and dexamethasone. Tissue Eng Part A. **2009** 15: 2491-2499

Lee Y., Ahn C., Han J., Choi H., Kim J., Yim J., Lee J., Provost P., Rådmark O., Kim S., Kim V.N. The nuclear RNase III Drosha initiates microRNA processing. Nature. **2003** 425: 415-419

Lee Y., Jeon K., Lee J.T., Kim S., Kim V.N. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. **2002** 21: 4663-4670

Lee Y., Kim M., Han J., Yeom K.H., Lee S., Baek S.H., Kim V.N. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. **2004** 23: 4051-4060

Li M.L., Aggeler J., Farson D.A., Hatier C., Hassell J., Bissell M.J. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc Natl Acad Sci. **1987** 84: 136-140

Li W., Wei W., Zhu S., Zhu J., Shi Y., Lin T., Hao E., Hayek A., Deng H., Ding S. Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell. **2009** 4: 16-19

Liao J., Cui C., Chen S., Ren J., Chen J., Gao Y., Li H., Jia N., Cheng L., Xiao H., Xiao L. Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell. **2009** 4: 11-15

Lin L., Xu W., Dai Y., Li N. DNA methylation changes in cell line from β-lactoglobulin gene targeted fetus. Anim Reprod Sci. **2009** 112: 402-408

Lin X., Ruan X., Anderson M.G., McDowell J.A., Kroeger P.E., Fesik S.W., Shen Y. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. Nucleic Acids Res. **2005** 33: 4527-4535

Linzer D.I., Levine A.J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell. **1979** 17: 43-52

Liu H., Zhu F., Yong J., Zhang P., Hou P., Li H., Jiang W., Cai J., Liu M., Cui K., Qu X., Xiang T., Lu D., Chi X., Gao G., Ji W., Ding M., Deng H. Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell. **2008** 3: 587-590

Liu J., Carmell M.A., Rivas F.V., Marsden C.G., Thomson J.M., Song J.J., Hammond S.M., Joshua-Tor L., Hannon G.J. Argonaute2 is the catalytic engine of mammalian RNAi. Science. **2004** 305: 1437-1441

Liu J., Rivas F.V., Wohlschlegel J., Yates J.R. 3rd, Parker R., Hannon G.J. A role for the P-body component GW182 in microRNA function. Nature Cell Biology. **2005b** 7: 1261 - 1266

Liu J., Valencia-Sanchez M.A., Hannon G.J., Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol. **2005a** 7: 719-723

Lois C., Hong E.J., Pease S., Brown E.J., Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science. **2002** 295: 868-872

López-Fraga M., Wright N., Jiménez A. RNA interference-based therapeutics: new strategies to fight infectious disease. Infect Disord Drug Targets. **2008** 8: 262-273

Lund E., Güttinger S., Calado A., Dahlberg J.E., Kutay U. Nuclear export of microRNA precursors. Science. **2004** 303: 95-98

Lunney J.K. Advances in swine biomedical model genomics. Int J Biol Sci. **2007** 3: 179-184

MacLennan D.H., Phillips M.S. Malignant hyperthermia. Science. **1992** 256: 789-794

Macrae I.J., Zhou K., Li F., Repic A., Brooks A.N., Cande W.Z., Adams P.D., Doudna J.A. Structural basis for double-stranded RNA processing by Dicer. Science. **2006** 311: 195-198

Maier B., Wendt S., Vanselow J.T., Wallach T., Reischl S., Oehmke S., Schlosser A., Kramer A. A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. Genes Dev. **2009** 23: 708-718

Malkin D., Li F.P., Strong L.C., Fraumeni J.F. Jr, Nelson C.E., Kim D.H., Kassel J., Gryka M.A., Bischoff F.Z., Tainsky M.A., Friend S.H. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. **1990** 250: 1233-1238

Malladi P., Xu Y., Yang G.P., Longaker M.T. Functions of vitamin D, retinoic acid, and dexamethasone in mouse adipose-derived mesenchymal cells. Tissue Eng. **2006** 12: 2031-2040

Mäntyjärvi R., Rautiainen J., Virtanen T. Lipocalins as allergens. Biochim Biophys Acta. **2000** 1482: 308-317

Manzini S., Vargiolu A., Stehle I.M., Bacci M.L., Cerrito M.G., Giovannoni R., Zannoni A., Bianco M.R., Forni M., Donini P., Papa M., Lipps H.J., Lavitrano M. Genetically modified pigs produced with a nonviral episomal vector. Proc Natl Acad Sci. **2006** 103: 17672-17677

Marión R.M., Strati K., Li H., Murga M., Blanco R., Ortega S., Fernandez-Capetillo O., Serrano M., Blasco MA. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature. **2009** 460: 1149-1153

Maroney P.A., Yu Y., Fisher J., Nilsen T.W. Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol. **2006** 13: 1102-1107

Martin G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci. **1981** 78: 7634-7638

Matranga C., Tomari Y., Shin C., Bartel D.P., Zamore P.D. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell. **2005** 123: 607-620

McBride J.L., Boudreau R.L., Harper S.Q., Staber P.D., Monteys A.M., Martins I., Gilmore B.L., Burstein H., Peluso R.W., Polisky B., Carter B.J., Davidson B.L. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. Proc Natl Acad Sci. **2008** 105: 5868-5873

McBurney M.W., Staines W.A., Boekelheide K., Parry D., Jardine K., Pickavance L. Murine PGK-1 promoter drives widespread but not uniform expression in transgenic mice. Dev Dyn. **1994** 200: 278-293

McCreath K.J., Howcroft J., Campbell K.H., Colman A., Schnieke A.E., Kind A.J. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature. **2000** 405: 1066-1069

McGee S.F., Lanigan F., Gilligan E., Groner B. Mammary gland biology and breast cancer. Conference on Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression. EMBO Rep. **2006** 7: 1084-1088

McGrath C.M. Cell Organization and Responsiveness to Hormones in Vitro: Genesis of Domes in Mammary Cell Cultures. Amer Zool. **1975** 15: 231-236

McKenzie H.A., Sawyer W.H. Effect of pH on beta-lactoglobulins. Nature. **1967** 214: 1101-1104

McManus M.T., Petersen C.P., Haines B.B., Chen J., Sharp P.A. Gene silencing using micro-RNA designed hairpins. RNA. **2002** 8: 842-850

Mead E.A., Tu Z. Cloning, characterization, and expression of microRNAs from the Asian malaria mosquito, Anopheles stephensi. BMC Genomics. **2008** 9:244

Medina D., Li M.L., Oborn C.J., Bissell M.J. Casein gene expression in mouse mammary epithelial cell lines: dependence upon extracellular matrix and cell type. Exp Cell Res. **1987** 172: 192-203

Mehta A.K., Majumdar S.S., Alam P., Gulati N., Brahmachari V. Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. Gene. **2009** 428: 20-24

Meister G., Landthaler M., Patkaniowska A., Dorsett Y., Teng G., Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. **2004** 15: 185-197

Melo E.O., Canavessi A.M., Franco M.M., Rumpf R. Animal transgenesis: state of the art and applications. J Appl Genet. **2007** 48: 47-61

Meng X., Noyes M.B., Zhu L.J., Lawson N.D., Wolfe S.A. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat Biotechnol. **2008** 26: 695-701

Mercier J.C., Hazé G., Addeo F., Gaye P., Hue D., Raymond M.N.  Amino terminal sequence of porcine pre-beta-lactoglobulin. Comparison with its ovine counterpart. Biochem Biophys Res Commun. **1980** 97: 802-810

Miller D.G., Petek L.M., Russell D.W. Human gene targeting by adeno-associated virus vectors is enhanced by DNA double-strand breaks. Mol Cell Biol. **2003** 23: 3550-3557

Miyagishi M. and Taira K. U6 promoter–driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nat Biotechnol. **2002** 20: 497 - 500

Momand J., Jung D., Wilczynski S., Niland J. The MDM2 gene amplification database. Nucleic Acids Res. **1998** 26: 3453-3459

Monti J.C., Mermoud A.F., Jollès P. Anti-bovine beta-lactoglobulin antibodies react with a human lactoferrin fragment and bovine beta-lactoglobulin present in human milk. Experientia. **1989** 45: 178-180

Myslinski E., Amé J.C., Krol A., Carbon P. An unusually compact external promoter for RNA polymerase III transcription of the human H1 RNA gene. Nucleic Acids Res. **2001** 29: 2502-2509

Napoli C., Lemieux C., Jorgensen R. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell. **1990** 2: 279-289

Naqvi A.R., Islam M.N., Choudhury N.R., Haq Q.M. The fascinating world of RNA interference. Int J Biol Sci. **2009** 5: 97-117

Niemann H., Verhoeyen E., Wonigeit K., Lorenz R., Hecker J., Schwinzer R., Hauser H., Kues W.A., Halter R., Lemme E., Herrmann D., Winkler M., Wirth D., Paul D. Cytomegalovirus early promoter induced expression of hCD59 in porcine organs provides protection against hyperacute rejection. Transplantation. **2001** 72: 1898-1906

Niwa H., Yamamura K., Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene. **1991** 108: 193-199

O'Hara S.P., Mott J.L., Splinter P.L., Gores G.J., Larusso N.F. MicroRNAs: Key modulators of posttranscriptional gene expression. Gastroenterology. **2009** 136: 17-25

Oh H.Y., Jin X., Kim J.G., Oh M.J., Pian X., Kim J.M., Yoon M.S., Son C.I., Lee Y.S., Hong K.C., Kim H., Choi Y.J., Whang K.Y. Characteristics of primary and immortalized fibroblast cells derived from the miniature and domestic pigs. BMC Cell Biol. **2007** 8: 20

Okamura K., Hagen J.W., Duan H., Tyler D.M., Lai E.C. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell. **2007** 130: 89-100

Okamura K., Ishizuka A., Siomi H., Siomi M.C. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. **2004** 18: 1655-1666

Okita K., Ichisaka T., Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. **2007** 448: 313-317

Okita K., Nakagawa M., Hyenjong H., Ichisaka T., Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. Science. **2008** 322: 949-953

Onishi A., Iwamoto M., Akita T., Mikawa S., Takeda K., Awata T., Hanada H., Perry A.C. Pig cloning by microinjection of fetal fibroblast nuclei. Science. **2000** 289: 1188-1190

Orban T.I., Izaurralde E. Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. RNA. **2005** 11: 459-469

Osada T., Toyoda A., Moisyadi S., Akutsu H., Hattori M., Sakaki Y., Yanagimachi R. Production of inbred and hybrid transgenic mice carrying large (> 200 kb) foreign DNA fragments by intracytoplasmic sperm injection. Mol Reprod Dev. **2005** 72: 329-335

Overhoff M., Alken M., Far R.K., Lemaitre M., Lebleu B., Sczakiel G., Robbins I. Local RNA target structure influences siRNA efficacy: a systematic global analysis. J Mol Biol. **2005** 348: 871-881

Paddison P.J., Caudy A.A., Bernstein E., Hannon G.J., Conklin D.S. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **2002** 16:948-958

Palmiter R.D., Brinster R.L. Transgenic mice. Cell. **1985** 41: 343-345

Palmiter R.D., Chen H.Y., Brinster R.L. Differential regulation of methallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. Cell. **1982** 29: 701-710

Pantschenko A.G., Woodcock-Mitchell J., Bushmich S.L., Yang T.J. Establishment and characterization of a caprine mammary epithelial cell line (CMEC). In Vitro Cell Dev Biol Anim. **2000** 36: 26-37

Papiz M.Z., Sawyer L., Eliopoulos E.E., North A.C., Findlay J.B., Sivaprasadarao R., Jones T.A., Newcomer M.E., Kraulis P.J. The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein. Nature. **1986** 324: 383-385

Pasquinelli A.E. MicroRNAs: deviants no longer. Trends Genet. **2002** 18: 171-173

Paul C.P., Good P.D., Winer I., Engelke D.R. Effective expression of small interfering RNA in human cells. Nat Biotechnol. **2002** 20: 505-508

Pérez M.D., Calvo M. Interaction of beta-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. J Dairy Sci. **1995** 78: 978-988

Pérez M.D., Díaz de Villegas C., Sánchez L., Aranda P., Ena J.M., Calvo M. Interaction of fatty acids with beta-lactoglobulin and albumin from ruminant milk. J Biochem. **1989** 106: 1094-1097

Pérez M.D., Sanchez L., Aranda P., Ena J.M., Oria R., Calvo M. Effect of beta-lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. Biochim Biophys Acta. **1992** 1123: 151-155

Perry A.C., Wakayama T., Kishikawa H., Kasai T., Okabe M., Toyoda Y., Yanagimachi R. Mammalian transgenesis by intracytoplasmic sperm injection. Science. **1999** 284: 1180-1183

Pervaiz S., Brew K. Purification and characterization of the major whey proteins from the milks of the bottlenose dolphin (Tursiops truncatus), the Florida manatee (Trichechus manatus latirostris), and the beagle (Canis familiaris). Arch Biochem Biophys. **1986** 246: 846-854

Petersen C.P., Bordeleau M.E., Pelletier J., Sharp P.A. Short RNAs repress translation after initiation in mammalian cells. Mol Cell. **2006** 21: 533-542

Pfeifer A. Lentiviral transgenesis. Transgenic Res. **2004** 13: 513-522

Piedrahita J.A. Gene targeting in domestic species: a new beginning. Transgenic Res. **2000** 9: 261-262

Pillai R.S., Bhattacharyya S.N., Artus C.G., Zoller T., Cougot N., Basyuk E., Bertrand E., Filipowicz W. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. **2005** 309: 1573-1576

Pillai R.S., Bhattacharyya S.N., Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. **2007** 17: 118-126

Polejaeva I.A., Chen S.H., Vaught T.D., Page R.L., Mullins J., Ball S., Dai Y., Boone J., Walker S., Ayares D.L., Colman A., Campbell K.H. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature. **2000** 407: 86-90

Poluri A., Sutton R.E. Titers of HIV-based vectors encoding shRNAs are reduced by a dicer-dependent mechanism. Mol Ther. **2008 1**6: 378-386

Pomp D., Good B.A., Geisert R.D., Corbin C.J., Conley A.J. Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of day-10 or -11 pig embryos. J Anim Sci. **1995** 73: 1408-1415

Porteus M.H., Carroll D. Gene targeting using zinc finger nucleases. Nat Biotechnol. **2005** 23: 967-973

Porteus M.H., Cathomen T., Weitzman M.D., Baltimore D. Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. Mol Cell Biol. **2003** 23: 3558-3565

Poueymirou W.T., Auerbach W., Frendewey D., Hickey J.F., Escaravage J.M., Esau L., Doré A.T., Stevens S., Adams N.C., Dominguez M.G., Gale N.W., Yancopoulos G.D., DeChiara T.M., Valenzuela D.M. F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. Nat Biotechnol. **2007** 25: 91-99

Prawitt D., Brixel L., Spangenberg C., Eshkind L., Heck R., Oesch F., Zabel B., Bockamp E. RNAi knock-down mice: an emerging technology for post-genomic functional genetics. Cytogenet Genome Res. **2004** 105: 412-421

Promega, Technical Bulletin #TB329, siCHECK Vectors, **06/09**

Provost P., Dishart D., Doucet J., Frendewey D., Samuelsson B., Rådmark O. Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J. **2002** 21: 5864-5874

Pruett-Miller S.M., Connelly J.P., Maeder M.L., Joung J.K., Porteus M.H.Comparison of zinc finger nucleases for use in gene targeting in mammalian cells. Mol Ther. **2008** 16: 707-717

Qin B.Y., Creamer L.K., Baker E.N., Jameson G.B. 12-Bromododecanoic acid binds inside the calyx of bovine beta-lactoglobulin. FEBS Lett. **1998** 438: 272-278

Qiu L., Wang H., Xia X., Zhou H., Xu Z. A construct with fluorescent indicators for conditional expression of miRNA. BMC Biotechnol. **2008a** 8:77

Qiu Y., Shen Y., Li X., Liu Q., Ma Z. Polyclonal antibody to porcine p53 protein: a new tool for studying the p53 pathway in a porcine model. Biochem Biophys Res Commun. **2008b** 377: 151-155

Qu C.Q., Zhang G.H., Zhang L.J., Yang G.S. Osteogenic and adipogenic potential of porcine adipose mesenchymal stem cells. In Vitro Cell Dev Biol Anim. **2007** 43: 95-100

Quarto N., Longaker M.T. FGF-2 inhibits osteogenesis in mouse adipose tissue-derived stromal cells and sustains their proliferative and osteogenic potential state. Tissue Eng. **2006** 12: 1405-1418

Ramkissoon S.H., Mainwaring L.A., Sloand E.M., Young N.S., Kajigaya S. Nonisotopic detection of microRNAs using digoxigenin labeled RNA probes. Mol Cell Probes. **2006** 20: 1-4

Ramsoondar J., Vaught T., Ball S., Mendicino M., Monahan J., Jobst P., Vance A., Duncan J., Wells K., Ayares D. Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs. Xenotransplantation. **2009** 16: 164-180

Rao D.D., Vorhies J.S., Senzer N., Nemunaitis J. siRNA vs. shRNA: similarities and differences. Adv Drug Deliv Rev. **2009** 61: 746-759

Rao M.K., Wilkinson M.F. Tissue-specific and cell type-specific RNA interference in vivo. Nat Protoc. **2006** 1: 1494-1501

Rao M.K., Pham J., Imam J.S., MacLean J.A., Murali D., Furuta Y., Sinha-Hikim A.P., Wilkinson M.F. Tissue-specific RNAi reveals that WT1 expression in nurse cells controls germ cell survival and spermatogenesis. Genes Dev. **2006** 20: 147-152

Reddy A.M., Zheng Y., Jagadeeswaran G., Macmil S.L., Graham W.B., Roe B.A., Desilva U., Zhang W., Sunkar R. Cloning, characterization and expression analysis of porcine microRNAs. BMC Genomics. **2009** 10: 65

Rehwinkel J., Behm-Ansmant I., Gatfield D., Izaurralde E. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA. **2005** 11: 1640-1647

Reinhart B.J., Slack F.J., Basson M, Pasquinelli A.E., Bettinger J.C., Rougvie A.E., Horvitz H.R., Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. **2000** 403: 901-906

Rettenberger G., Klett C., Hameister H. Localization of the tumour protein, TP53, on porcine chromosome 12q12-q14. Anim Genet. **1993** 24: 307-309

Reynolds A., Leake D., Boese Q., Scaringe S., Marshall W.S., Khvorova A.  Rational siRNA design for RNA interference. Nat Biotechnol. **2004** 22: 326-330

Ringe J., Kaps C., Schmitt B., Büscher K., Bartel J., Smolian H., Schultz O., Burmester G.R., Häupl T., Sittinger M. Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res. **2002** 307: 321-327

Ro S., Park C., Young D., Sanders K.M., Yan W. Tissue-dependent paired expression of miRNAs. Nucleic Acids Res. **2007** 35: 5944-5953

Robl J.M., Wang Z., Kasinathan P., Kuroiwa Y. Transgenic animal production and animal biotechnology. Theriogenology. **2007** 67: 127-133

Rodriguez-Campos A, Ruiz-Enríquez P, Faraudo S, Badimon L. Mitogen-induced p53 downregulation precedes vascular smooth muscle cell migration from healthy tunica media and proliferation. Arterioscler Thromb Vasc Biol. **2001** 21: 214-219

Rogers C.S., Hao Y., Rokhlina T., Samuel M., Stoltz D.A., Li Y., Petroff E., Vermeer D.W., Kabel A.C., Yan Z., Spate L., Wax D., Murphy C.N., Rieke A., Whitworth K., Linville M.L., Korte S.W., Engelhardt J.F., Welsh M.J., Prather R.S. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest. **2008** 118: 1571-1577

Romano N., Macino G. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol Microbiol. **1992** 6: 3343-3353

Roth-Walter F., Berin M.C., Arnaboldi P., Escalante C.R., Dahan S., Rauch J., Jensen-Jarolim E., Mayer L. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy. **2008** 63: 882-890

Rubinson D.A., Dillon C.P., Kwiatkowski A.V., Sievers C., Yang L., Kopinja J., Rooney D.L., Zhang M., Ihrig M.M., McManus M.T., Gertler F.B., Scott M.L., Van Parijs L. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet. **2003** 33: 401-406

Ruby J.G., Jan C.H., Bartel D.P. Intronic microRNA precursors that bypass Drosha processing. Nature. **2007** 448: 83-86

Rutz S., Scheffold A. Towards in vivo application of RNA interference - new toys, old problems. Arthritis Res Ther. **2004** 6: 78-85

Sakurai K., Goto Y. Manipulating monomer-dimer equilibrium of bovine Beta -lactoglobulin by amino acid substitution. J Biol Chem. **2002** 277: 25735-25740

Salzman D.W., Shubert-Coleman J., Furneaux H. P68 RNA helicase unwinds the human let-7 microRNA precursor duplex and is required for let-7-directed silencing of gene expression. J Biol Chem. **2007** 282: 32773-32779

Santiago Y., Chan E., Liu P.Q., Orlando S., Zhang L., Urnov F.D., Holmes M.C., Guschin D., Waite A., Miller J.C., Rebar E.J., Gregory P.D., Klug A., Collingwood T.N. Targeted gene

knockout in mammalian cells by using engineered zinc-finger nucleases. Proc Natl Acad Sci. **2008** 105: 5809-5814

Santoro R., Lienemann P., Fussenegger M. Epigenetic engineering of ribosomal RNA genes enhances protein production. PLoS One. **2009** 4: e6653

Sasaguri H., Mitani T., Anzai M., Kubodera T., Saito Y., Yamada H., Mizusawa H., Yokota T. Silencing efficiency differs among tissues and endogenous microRNA pathway is preserved in short hairpin RNA transgenic mice. FEBS Lett. **2009** 583: 213-218

Sawyer L., Kontopidis G. The core lipocalin, bovine beta-lactoglobulin. Biochim Biophys Acta. **2000** 1482: 136-148

Schnieke A.E., Kind A.J., Ritchie W.A., Mycock K., Scott A.R., Ritchie M., Wilmut I., Colman A., Campbell K.H. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science. **1997** 278: 2130-2133

Schwarz D.S., Hutvágner G., Du T., Xu Z., Aronin N., Zamore P.D. Asymmetry in the assembly of the RNAi enzyme complex. Cell. **2003** 115: 199-208

Sélo I., Négroni L., Créminon C., Yvon M., Peltre G., Wal J.M. Allergy to bovine beta-lactoglobulin: specificity of human IgE using cyanogen bromide-derived peptides. Int Arch Allergy Immunol. **1998** 117: 20-28

Serrano M., Lin A.W., McCurrach M.E., Beach D., Lowe S.W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. **1997** 88: 593-602

Shu K.X., Li B., Wu L.X. **The** p53 network: p53 and its downstream genes. Colloids Surf B Biointerfaces. **2007** 55: 10-18

Shukla V.K., Doyon Y., Miller J.C., DeKelver R.C., Moehle E.A., Worden S.E., Mitchell J.C., Arnold N.L., Gopalan S., Meng X., Choi V.M., Rock J.M., Wu Y.Y., Katibah G.E., Zhifang G., McCaskill D., Simpson M.A., Blakeslee B., Greenwalt S.A., Butler H.J., Hinkley S.J., Zhang L., Rebar E.J., Gregory P.D., Urnov F.D. Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature. **2009** 459: 437-441

Sinn P.L., Sauter S.L., McCray P.B. Jr. Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. Gene Ther. **2005** 12: 1089-1098

Siomi H. and Siomi M.C. On the road to reading the RNA-interference code. Nature. **2009** 457: 396-404

Sledz C.A., Holko M., de Veer M.J., Silverman R.H., Williams B.R. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol. **2003** 5: 834-839

Song E., Lee S.K., Wang J., Ince N., Ouyang N., Min J., Chen J., Shankar P., Lieberman J. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. **2003** 9: 347-51

Sonoda E., Hochegger H., Saberi A., Taniguchi Y., Takeda S. Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. DNA Repair (Amst). **2006** 5: 1021-1029

Soussi T., Dehouche K., Béroud C. p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis. Hum Mutat. **2000** 15: 105-113

Stark G.R., Kerr I.M., Williams B.R., Silverman R.H., Schreiber R.D. How cells respond to interferons. Annu Rev Biochem. **1998** 67: 227-264

Stephen C.W., Helminen P., Lane D.P. Characterisation of epitopes on human p53 using phage-displayed peptide libraries: insights into antibody-peptide interactions. J Mol Biol. **1995** 248: 58-78

Stern P., Astrof S., Erkeland S.J., Schustak J., Sharp P.A., Hynes R.O. A system for Cre-regulated RNA interference in vivo. Proc Natl Acad Sci. **2008** 105: 13895-13900

Stewart C.K., Li J., Golovan S.P. Adverse effects induced by short hairpin RNA expression in porcine fetal fibroblasts. Biochem Biophys Res Commun. **2008** 370: 113-117

Strano S., Dell'Orso S., Di Agostino S., Fontemaggi G., Sacchi A., Blandino G. Mutant p53: an oncogenic transcription factor. Oncogene. **2007** 26: 2212-2219

Stürzbecher H.W., Brain R., Addison C., Rudge K., Remm M., Grimaldi M., Keenan E., Jenkins J.R. A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization. Oncogene. **1992** 7: 1513-1523

Sun G., Rossi J.J. Problems associated with reporter assays in RNAi studies. RNA Biol. **2009** 6: 406-411

Sun X., Yan Z., Yi Y., Li Z., Lei D., Rogers C.S., Chen J., Zhang Y., Welsh M.J., Leno G.H., Engelhardt J.F. Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. J Clin Invest. **2008** 118: 1578-1583

Sun Y.L., Lin C.S., Chou Y.C. Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. Cell Biol Int. **2006** 30: 970-976

Sun Y.L., Lin C.S., Chou Y.C. Gene transfection and expression in a primary culture of mammary epithelial cells isolated from lactating sows. Cell Biol Int. **2005** 29: 576-582

Sutherland H.G., Kearns M., Morgan H.D., Headley A.P., Morris C., Martin D.I., Whitelaw E. Reactivation of heritably silenced gene expression in mice. Mamm Genome. **2000** 11: 347-355

Sutton, L.F., Alston-Mills B. β-lactoglobulin as a potential modulator of intestinal activity and morphology in neonatal piglets. Anat Rec A Discov Mol Cell Evol Biol. **2006** 288: 601-608

Suzuki H.I., Yamagata K., Sugimoto K., Iwamoto T., Kato S., Miyazono K. Modulation of microRNA processing by p53. Nature. **2009** 460: 529-533

Svoboda P., Stein P., Hayashi H., Schultz R.M. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development. **2000** 127: 4147-4156.

Takahashi K., Tanabe K., Ohnuki M., Narita M., Ichisaka T., Tomoda K., Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. **2007** 131: 861-872

Takahashi K., Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. **2006** 126: 663-676

Talbot N.C., Blomberg le A. The pursuit of ES cell lines of domesticated ungulates. Stem Cell Rev. **2008** 4: 235-254

Talhouk R.S., Neiswander R.L., Schanbacher F.L. Effect of substratum on growth, cell morphology and lactoferrin synthesis and secretion in bovine mammary cell culture. Tissue Cell. **1998** 30: 226-235

Talhouk R.S., Neiswander R.L., Schanbacher F.L. Morphological and functional differentiation of cryopreserved lactating bovine mammary cells cultured on floating collagen gels. Tissue Cell. **1993** 25: 799-816

Thomas K.R., Folger K.R., Capecchi M.R. High frequency targeting of genes to specific sites in the mammalian genome. Cell. **1986** 44: 419-428

Thomson A.J., Marques M.M., McWhir J. Gene targeting in livestock. Reprod Suppl. **2003** 61: 495-508

Thomson J.A., Itskovitz-Eldor J., Shapiro S.S., Waknitz M.A., Swiergiel J.J., Marshall V.S., Jones J.M. Embryonic stem cell lines derived from human blastocysts. Science. **1998** 282: 1145-1147

Tomii R., Kurome M., Ochiai T., Wako N., Ueda H., Hirakawa K., Kano K., Nagashima H. Production of cloned pigs by nuclear transfer of preadipocytes established from adult mature adipocytes. Cloning Stem Cells. **2005** 7: 279-288

Tomii R., Kurome M., Wako N., Ochiai T., Matsunari H., Kano K., Nagashima H. Production of cloned pigs by nuclear transfer of preadipocytes following cell cycle synchronization by differentiation induction. J Reprod Dev. **2009** 55: 121-127

Townsend J.A., Wright D.A., Winfrey R.J., Fu F., Maeder M.L., Joung J.K., Voytas D.F. High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature. **2009** 459: 442-445

Tsutsumi S., Shimazu A., Miyazaki K., Pan H., Koike C., Yoshida E., Takagishi K., Kato Y. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun. **2001** 288: 413-419

Uchida M., Shimatsu Y., Onoe K., Matsuyama N., Niki R., Ikeda J.E., Imai H. Production of transgenic miniature pigs by pronuclear microinjection. Transgenic Res. **2001** 10: 577-582

Ugolini R., Ragona L., Silletti E., Fogolari F. Visschers R.W., Alting A.C., Molinari H. Dimerization, stability and electrostatic properties of porcine β-lactoglobulin. Eur J Biochem. **2001** 268: 4477-4488

Uhrínová S., Smith M.H., Jameson G.B., Uhrín D., Sawyer L., Barlow P.N. Structural changes accompanying pH-induced dissociation of the beta-lactoglobulin dimer. Biochemistry. **2000** 39: 3565-3574

Ui-Tei K., Naito Y., Saigo K. Essential Notes Regarding the Design of Functional siRNAs for Efficient Mammalian RNAi. J Biomed Biotechnol. **2006** 2006: 65052

Ui-Tei K., Naito Y., Takahashi F., Haraguchi T., Ohki-Hamazaki H., Juni A., Ueda R., Saigo K. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. **2004** 32: 936-948

Umeyama K., Watanabe M., Saito H., Kurome M., Tohi S., Matsunari H., Miki K., Nagashima H. Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic Res. **2009** 18: 697-706

Utikal J., Polo J.M., Stadtfeld M., Maherali N., Kulalert W., Walsh R.M., Khalil A., Rheinwald J.G., Hochedlinger K. Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature. **2009** 460: 1145-1148

van den Bos C., Mosca J.D., Winkles J., Kerrigan L., Burgess W.H., Marshak D.R. Human mesenchymal stem cells respond to fibroblast growth factors. Hum Cell. **1997** 10: 45-50

Vasileva A., Jessberger R. Precise hit: adeno-associated virus in gene targeting. Nat Rev Microbiol. **2005** 3: 837-847

Vermeulen A., Behlen L., Reynolds A., Wolfson A., Marshall W.S., Karpilow J., Khvorova A. The contributions of dsRNA structure to Dicer specificity and efficiency. RNA. **2005** 11: 674-682

Vickers T.A., Koo S., Bennett C.F., Crooke S.T., Dean N.M., Baker B.F. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J Biol Chem. **2003** 278: 7108-7118

Vousden K.H., Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer. **2002** 2: 594-604

Wal J.M. Cow's milk allergens. Allergy. **1998a** 53: 1013-1022

Wal J.M. Immunochemical and molecular characterization of milk allergens. Allergy. **1998b** 53: 114-117

Wang B., Zhou J. Specific genetic modifications of domestic animals by gene targeting and animal cloning. Reprod Biol Endocrinol. **2003** 1: 103-110

Wang K.H., Kao A.P., Wangchen H., Wang F.Y., Chang C.H., Chang C.C., Lin S.D. Optimizing proliferation and characterization of multipotent stem cells from porcine adipose tissue. Biotechnol Appl Biochem. **2008** 51: 159-166

Wang P., Reed M., Wang Y., Mayr G., Stenger J.E., Anderson M.E., Schwedes J.F., Tegtmeyer P. p53 domains: structure, oligomerization, and transformation. Mol Cell Biol. **1994** 14: 5182-5191

Wei J.X., Yang J., Sun J.F., Jia L.T., Zhang Y., Zhang H.Z., Li X., Meng Y.L., Yao L.B., Yang A.G. Both strands of siRNA have potential to guide posttranscriptional gene silencing in mammalian cells. PLoS ONE. **2009** 4:e5382

Wernersson R., Schierup M.H., Jørgensen F.G., Gorodkin J., Panitz F., Staerfeldt H.H., Christensen O.F., Mailund T., Hornshøj H., Klein A., Wang J., Liu B., Hu S., Dong W., Li W., Wong G.K., Yu J., Wang J., Bendixen C., Fredholm M., Brunak S., Yang H., Bolund L. Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. BMC Genomics. **2005** 6: 70

Westbrook T.F., Martin E.S., Schlabach M.R., Leng Y., Liang A.C., Feng B., Zhao J.J., Roberts T.M., Mandel G., Hannon G.J., Depinho R.A., Chin L., Elledge S.J. A genetic screen for candidate tumor suppressors identifies REST. Cell. **2005** 121: 837-848

Weston M.D., Pierce M.L., Rocha-Sanchez S., Beisel K.W., Soukup G.A. MicroRNA gene expression in the mouse inner ear. Brain Res. **2006** 1111: 95-104

Whitehead K.A., Langer R., Anderson D.G. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov. **2009** 8: 129-138

Whitelaw C.B., Radcliffe P.A., Ritchie W.A., Carlisle A., Ellard F.M., Pena R.N., Rowe J., Clark A.J., King T.J., Mitrophanous K.A. Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett. **2004** 571: 233-236

Wianny F., Zernicka-Goetz M. Specific interference with gene function by double-stranded RNA in early mouse development. Nat Cell Biol. **2000** 2: 70-75

Wienholds E., Kloosterman W.P., Miska E., Alvarez-Saavedra E., Berezikov E., de Bruijn E., Horvitz H.R., Kauppinen S., Plasterk R.H. MicroRNA expression in zebrafish embryonic development. Science. **2005** 309: 310-311

Wightman B., Ha I., Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. **1993** 75: 855-862

Willis A., Jung E.J., Wakefield T., Chen X. Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes. Oncogene. **2004** 23: 2330-2338

Wilmut I., Schnieke A.E., McWhir J., Kind A.J., Campbell K.H. Viable offspring derived from fetal and adult mammalian cells. Nature. **1997** 385: 810-813

Wischhusen J., Melino G., Weller M. p53 and its family members -- reporter genes may not see the difference. Cell Death Differ. **2004** 11: 1150-1152

Wright D.A., Townsend J.A., Winfrey R.J. Jr, Irwin P.A., Rajagopal J., Lonosky P.M., Hall B.D., Jondle M.D., Voytas D.F. High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J. **2005** 44: 693-705

Wu S.C. RNA interference technology to improve recombinant protein production in Chinese hamster ovary cells. Biotechnol Adv. **2009b** 27: 417-422

Wu S.Y., Pérez M.D., Puyol P., Sawyer L. beta-lactoglobulin binds palmitate within its central cavity. J Biol Chem. **1999** 274: 170-174

Wu Z., Chen J., Ren J., Bao L., Liao J., Cui C., Rao L., Li H., Gu Y., Dai H., Zhu H., Teng X., Cheng L., Xiao L. Generation of Pig-Induced Pluripotent Stem Cells with a Drug-Inducible System. J Mol Cell Biol. **2009a** 1: 46-54

Xia X.G., Zhou H., Samper E., Melov S., Xu Z. Pol II-expressed shRNA knocks down Sod2 gene expression and causes phenotypes of the gene knockout in mice. PLoS Genet. **2006** 2: e10

Yamada H., Shinmura K., Okudela K., Goto M., Suzuki M., Kuriki K., Tsuneyoshi T., Sugimura H. Identification and characterization of a novel germ line p53 mutation in familial gastric cancer in the Japanese population. Carcinogenesis. **2007** 28: 2013-2018

Yamamoto M., Maehara Y., Oda S., Ichiyoshi Y., Kusumoto T., Sugimachi K. The p53 tumor suppressor gene in anticancer agent-induced apoptosis and chemosensitivity of human gastrointestinal cancer cell lines. Cancer Chemother Pharmacol. **1999** 43: 43-49

Yan D., Zhou X., Chen X., Hu D.N., Dong X.D., Wang J., Lu F., Tu L., Qu J. MicroRNA-34a inhibits uveal melanoma cell proliferation and migration through downregulation of c-Met. Invest Ophthalmol Vis Sci. **2009** 50: 1559-1565

Yang M.C., Guan H.H., Liu M.Y., Lin Y.H., Yang J.M., Chen W.L., Chen C.J., Mao S.J. Crystal structure of a secondary vitamin D3 binding site of milk beta-lactoglobulin. Proteins. **2008** 71: 1197-1210

Yang S., Tutton S., Pierce E., Yoon K. Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. Mol Cell Biol. **2001** 21: 7807-7816

Yi R., Qin Y., Macara I.G., Cullen B.R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. **2003** 17: 3011-3016

Yu J., Vodyanik M.A., Smuga-Otto K., Antosiewicz-Bourget J., Frane J.L., Tian S., Nie J., Jonsdottir G.A., Ruotti V., Stewart R., Slukvin I.I., Thomson J.A. Induced pluripotent stem cell lines derived from human somatic cells. Science. **2007** 318: 1917-1920

Yu J.Y., DeRuiter S.L., Turner D.L RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci. **2002** 99:6047-6052

Zamore P.D. Ancient pathways programmed by small RNAs. Science. **2002** 296: 1265-1269

Zamore P.D., Tuschl T., Sharp P.A., Bartel D.P. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. **2000** 101: 25-33

Zavizion B., van Duffelen M., Schaeffer W., Politis I. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. In Vitro Cell Dev Biol Anim. **1996** 32: 138-148

Zender L., Hutker S., Liedtke C., Tillmann H.L., Zender S., Mundt B., Waltemathe M., Gosling T., Flemming P., Malek N.P., Trautwein C., Manns M.P., Kuhnel F., Kubicka S. Caspase 8 small interfering RNA prevents acute liver failure in mice. Proc Natl Acad Sci. **2003** 100: 7797-7802

Zeng Y., Wagner E.J., Cullen B.R. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol Cell. **2002** 9: 1327-1333

Zhang H., Kolb F.A., Jaskiewicz L., Westhof E., Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. Cell. **2004** 118: 57-68

Zhao X.Y., Li W., Lv Z., Liu L., Tong M., Hai T., Hao J., Guo C.L., Ma Q.W., Wang L., Zeng F., Zhou Q. iPS cells produce viable mice through tetraploid complementation. Nature. **2009** 461: 86-90

Zhao Y., Yin X., Qin H., Zhu F., Liu H., Yang W., Zhang Q., Xiang C., Hou P., Song Z., Liu Y., Yong J., Zhang P., Cai J., Liu M., Li H., Li Y., Qu X., Cui K., Zhang W., Xiang T., Wu Y., Zhao Y., Liu C., Yu C., Yuan K., Lou J., Ding M., Deng H. Two supporting factors greatly improve the efficiency of human iPSC generation. Cell Stem Cell. **2008** 3: 475-479

Zhou H., Wu S., Joo J.Y., Zhu S., Han D.W., Lin T., Trauger S., Bien G., Yao S., Zhu Y., Siuzdak G., Schöler H.R., Duan L., Ding S. Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell. **2009** 4: 381-384

Zou J., Maeder M.L., Mali P., Pruett-Miller S.M., Thibodeau-Beganny S., Chou B.K., Chen G., Ye Z., Park I.H., Daley G.Q., Porteus M.H., Joung J.K., Cheng L. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell. **2009** 5: 97-110

Zufferey R., Donello J.E., Trono D., Hope T.J. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol. **1999** 73: 2886-2892

# 10 Acknowledgement

I would like to thank my supervisor Prof. A. Schnieke for giving me the opportunity to perform this work at her institute, for all the helpful discussions, ideas and her continous support.

Further I would like to thank Prof. Dr. M. Klingenspor for evaluating this thesis as second referee.

Also I would like to thank Dr. A. Kind for all the help he offered during my doctorate and with correcting the manuscript of this thesis.

I would like to thank Prof. Dr. O. Rottmann, Steffen and Viola Löbnitz and everyone else at the animal research station in Thalhausen as well as Dr. B. Keßler and Prof. Dr. E. Wolf for the help with the embryo transfer and the sourcing of porcine tissues from the abattoir.

In addition, I would like to thank all my students working on a variety of topics in their Master and Bachelor projects or internships. My special thanks go to Anja Saalfrank, Davide Cavanna, Bernadette Antoni, Joachim Weidmann, Christian Bogen and Simon Leuchs. You rock!

My fellow Ph.D. students Simon Leuchs, Marina Durkovic, Alex Tschukes, Xinxin Cui and Tobias Richter I would like to thank all of you for the great time, all the fun, help and the familial atmosphere. My special thank you goes to Tina Landmann for being there, trying to make me eat fruit and everything else! It was great to work with you!

Further, I would like to thank Dr. Hagen Wieland, Dr. Tatiana Flisikowska, Dr. Nousin Rezaei and Dr. Simone Kraner for all the scientific and non-scientific help and the great time.

Furthermore, I would like to thank Kristina Mosandl, Angela Zaruba, Marlene Edlinger, Sulith Christan, Andi Braun, Tobi Kranz and Barbara Bauer for the technical and non-technical help and the nice coffee breaks. Moreover I would like to thank Margret Bahnweg and especially Peggy Müller, this work would not have been possible without you!

I thank my parents for all their support and all their love! You were always there for me!

Last but not least, I would like to thank you, Dave, for all the tolerance, patience, support and love!

# 11 Curriculum vitae

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